

RESEARCH PAPER

# Anther-specific carbohydrate supply and restoration of metabolically engineered male sterility

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## Abstract

Male-sterile plants are used in hybrid breeding as well as for gene confinement for genetically modified plants in field trials and agricultural production. Apart from naturally occurring mutations leading to male sterility, biotechnology has added new possibilities for obtaining male-sterile plants, although so far only one system is used in practical breeding due to limitations in propagating male-sterile plants without segregations in the next generation or insufficient restoration of fertility when fruits or seeds are to be harvested from the hybrid varieties. Here a novel mechanism of restoration for male sterility is presented that has been achieved by interference with extracellular invertase activity, which is normally specifically expressed in the anthers to supply the developing microspores with carbohydrates. Microspores are symplastically isolated in the locular space of the anthers, and thus an unloading pathway of assimilates via the apoplasmic space is mandatory for proper development of pollen. Antisense repression of the anther-specific cell wall invertase or interference with invertase activity by expressing a proteinacious inhibitor under the control of the anther-specific invertase promoter results in a block during early stages of pollen development, thus causing male sterility without having any pleiotropic effects. Restoration of fertility was successfully achieved by substituting the down-regulated endogenous plant invertase activity by a yeast invertase fused to the N-terminal portion of potato-derived vacuolar protein proteinase II (Pill-ScSuc2), under control of the orthologous anther-specific invertase promoter *Nin88* from tobacco. The chimeric fusion Pill-ScSuc2 is known to be N-glycosylated and efficiently secreted from plant cells, leading to its apoplastic location. Furthermore, the *Nin88::Pill-ScSuc2* fusion does not show effects on pollen development in the wild-type background. Thus, such plants can be used as paternal parents of a hybrid variety, thereby the introgression of *Nin88::Pill-ScSuc2* to the hybrid is obtained and fertility is restored. In order to broaden the applicability of this male sterility/restoration system to other plant species, a phylogenetic analysis of plant invertases ( $\beta$ -fructofuranosidases) and related genes of different species was carried out. This reveals a specific clustering of the cell wall invertases with anther-specific expression for dicotyl species and another cluster for monocotyl plants. Thus, in both groups of plants, there seems to be a kind of co-evolution, but no recent common ancestor of these members of the gene family. These findings provide a helpful orientation to classify corresponding candidate genes in further plant species, in addition to the species analysed so far (*Arabidopsis*, tobacco, tomato, potato, carrots, rice, and wheat).

**Key words:** Genetic engineering, hybrid breeding, invertase, male sterility, restoration.

## Introduction

Among breeding strategies,  $F_1$  hybrids have several advantages over open-pollinated varieties.  $F_1$  hybrids are obtained by crossing parental inbred lines, and their special importance is due to the uniformity that is based

on the resulting genetic homogeneity as well as on higher yields through heterosis or 'hybrid vigour', due to their heterozygous nature (Brewbaker, 1964; Feistritzer and Kelly, 1987). The heterozygous nature of the

hybrids requires seeds to be obtained from breeding companies.

A further important application for male sterility systems, apart from hybrid breeding, is their use for gene confinement for the increasing number of genetically modified crop plants used in field trials and agricultural production (Daniell, 2002). Although the risk of outcrossing to wild species depends on the mode of pollination (self-pollination or outcrossing by wind or insects) as well as the presence of related species in the surrounding ecosystem, the availability and use of such biological safety precautions will help to cope with public awareness and fears regarding the potential spread of transgenes, and concerns about the environmental impact of genetically modified plants (GMPs). Nowadays, the most widespread are 'first-generation' GMPs such as herbicide-tolerant soybean and rapeseed, and insect-tolerant corn and cotton. Perhaps the public awareness and fears of potential risks of GMPs arise from these modifications, since they are associated with toxins and the commercial interests of a few companies. The so-called second generation of GMPs is that of plants in which the quality of the product has been changed and improved; examples are rice with increased provitamin A levels, rapeseed varieties with modified fatty acid composition, or lysine-rich soybean. The 'third generation' of GMPs producing pharmaceutical or industrial proteins or vaccines is under development.

Naturally occurring genetic systems such as sex inheritance, self-incompatibility, and, in the first instance, cytoplasmic male sterility (CMS), have been used for a long time in practical breeding strategies. In the case of CMS, the maternal transmission of sterility-inducing cytoplasm, specified by mitochondrial mutations, in combination with Mendelian nuclear genes, permits the efficient control of pollination. However, CMS is not available in all crops and, if available, 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 also arise due to unstable 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 decreased fruit or seed setting (reviewed in Engelke *et al.*, 2004a, b). Biotechnology has added new possibilities of obtaining male-sterile plants. Various successful approaches to engineer male sterility have been described, although only a single system is ready to be used in agriculture and indeed is already in practical use (reviewed in Roitsch and Engelke 2006).

Developing microspores are symplastically isolated in the locular space of the anthers, and thus an unloading pathway of assimilates via the apoplasmic space is mandatory for proper development of pollen. Therefore, sucrose, the most ubiquitous transport sugar, 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 (Roitsch and González, 2004). Uptake of the hexose monomers (glucose and, with a lower preference, fructose) into the sink cell is realized by high-affinity hexose

transporters. The importance of cell wall-bound invertases (cwINVs) during this process was demonstrated in previous studies involving model plant species (Goetz *et al.*, 2001; Proels *et al.*, 2006; Hirsche *et al.*, 2009). In the plants investigated so far, *Nicotiana tabacum*, *Solanum lycopersicum*, and *Arabidopsis thaliana*, repression of cwINV activity (Nin88, Lin7, and AtcwINV2, respectively) by anther-specific RNA interference turned out to be an efficient method to circumvent carbohydrate supply of the symplastically isolated pollen with a subsequent strong decrease of pollen germination ability and seed setting. Comparable results were also obtained by expressing a proteinaceous invertase inhibitor. The specific involvement of invertases during anther development was also suggested for other plant species, for example *Lilium longiflorum* (Clément *et al.*, 1996; Ranwala and Miller, 1998; Hsu *et al.*, 2007), potato (Maddison *et al.*, 1999), and carrot (Lorenz *et al.*, 1995), indicating the crucial function of cwINVs in providing carbohydrates for male gametophyte development. In wheat, an arrest in pollen development because of water deficiency correlated with alterations in carbohydrate metabolism, a drastic decrease in invertase activity, and subsequently the failure to accumulate starch (Saini *et al.*, 1984; Dorion *et al.*, 1996; Lalonde *et al.*, 1997). Comparable results were found for rice under cold stress (Oliver *et al.*, 2005). These findings demonstrate the highly specific effects leading to male sterility in different dicotyledonous and monocotyledonous plants, and support the contention that generating male-sterile plants by anther-specific interference with cwINV activity should be generally applicable to different plant species.

The question arises of whether the cwINVs with anther-specific expression are closely related to each other, or if there is some kind of co-evolution in different plant species. To answer this question, the number of known sequences coding for acid invertases in *N. tabacum* was broadened and an alignment was carried out of the anther-specific invertases of different species with other members of the gene family ( $\beta$ -fructofuranosidases EC 3.2.1.26) found in the cell wall (cwINV), the vacuole (vacINV), or the cytoplasm (cytINV), as well as with related genes coding for fructan-building enzymes (FBEs) and fructan exohydrolases (FEHs). The consequences of the phylogenetic relationships for future applications of the described transgenic approach to produce male sterility in other species are discussed.

