Running Head Sugars and invertases in tobacco pollination

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Metabolic control of tobacco pollination by sugars and invertases

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Summary Invertases and hexose transporters, as essential components of carbohydrate supply, are regulated in a spatiotemporally coordinated manner to maintain functionality of tobacco pollination.

Footnotes:

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ABSTRACT

Pollination in flowering plants is initiated by germination of pollen grains on stigmas followed by ~~the~~ fast growth of pollen tubes representing highly energy-consuming processes. The symplastic isolation of pollen grains and tubes requires ~~the~~ import of sucrose available in the apoplast. We show that the functional coupling of sucrose cleavage by ~~extracellular~~ invertases and uptake of the ~~resulting~~released hexoses by monosaccharide transporters are critical for pollination in tobacco. ~~The spatiotemporally coordinated e~~Transcript profiling, *in situ* hybridization and immunolocalization ~~xpression~~ of extracellular invertases and two monosaccharide transporters *in vitro* and *in vivo* support the functional coupling in supplying carbohydrates for pollen germination and tube growth evidenced by spatiotemporally coordinated expression. ~~The requirement for carbohydrate supply during pollination is further supported by~~ Detection of vacuolar invertases~~-~~ ~~activity~~ in ~~the~~ maternal tissues~~,~~ by these approaches revealed~~