As a pre-condition for practical application of the described male sterility in hybrid breeding or as a biological safety precaution, the system has to be extended to include a mechanism that allows the multiplication of male-sterile plants without segregation in the offspring (maintenance) as well as for a restorer mechanism when seeds or fruits are to be harvested from the crops. To achieve these goals, a strategy is devised in the present study: replacement of the down-regulated natural plant invertase activity by expressing the distantly related isoenzyme Suc2 of yeast (*Saccharomyces cerevisiae*), which was not expected to interfere with the plant antisense invertase or with the plant invertase inhibitor due to low sequence homology.

## Materials and methods

*Male-sterile tobacco plants, generated by expressing an antisense construct of anther-specific cell wall invertase (Nin88::Nin88-antisense) or a proteinacious invertase inhibitor (Nin88::NtCIF)*

Two male-sterile plants expressing the antisense construct *Nin88::Nin88-antisense* (102K and 102KK) as well as one plant expressing the proteinacious inhibitor *Nin88::NtCIF* (105L) were selected in the 'SNN' background. As has been previously described, these plants are characterized by strongly reduced total pollen amount, pollen vitality, and germination efficiency, as well as their inability to produce seeds by self-pollination (Hirsche *et al.*, 2009).

*Construction of anther-specific promoter-yeast invertase fusions (Nin88::ScSuc2 and Nin88::PiII-ScSuc2) and transformation into tobacco*

The constructs were derived from the previously described reporter construct originally used for identification of tobacco *Nin88* promoter activity (*Nin88* fused to the *uidA* gene in the pBI101 binary vector; Goetz *et al.*, 2001) by replacing the *uidA* gene with the cDNA of the yeast (*S. cerevisiae*) invertase *Suc2*.

In yeast, the invertase gene *Suc2* encodes two differently regulated mRNAs (1.8 kb and 1.9 kb) that differ at their 5' ends. The smaller RNA encodes an intracellular form that is constitutively expressed at low levels in yeast. The larger RNA contains a signal peptide-coding sequence leading to a secreted form of invertase (Fig. 1) (Carlson *et al.*, 1983). Two different constructs have been made under control of the anther-specific invertase promoter of tobacco, *Nin88*, using either *Suc2* with its native yeast signal peptide (*Nin88::ScSuc2*) or a shorter part of *Suc2* fused to the N-terminal portion of potato-derived vacuolar protein proteinase II (*Nin88::PiII-ScSuc2*; Fig. 1). This latter chimeric fusion is known to be N-glycosylated and efficiently secreted from plant cells, leading to its apoplastic location (von Schaewen *et al.*, 1990). These constructs were used for *Agrobacterium tumefaciens* strain LBA4404-mediated transformation of tobacco leave discs (*N. tabacum* cv. Samsun) by using standard procedures (Horsch *et al.*, 1985). Transformed plants were selected on Murashige-Skoog

(MS) medium containing 2% sucrose, 0.3% gelrite, and 100 mg l<sup>-1</sup> kanamycin. In a first approach both constructs have been tested in the wild-type tobacco background, in order to see the effects on pollen germination efficiencies.

Further investigations focused on the *PiII-ScSuc2* sequence, fused to the *Nin88* promoter to obtain stable restoration of fertility in the formerly male-sterile plants. Supertransformation of the kanamycin-resistant male-sterile plants expressing *Nin88::Nin88-antisense* (102K and 102KK) or *Nin88::NtCIF* (105L) was achieved by transferring the cassette *Nin88::PiII-Suc2-nosTerm* to pCambia1300, a vector that allows selection on hygromycin (50 mg l<sup>-1</sup> in MS as described above).

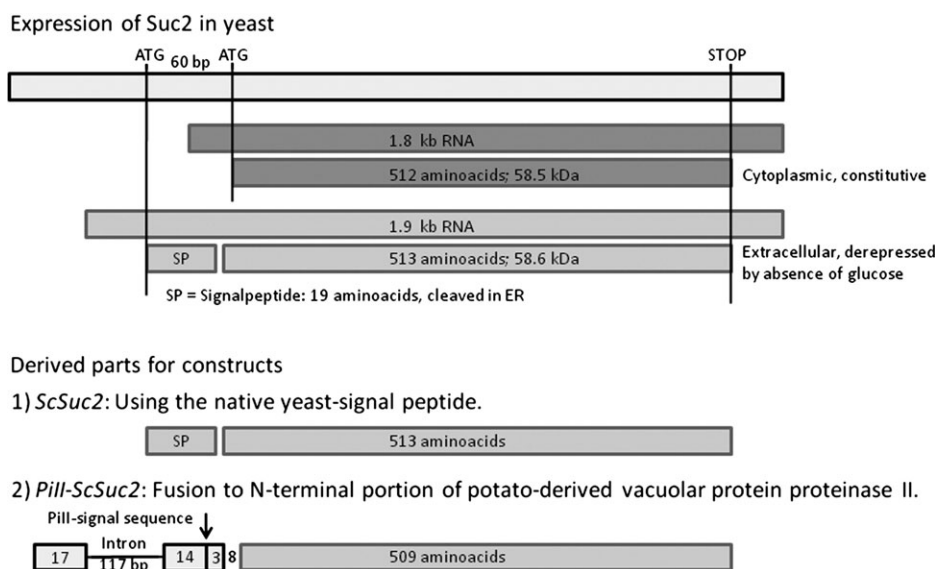
Plants were grown in the greenhouse at 22 °C in the light and 18 °C in the dark with 12 h light under additional illumination. Transformation was confirmed by PCR, amplifying parts of the specific constructs.

*In vitro germination tests of tobacco pollen*

The germination of pollen was tested on sitting drops of 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 sulphate, and 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.

*In situ staining of invertase activity in tobacco pollen*

Anthers were collected, frozen in liquid nitrogen, and stored at -80 °C. Before staining, 500 µl of buffer (200 mM HEPES, 3 mM MgCl<sub>2</sub>, 1 mM EDTA, and 2% glycerin) were added to the anthers, giving a pollen suspension from which anthers were removed by using a pipette tip. Pollen were centrifuged for 5 min at 8000 g, and the pellet resuspended in 500 µl of buffer. This washing step was repeated to remove sugars which might give false-positive results. Washed pollen was resuspended in 40 µl of 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 incubation medium contained 70 mM K<sub>2</sub>HPO<sub>4</sub>+40 mM citrate, resulting in pH 4.5, 20 U ml<sup>-1</sup> glucose oxidase (GOD; Boehringer), 0.014% phenazine methosulphate



**Fig. 1.** Scheme showing the expression of yeast (*Saccharomyces cerevisiae*) invertase *Suc2* (Carlson *et al.*, 1983, with modifications) and the derived parts of the constructs used for supertransformation of male-sterile plants (*ScSuc2* with the native signal sequence leading to extracellular location in yeast; *PiII-ScSuc2*, that is secreted from the plant cells leading to its apoplastic location, according to PI-3-INV in von Schaewen *et al.*, 1990).

(PMS), 0.024% nitroblue tetrazolium salt (NBT), and 0.5% sucrose. A 1 ml aliquot 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. After centrifugation for 5 min at 8000 g, the incubation medium was replaced with 500 µl of 70% EtOH. For light microscopic investigation of the staining, pollen was centrifuged again and resuspended in 100 µl of water. Photographs were taken with 100-fold magnification.

#### Partial cloning of additional tobacco invertases

In order to broaden knowledge of the invertase gene family of tobacco, parts of exon 3 of invertases from *N. tabacum* were amplified from genomic DNA or cDNA from anthers using degenerate primers that have been designed by alignment of different known invertase sequences. Primers oin3 (CCTTCA-CYNTNTTYTAYCARTAYAAAYCC) and oin4 (CCTTTCRWAR-AARGTYTTDGGWGCCTA) amplified PCR products of ~750 bp in size (Roitsch et al., 1995). The PCR products were ligated in pGEM-T Easy vector and transformed in *Escherichia coli* DH5a, according to the instructions of the supplier (Promega, Madison, WI, USA). Due to the degenerate 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, and plasmids were isolated and characterized by test restriction with enzymes having 4 bp recognition sites (*Bsu*RI, *Hpa*II, and *Taq*I; MBI Fermentas, St Leon-Rot, Germany). Plasmids with different restriction patterns were sequenced. Putative invertase sequences of *N. tabacum* were identified using the Blast N program (NCBI; Altschul et al., 1997) and aligned with the reported coding sequences (CDS) from other species, using the Clustal W program (Higgins et al., 1994).

#### Phylogenetic tree construction of anther-specific invertases and related enzymes of different monocotyledonous and dicotyledonous plants

The phylogram is a branching diagram (tree) assumed to be an estimate of a phylogeny; branch lengths are proportional to the amount of inferred evolutionary change (EMBL; Larkin et al., 2007). After the abbreviation for the coding sequences, the pI value of the corresponding protein is given, calculated with Protein Calculator v3.3 (www.scripps.edu/~cdputnam/protcalc.html). The complete invertase gene families (acid and cytoplasmic) from *A. thaliana* and *Oryza sativa* are the backbone of the phylogenetic analyses, extended with already described and newly isolated sequences of the acid invertases from tobacco, as well as related sequences from the well-studied fructan-accumulating species *Cichorium intybus* and *Triticum aestivum* (with FBE and FEH genes). CwINVs with known anther-specific expression from *S. lycopersicum*, *Solanum tuberosum*, and *Daucus carota* are added. The accession numbers of the sequences used to construct the phylogenetic tree are given below

**Non-fructan plants:** Cell wall invertases. *A. thaliana*: *AtcwINV1–AtcwINV6* (Sherson et al., 2003), At3g13790, At3g52600, At1g55120, At2g36190, At3g13784, At5g11920. *O. sativa*: *OsCIN1–OsCIN8* (Cho et al., 2005), AY578158–65 [corresponding to *OsCIN1–OsCIN3* and *OsCIN5–OsCIN9* in Ji et al. (2005) since an additional pseudogene is integrated as *OsCIN4* in Ji et al. (2005)]; AY575551 (Os03g0735800) referred to as *OsCIN4(Ji)\_pseudo*. *N. tabacum*: *Ntβfruc1 (NtcwINV1)* (Greiner et al., 1995), X81834; *NtNin88 (NtcwINV2)* (Goetz et al., 2001), AF376773; and newly isolated *NtcwINV3*, HM022265; *NtcwINV4*, HM022266; *NtcwINV5-FEH?*, HM022267; *NtcwINV6-FEH?*, HM022268. *S. lycopersicum*: *SILin7* (Godt and Roitsch, 1997; Proels et al.,

2003, 2006), AY173050. *S. tuberosum*: *StInvGF* (Maddison et al., 1999), AJ133765. *D. carota*: *DcInvDe2* (Lorenz et al., 1995), X78424.

**Vacuolar invertases.** *A. thaliana*: *Atβfruct3–Atβfruct4* (Tymowska-Lalanne and Kreis, 1998), At1g62660, At1g12240. *O. sativa*: *OsVIN1–OsVIN2* (Ji et al., 2005), AF276703, AF276704). *N. tabacum*: *NtVI (NtvacINV1)* (Lauer, 2006), AJ305044, and newly isolated *NtvacINV2*, HM022269.

**Cytoplasmic (alkaline/neutral) invertases.** *A. thaliana*: *At-A1N-InvA–At-A1N-InvI* (Vargas et al., 2003), At1g56560, At4g34860, At3g06500, At1g22650, At5g22510, At1g72000, At1g35580, At3g05820, At4g09510. *O. sativa*: *OsNIN1–OsNIN8* (Ji et al., 2005), NM\_001056471, NM\_001049466, NM\_001053547, NM\_001059261, AP005311, NM\_001072410, NM\_001059365, NM\_001053630.

**Fructan-accumulating plants:** Cell wall invertases. *T. aestivum*: *TaIVR1* and *TaIVR3* (Minhas and Saini, 1998; Koonjul et al., 2004), AF030420, AF030421.

**Vacuolar invertases.** *C. intybus*: *CivacINV* (van den Ende et al., 2002), AJ419971. *T. aestivum*, *TaVIN2* (L Schroeven, personal communication), AJ635225, *TaVIN3 (=TaIVR5)*, Koonjul et al., 2004), AF069309.

**Cytoplasmic (alkaline/neutral) invertases.** *T. aestivum*: *TaAInv* (Vargas et al., 2007), AM295169.

**Fructan-building enzymes (FBEs).** *C. intybus*: *Cil-FFT* (JP Goblet, L Canon, and Van PJ Cutsem, personal communication), U84398; *Cil-SST* (de Halleux and van Cutsem, 1997), U81520. *T. aestivum*: *Ta1-SST* and *Ta6-FST* (Kawakami and Yoshida, 2002), AB029888, AB029887; *Ta1-FFT* (Kawakami and Yoshida, 2005), B088409.

**Fructan exohydrolases (FEHs).** *C. intybus*: *Cil-FEH-1* (van den Ende et al., 2000), AJ242538; *Cil-FEH-IIa* and *Cil-FEH-IIb* (van den Ende et al., 2001), AJ295033, AJ295034. *T. aestivum*: *Ta1-FEHw1–Ta1-FEHw3* (Zhang et al., 2008), FJ184989, FJ184991, FJ184990; *Ta6-FEH* (van Riet et al., 2006), AM075205; *Ta1&6-FEH* (Kawakami et al., 2005), AB089269.

## Results

### Transformation of wild-type tobacco SNN with two different anther-specific promoter–yeast invertase fusions (Nin88::ScSuc2 or Nin88::Pill-ScSuc2) does not result in drastic changes in pollen germination

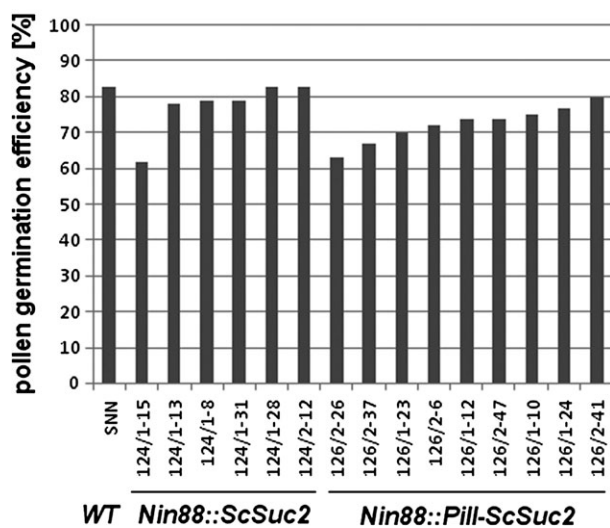
Repression of cwINV activity by anther-specific RNA interference turned out to be an efficient method to circumvent carbohydrate supply of the symplastically isolated pollen with a subsequent strong decrease of pollen germination ability and seed setting. Comparable results were also obtained by expressing a proteinaceous invertase inhibitor (see Introduction). The present study devises a strategy for restoration of fertility by replacing the down-regulated natural plant invertase activity through the distantly related isoenzyme Suc2 of yeast. Therefore, two constructs have been made and tested in the wild-type background in a first approach.

In yeast, the invertase gene *Suc2* encodes two differently regulated mRNAs (1.8 kb and 1.9 kb) that differ at their 5' ends. The smaller RNA encodes an intracellular form that is constitutively expressed at low levels in yeast. The larger RNA contains a signal peptide-coding sequence leading to a secreted form of invertase that is regulated by derepression

of glucose (Fig. 1) (Carlson *et al.*, 1983). The secreted form is glycosylated, with ~50% of the molecular mass attributed to *N*-linked oligosaccharide residues (in the form of nine high-mannose oligosaccharide chains, each linked to an asparagine residue on the 60 kDa polypeptide backbone). In yeast, the secreted form has been found in the periplasmic space as oligomers which result from an aggregation of the dimer, the smallest enzymically active unit. Dimers, tetramers, and hexamers have a molecular mass of ~260, 360, and 560 kDa, respectively (Chu *et al.*, 1983).

Using this information, two different constructs with the yeast invertase gene *Suc2* under the control of the anther-specific invertase promoter of tobacco, *Nin88*, have been engineered (Fig. 1): (i) the yeast invertase *Suc2* with its native signal peptide resulting in the construct *Nin88::ScSuc2*; and (ii) an N-terminally shortened version of *Suc2* fused to the N-terminal portion of potato-derived vacuolar protein proteinase II (von Schaewen *et al.* 1990), giving rise to *Nin88::PiII-ScSuc2*. The chimeric fusion of *Suc2* to the N-terminal portion of potato-derived vacuolar protein proteinase II was known to be *N*-glycosylated and efficiently secreted from plant cells, leading to its apoplastic location (von Schaewen *et al.*, 1990).

Both these constructs were transformed into wild-type tobacco SNN, and six and nine independently regenerated plants were investigated, respectively. Phenotypic analyses of vegetative growth and determination of pollen germination efficiencies, calculated as the percentage of germinating pollen in the visible pollen population, did not reveal any apparent changes in relation to the wild-type controls. The pollen germination efficiency of the wild type was determined to be ~80%, while the transformants, independently from the construct used, showed slight variations of 60–80% (Fig. 2).



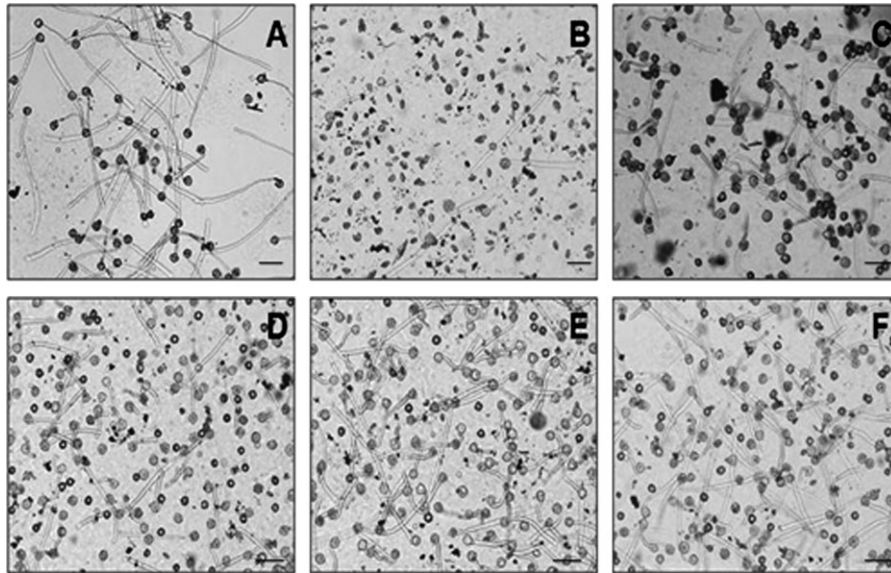
**Fig. 2.** Influence of the constructs *Nin88::ScSuc2* and *Nin88::PiII-ScSuc2* on pollen germination efficiency in the wild-type SNN background.

### *Male sterility due to antisense or inhibitor interference with anther-specific cwINV is restored by supertransformation with the chimeric yeast invertase construct Nin88::PiII-ScSuc2*

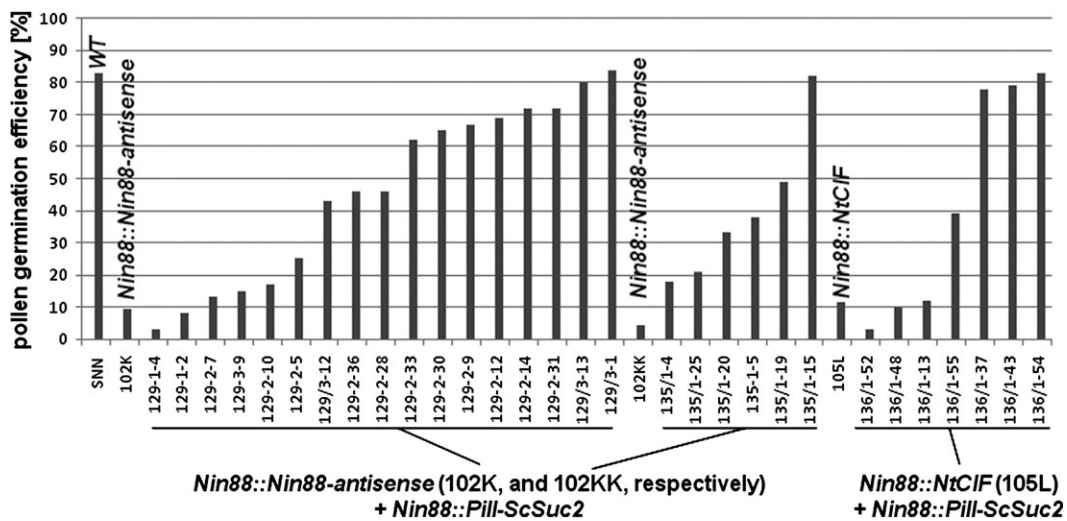
Since neither construct, either that with the native yeast signal peptide (*Nin88::ScSuc2*) or that with the potato-derived leader sequence (*Nin88::PiII-ScSuc2*), had an apparent effect when transformed into wild-type tobacco (see above), the construct *Nin88::PiII-ScSuc2* was investigated in order to test the strategy for restoration of fertility, for the following reason: the fusion of the N-terminal portion of potato-derived vacuolar protein proteinase II (leader sequence *PiII*) with *Suc2* was already known to be efficiently secreted from plant cells, as previous studies with protoplasts had revealed (von Schaewen *et al.*, 1990). Thus, this construct was the more promising one for replacing the down-regulated natural plant cwINV activity through the distantly related isoenzyme *Suc2* of yeast.

This construct was supertransformed into three originally male-sterile tobacco plants which were selected from a previous study (Hirsche *et al.*, 2009). These three male-sterile plants had originated by transformation with the antisense construct against the anther-specific cwINV *Nin88* (*Nin88::Nin88-antisense*), plant 102K and 102KK, or by expressing the proteinacious invertase inhibitor *NtCIF* (*Nin88::NtCIF*), plant 105L. The strong phenotypes of these three plants have been characterized by reduced total numbers of pollen per anther (from ~50 000 in the wild type to <10 000) and, furthermore, by drastically reduced pollen germination efficiencies in the visible pollen population [from ~80% in the wild type to <10% in the male-sterile plants; Hirsche *et al.* (2009), see also Figs 3a, b, 4]. These three male-sterile plants were supertransformed with the yeast invertase construct *Nin88::PiII-ScSuc2* and 17 plants from 102K, six from 102KK, and seven from 105L regenerated, and the pollen germination efficiencies were determined. Among the supertransformed plants, fertility is restored to different extents (Figs 3c–f, 4). In about two-thirds of the plants, pollen germination efficiencies of >30% were determined. Independently from the construct originally used for creating the male-sterile plants (antisense or inhibitor), the best phenotypes of the supertransformants showed pollen germination efficiencies of ~80%, thus reaching the level of the wild type (Fig. 4).

In order to visualize the reduced endogenous invertase activity in the male-sterile plants and its substitution in the supertransformants by the yeast invertase, histochemical *in situ* invertase activity stains were performed. The staining method uses sucrose as substrate. The glucose liberated by invertase activity is oxidized by GOD, thereby reducing PMS and finally NBT. The reaction yields an intensely blue insoluble formazan which is easily visible microscopically, while NBT itself is soluble and yields a practically colourless (slightly yellow) solution (Dahlqvist and Brun, 1962). While pollen of the wild type SNN (Fig. 5A, lower row) shows a dark blue-black staining in the incubation medium, pollen of male-sterile plants expressing *Nin88::Nin88-antisense*



**Fig. 3.** *In vitro* pollen germination of wild-type tobacco SNN (A), *Nin88::Nin88-antisense* (B, plant Nt102K), and of plants supertransformed with *Nin88::Pill-ScSuc2* (C–F, plants Nt129-2-5, Nt129-2-28, Nt129-2-30, and Nt129-2-31). Bars=100 µm.

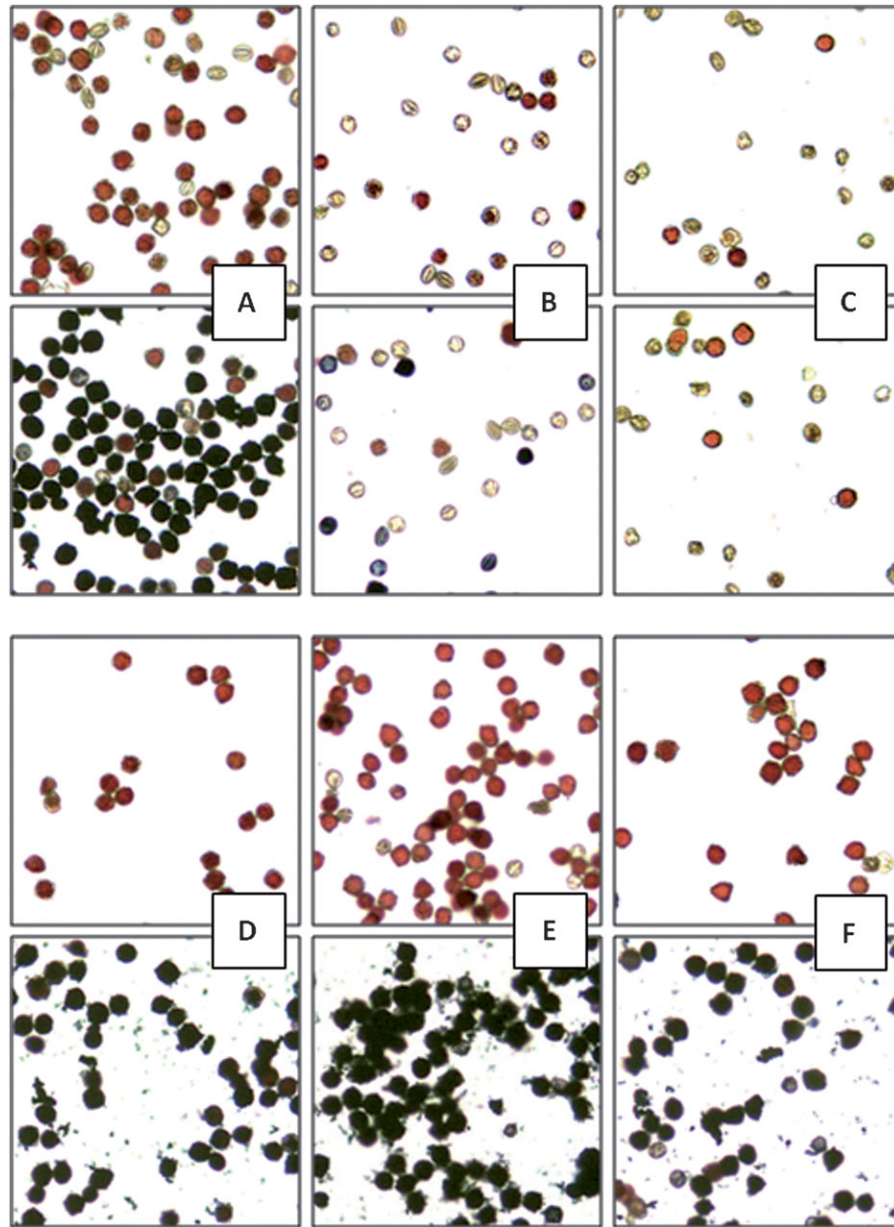


**Fig. 4.** Pollen germination efficiencies (calculated as the relative part of germinating pollen in the visible pollen population) of wild-type tobacco SNN, male-sterile plants 102K, 102KK (*Nin88::Nin88-antisense*), and 105L (*Nin88::NtCIF*), as well as their supertransformed (*Nin88::Pill-ScSuc2*) descendants.

(Fig. 5B) or *Nin88::NtCIF* (Fig. 5C) largely failed to become stained. Even in the control, where sucrose as the substrate is omitted, pollen of male-sterile plants do not show the reddish background staining normally observed in fertile pollen. This verifies the degradation of the pollen in the male-sterile plants previously detected by acetocarmine staining (Hirsche et al., 2009). However, when supertransformed with the yeast invertase (*Nin88::Pill-ScSuc2*), the staining in the control (Fig. 5D–F, upper row) as well as in the incubation medium (Fig. 5D–F, lower row) resembles that of the wild type. Thus, invertase activity is restored to the level of the wild type through tissue-specific expression of the heterologous invertase, reflecting the normal development of pollen and the ability for normal pollen germination efficiencies observed in these genotypes (see above).

*Nicotiana tabacum* has a similar number of genes in the acid invertase gene family to *Arabidopsis*

So far the investigations of anther-specific cwINVs have focused on the model plants *N. tabacum*, *S. lycopersicum*, and *A. thaliana* (Goetz et al., 2001; Proels et al., 2006; Hirsche et al., 2009). However, candidates for invertases with an impact on pollen development are known from other dicotyledonous as well as from monocotyledonous plants (see Introduction). A phylogenetic analysis of these genes within the invertase gene family was done with the aim to provide insights into whether the cwINVs with anther-specific expression are closely related to each other, or if there is some type of co-evolution in the different plant species. This knowledge will provide an impact on future



**Fig. 5.** *In situ* stains of invertase activity of wild-type tobacco, male-sterile plants, and supertransformed plants. Upper rows, controls (– sucrose); lower rows, dark staining indicates sucrose cleavage. (A) Wild type SNN; (B) male-sterile plant 102K, expressing *Nin88::Nin88-antisense*; (C) male-sterile plant 105L, expressing *Nin88::Nin88-NtCIF*; (D) Nt129-2-14 (antisense plant 102K, supertransformed with *Nin88::PillScSuc2*); (E) Nt135-1-15 (antisense plant 102KK, supertransformed with *Nin88::PillScSuc2*); (F) Nt136-1-54 (inhibitor plant 105L, supertransformed with *Nin88::PillScSuc2*).

applications of the described transgenic approach to produce male sterility in other species.

Therefore, it was decided to extend knowledge about the acid invertase family from tobacco in order to broaden the basis for the phylogenetic analysis. This basis is provided by the known invertase gene families from *Arabidopsis* and rice.

In the genome of *A. thaliana* six putative cwINV genes have been identified. Two of them, originally referred to as *Atβfruct1* and *Atβfruct2* (Tymowska-Lalanne and Kreis, 1998), were renamed *AtcwINV1* and *AtcwINV2*, respectively by Sherson *et al.* (2003). Later, *AtcwINV3* and

*AtcwINV6* with lower pI values of 5.5 and 4.8 compared with 8.1–9.7 for the remaining cwINVs, turned out to be FEHs that split one terminal fructose unit from a longer fructan chain, instead of sucrose cleavage (de Coninck *et al.*, 2005). It was decided to adapt the nomenclature of the *Arabidopsis* invertases suggested by Sherson *et al.* (2003) for the tobacco invertases, but, in order to accommodate the findings of de Coninck *et al.* (2005), FEH<sup>?</sup> was added after the name of such tobacco sequences with lower pI values to indicate that these are most probably FEHs. A set of four cwINVs and two cwINVs(FEH<sup>?</sup>) comparable with that in *Arabidopsis* was found in tobacco. Two cwINVs

have previously been described: following the numbering in *Arabidopsis*, *Ntβfruc1* (Greiner et al., 1995, accession no. X81834) would correspond to *NtcwINV1*, and *NtNin88* (Goetz et al., 2001, accession no. AF376773) would correspond to *NtcwINV2*. Four additional sequences have been identified during this study: *NtcwINV3* (accession no. HM022265), *NtcwINV4* (accession no. HM022266), *NtcwINV5-FEH?* (accession no. HM022267), and *NtcwINV6-FEH?* (accession no. HM022268). The latter two most probably have to be considered as FEHs, according to their low pI values (Table 1).

vacINVs have lower pI values of ~5–6, compared with cwINVs with pI values of 8–9 and they can be distinguished from each other 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 possess a valine residue (Goetz and Roitsch, 1999). In *Arabidopsis*, two vacINV genes are known (Haouazine et al., 1997; Tymowska-Lalanne and Kreis, 1998) and have been referred to as *Atβfruct3* and *Atβfruct4* (also by Sherson et al., 2003). Since both, cwINVs and vacINVs are β-fructofuranosidases, and the numbers 3 and 4 in *Arabidopsis* are somewhat misleading because only two vacINVs are present, it is suggested to designate the tobacco invertases genes as *NtvacINV*. A comparable set of two vacINV genes has also been identified by the same approach in tobacco: *NtvacINV1* (previously described as *NtVI*; Lauer, 2006, accession no. AJ305044) and *NtvacINV2* (accession no. HM022269) (Table 1).

#### Phylogenetic relationship of the anther-specific invertases in comparison with related enzymes from different species

The complete invertase gene families (acid and cytoplasmic) from *A. thaliana* and *O. sativa* are the backbone of the phylogenetic analyses, extended with the sequences of the acid invertases from tobacco, as well as related sequences from the well-studied fructan-accumulating species *C. intybus* and *T. aestivum* with FBE and FEH genes. CwINVs with

known anther-specific expression from *S. lycopersicum*, *S. tuberosum*, and *D. carota* are also added.

All anther-specific invertases of dicotyledenous plants cluster within one phylogenetic group, referred to as dicot-A. The anther-specific invertases of monocotyledenous plants cluster within another phylogenetic group, referred to as monocot-A (Fig. 6, marked with arrowheads). Although these clusters are only distantly related to each other, both consist of cwINVs only, some of them with other expression profiles; however, no other enzymes investigated are located within these clusters.

The remaining cwINVs of dicotyledenous and monocotyledenous plants group in two different clusters each, together with FEHs (Fig. 6, dicot-B and -C, monocot-B and -C). In the case of the monocot-B and -C clusters, all enzymes have low pI values, thus it is debatable whether the enzymes described as cwINVs are in fact FEHs (this problem is indicated by *cwINVs?* in Fig. 6).

The vacINVs cluster together with FBEs in the two specific clusters dicot-D and monocot-D.

Finally, the cytoplasmic INVs (cytINVs) of *Arabidopsis* and rice form a common cluster that shows only a distant relationship to the sequences mentioned above and thus this cluster was designated *cytINVs* (monocot+dicot) (Fig. 6).

## Discussion

### The phylogenetic classification of invertases to related genes in different species

In plant cells, invertases (β-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 ~5.0 and their amino acid sequences are more closely related to each other than to that of cytINV (alkaline/neutral, optimum pH between 6.5 and 8.0), whose origin is believed to be in cyanobacteria (Sturm, 1999; Sturm and Tang, 1999; Vargas et al., 2003). This distinct evolutionary origin is clearly

**Table 1.** The acid invertase gene family from *Nicotiana tabacum*

Suggested nomenclature	GenBank accession no.	pI (calculated from the region amplified)	Highest homology	
			% homology	Gene
NtcwINV1	Identical to <i>Ntβfruc1</i> <sup>a</sup>	–	–	<i>Ntβfruc1</i> <sup>a</sup> (X81834) pI 9.1
NtcwINV2	Identical to <i>Nin88</i> <sup>b</sup>	9.1	100 %	<i>Nin88</i> <sup>b</sup> (AF376773) pI 8.4
NtcwINV3	HM022265	9.3	100 %	<i>Nin77</i> <sup>c</sup> pI 9.3
NtcwINV4	HM022266	8.9	98 %	<i>CIN1</i> (X81792) pI 9.1
NtcwINV5 FEH?	HM022267	6.2	60 %	<i>GmCWINV</i> (CAD91338) pI 8.7
NtcwINV6 FEH?	HM022268	4.6	–	–
NtvacINV1	Identical to <i>Nt-VI</i> <sup>d</sup>	5.0	100 %	<i>Nt-VI</i> <sup>d</sup> (AJ305044) pI 5.8
NtvacINV2	HM022269	5.4	86 %	<i>Lin9</i> (AM050394) pI 6.7

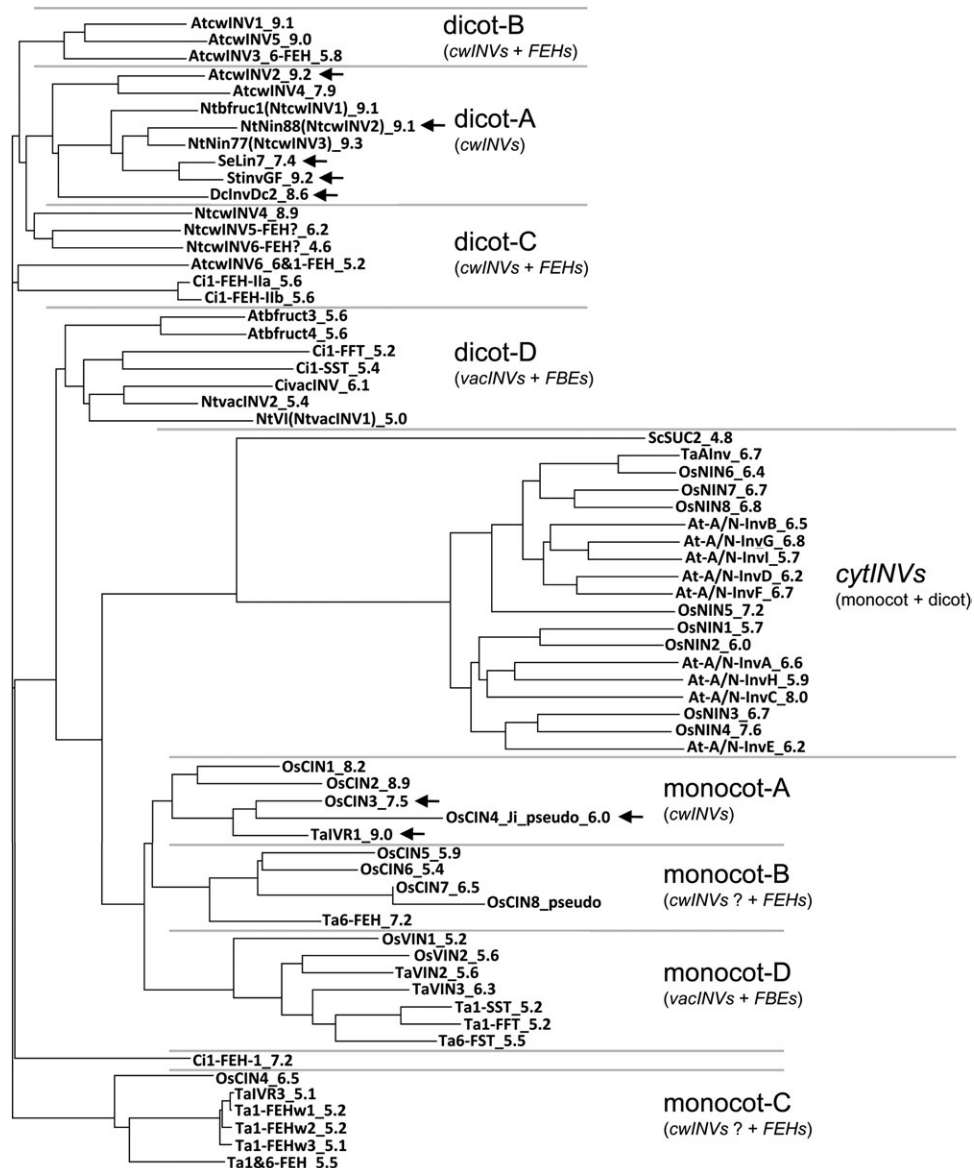
<sup>a</sup> Previously identified by Greiner et al. (1995) (accession no. X81834).

<sup>b</sup> Previously identified by Goetz et al. (2001) (accession no. AF376773).

<sup>c</sup> Previously identified by D Godt; sequence not available in databases.

<sup>d</sup> Previously identified by Lauer (2006) (accession no. AJ305044).





**Fig. 6.** Phylogenetic tree showing the evolutionary relationships among invertases, fructan exohydrolases, and fructosyltransferases. Each node with descendants represents the most recent common ancestor of the invertases, and the branch lengths are proportional to the amount of inferred evolutionary change and thus correspond to time estimates. Arrowheads indicate anther-specifically expressed cell wall-bound invertases. *CwINVs*, cell wall-bound invertases; *vacINVs*, vacuolar invertases; *cytINVs*, cytoplasmic invertases; *FEHs*, fructan exohydrolases; *FBEs*, fructan-building enzymes. For abbreviations and references for the single coding sequences of the different species see Materials and methods; the number at the end of the abbreviation indicates the pI value of the corresponding protein.

visible in Fig. 6, where *cytINVs* from monocotyledons and dicotyledons build a common cluster apart from acid invertases. While *cytINVs* were thought for a long time to be exclusively localized in the cytosol, 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 *cwINVs* 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 FEHs that split one terminal fructose unit from a longer fructan chain, instead of carrying out sucrose cleavage (de Coninck *et al.*, 2005). They possess lower pI

values of 5.5 and 4.8 compared with 8.1–9.7 for the remaining *cwINVs*. Comparable results were found in the present study by analysing the tobacco sequences: among the group of six *cwINVs* in tobacco, two possess lower pI values and most probably have to be considered as FEHs: NtcwINV5-FEH? and NtcwINV6-FEH?. As in *A. thaliana* and as predicted for *N. tabacum*, other species such as sugar beet (*Beta vulgaris*) also 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 of bacterial origin (van den Ende *et al.*, 2003).

In contrast to non-fructan plants, ~15% of flowering plants use fructans as reserve carbohydrates. Fructan-accumulating species mainly belong to the dicot families Asteraceae, Campanulaceae, and Boraginaceae [with (2,1)-type fructans, referred to as inulin] and the monocot families Poaceae and Liliaceae [with predominantly (2,6)-type fructans, referred to as levans, as in bacteria] (reviewed in van den Ende *et al.*, 2004). The close relationship between the functions of the genes encoding 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 an FEH function. Both types of enzymes, cwINVs and FEHs, partly do not cluster in different branches of the phylogenetic tree, indicating that they evolved from each other rather than from different ancestors.

The cwINVs and FEHs from monocotyl plants form clusters, which can be distinguished from the clusters from dicotyledenous plants, a further hint that FEHs evolved independently from each other in both groups of plants. The rice cwINVs form one main cluster that is separated into two subclusters monocot-A and monocot-B (Fig. 6). Both groups have been previously distinguished (Ji *et al.*, 2005, designated as  $\alpha$  and  $\beta$ ). Interestingly, only the sequences in monocot-A possess pI values of 7.5–9.0, while the remaining rice sequences of the neighbouring monocot-B cluster have pI values between 5.9 and 6.5. The low pI values might be a hint that these latter genes encode FEHs rather than cwINVs. Indeed, these sequences show high similarities to *Ta6-FEH* from wheat. *OsCIN4* was located in the  $\beta$ -group by Ji *et al.* (2005; designated as *OsCIN5*, cf. Materials and methods); however, in the present broader phylogenetic analysis, this gene groups in the cluster monocot-C together with *TaIVR3* from wheat. *TaIVR3* (originally described as cell wall invertase in Koonjul *et al.*, 2004) corresponds to *Tal-FEHw1* (Zhang *et al.*, 2008) and thus turned out to be an FEH gene, as all the remaining genes of this cluster in Fig. 6 are FEH genes from wheat (Zhang *et al.*, 2008). The calculated pI for the related *OsCIN4* is 6.5, a hint that this might also be a FEH rather than an invertase. Though the well characterized FEHs clearly evolved from cwINVs in both monocotyledenous and dicotyledenous plants, recently high FEH activities were also described for both rice vacINVs, especially for *OsVIN1* (Ji *et al.*, 2007).

The vacINVs from both *Arabidopsis* and tobacco are characterized by lower pI values of ~5–6 and a single amino acid difference in their cysteine catalytic sites (WECV-DF, see above), and together compose the cluster dicot-D (Fig. 6). Interestingly, the same number of vac-INVs was described in the monocotyledenous species rice (*OsVIN1* and *OsVIN2*; Ji *et al.*, 2005). Thus the set of vacINVs seems to be somewhat conserved in monocots and dicots, though the sequences do not group in the same cluster. Closely related to vacINVs are FBEs (fructosyltransferases) which are involved in the biosynthesis of fructan in fructan-accumulating plants, whereas these genes are lacking in non-fructan plants such as *Arabidopsis* and rice. The

corresponding enzymes from *C. intybus* and *T. aestivum* were integrated in the phylogenetic analyses, and, as expected, they group together with vacINVs in the clusters dicot-D and monocot-D, respectively (Fig. 6). The evolutionary steps from vacINVs to fructosyltransferases occurred independently in different species. The initial step started with water in a vacINV ancestor as the fructosyl group acceptor was replaced by a second sucrose molecule, resulting in sucrose:sucrose-fructosyltransferases 1-SST (Vijn *et al.*, 1998; Ritsema *et al.*, 2006; Ji *et al.*, 2007). In further evolution, there were changes in the donor substrate and/or the acceptor substrate to give the remaining forms of fructosyltransferases.

#### *Relationship of anther-specific cwINV genes and consequences for identifying corresponding genes in other species in order to engineer male sterility*

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 (Nin88; Goetz *et al.*, 2001; Hirsche *et al.*, 2009), and tomato (ScLin7; Godt and Roitsch, 1997; Fridman and Zamir, 2003; Proels *et al.*, 2006). Interestingly, all these anther-specific cwINVs from dicotyledenous plants cluster in the distinct group dicot-A in the phylogenetic tree (Fig. 6). Within this cluster, additional cwINVs with other expression profiles are found, but no FEHs, which group together with the remaining cwINVs in the additional clusters dicot-B and -C.

In monocotyledenous plants most investigations of the involvement of invertases in pollen development have been done in wheat and rice, considering abiotic stresses (drought and cold). Although vacINV and cwINV activities are involved in pollen abortion due to drought stress in wheat, the most pollen- and tapetum-specific expression profile was evident for the cell wall-bound *TaIVR1* (Koonjul *et al.*, 2004). Comparable results were found in rice under drought stress (Saini, 1997) as well as under cold stress (Oliver *et al.*, 2005). Cho *et al.* (2005) suggested that *OsCIN3* is the most important cwINV gene for pollen development in rice, comparable with *Nin88* in tobacco, since all the other cwINV genes are not specifically expressed in the flower. These findings were supported by further investigations showing the specific expression profile of *OsCIN3* in tapetum and pollen and a distinct cold stress reaction (Oliver *et al.*, 2005; designated as *OsINV4*). Furthermore *OsCIN3* and *TaIVR1* show a close relationship to each other (Fig. 6, cluster monocot-A). Within this cluster of the monocotyledenous species two further cwINV genes (*OsCIN1* and *OsCIN2*) are located, with their transcripts all having the characteristic high pI values. In contrast to the findings of Oliver *et al.* (2005) and Cho *et al.* (2005), Ji *et al.* (2005) also detected transcripts of *OsCIN3* in other tissues by RT-PCR; nevertheless the highest transcript level was also found in the panicle. Apart from *OsCIN3*, Ji *et al.*

(2005) found a flower-specific expression for the truncated cwINV gene *OsCIN4(Ji)\_pseudo* (designated as *OsCIN4* in Ji *et al.*, 2005). The coding sequence of this truncated invertase shows a close relationship to *OsCIN3* and *TaIVRI*, all grouping in cluster monocot-A, which can be clearly distinguished from the cluster with the anther-specific cwINVs of dicotyl plants (Fig. 6).

Summing up the above-mentioned findings on the phylogenetic relationships of anther-specific cwINVs, the most important is the fact that sequences of monocotyl and dicotyl plants fall in one specific cluster each, monocot-A and dicot-A, respectively, but these clusters can be clearly distinguished from each other and both contain further cwINV genes with different expression profiles. Thus, in both groups of plants, there is some kind of co-evolution, but no recent common ancestor of anther-specific cwINVs. This knowledge of the relationship might be helpful for identifying corresponding genes from other species by sequence alignment and thus for the application of the male sterility system by anther-specific interference with cwINV activity. It should be mentioned that in spite of the close relationship among the anther-specific cwINVs within both clusters, a promoter compatibility of these invertases cannot necessarily be assumed for distantly related species, as previous studies revealed for *Arabidopsis* and tobacco (Hirsche *et al.*, 2009).

#### *Development of a restorer system for metabolically engineered male sterility*

For the practical application of engineered male sterility in a hybrid breeding programme, the F<sub>1</sub> hybrid varieties in the farmer's field usually have to be fertile to produce the required grain or seed. In naturally occurring CMS systems, this restoration of fertility is achieved by introducing a restorer gene from the paternal parent to the hybrid. By far most restorer genes suppress male-sterile phenotypes by alterations of transcript patterns of the mitochondrial mutations causing CMS, thereby decreasing the expression of the related proteins, and they belong to the pentatricopeptide repeat-containing gene family (PPR), as in petunia [*Rf* of petunia (Bentolila *et al.*, 2002); *Rfo* of Ogura radish (Brown *et al.*, 2003); *Rfk1* of Kosenia radish (Koizuka *et al.*, 2003); *Rf-1* in BT rice (Kazama and Toriyama, 2003; Komori *et al.*, 2004)]. Although various successful approaches to engineer male sterility by transformation of the nucleus or the plastids have been described, most of them fail to provide an effective means of restoration, comparable with the natural restorer genes of CMS (reviewed in Roitsch and Engelke, 2006). Thus, the only system that is already in practical use is the barstar–barnase system. Mariani *et al.* (1990) have interfered with tapetal development using the 5' region of a tobacco tapetum-specific gene (TA29) to drive expression of recombinant RNase genes (RNase T1 and barnase) within the tapetal cells of transgenic tobacco and oilseed rape plants, thus preventing pollen formation. To produce a fertile hybrid for sale to farmers, the male-sterile line is grown alongside

a second line containing the barstar gene which codes for a protein that blocks the action of barnase. This system was used by PlantGeneticSystems (Ghent, Belgium) for oilseed rape that is grown commercially mainly in Canada.

Here a restorer system is presented for the previously described engineered male sterility system, using anther-specific interference with extracellular invertase activity. The applicability of this system has been shown for tobacco, tomato, and *Arabidopsis* (Goetz *et al.*, 2001; Proels *et al.*, 2006; Hirsche *et al.*, 2009), and the crucial function of cwINVs in providing carbohydrates for male gametophyte development and the specific involvement of invertases during anther development was also suggested for other dicotyledonous and monocotyledonous plant species (see above). Restoration of fertility is now successfully achieved by substituting the down-regulated natural plant invertase activity for a yeast invertase under the control of the same promoter. The yeast invertase is fused to the N-terminal portion of potato-derived vacuolar protein proteinase II (*PiII-ScSuc2*), leading to its apoplastic location. The bypassing of the anther-specific reduced carbohydrate supply results in a drastic increase of pollen germination rates; the best phenotypes in the present investigation showed pollen germination efficiencies comparable with those of untransformed wild types. Moreover, the transgene does not affect pollen germination rates when transformed to wild types, thus such plants can be used as paternal parents in a hybrid breeding programme in order to introgress the yeast invertase leading to restoration of fertility in the hybrid variety.

Apart from difficulties concerning the restoration of the hybrid, further difficulties in propagating the male-sterile lines seriously limit the practical application of many transgenic male sterilities. The challenge of propagating male-sterile plants was elegantly solved by Ruiz and Daniell (2005) by imitating the naturally occurring CMS systems. In many (but not all) plants, during formation of pollen, plastids are excluded or degraded so pollen does not contain plastid DNA which is inherited maternally through the ovum. Since transformation of plastids was successful at least for some species, engineering CMS via the chloroplast genome was suggested: a  $\beta$ -keto-thiolase gene was introduced and hyperexpressed in chloroplasts, leading to male sterility by depleting a substrate needed for fatty acid synthesis essential for pollen production (Ruiz and Daniell, 2005). This male sterility was reversible by prolonged light exposure, a mechanism which can theoretically be used for restoration of fertility in the hybrid; however, this approach seems not to be feasible under field conditions and limits the practical use of this system.

The male-sterile line can also be reproduced and maintained by selection of male-sterile plants from a segregating offspring, as is done with the above-mentioned barstar–barnase system: linkage of the barnase gene with the bar (*Streptomyces hygroscopicus*) marker gene, which encodes a phosphinothricin acetyltransferase enzyme that inactivates glufosinate, permits identification of the male-sterile line before flowering. Another possibility would be a reversible

suppression of the sterility. Since induction, especially under field conditions, is always leaky to some extent, an induction of the sterility-inducing gene, in this case the plant invertase antisense or invertase inhibitor, would lead to pollen production to some extent and the hybrids would be wasted with sibs. On the other hand, this leakiness is a minor problem when the gene bringing back the fertility, in the present case the yeast invertase, is connected to an inducible promoter. For propagating the male-sterile line it does not matter whether pollen production is complete or not, as long as pollination is ensured. This offers a way for propagation of the male-sterile line, as an alternative to the linkage to herbicide tolerance.

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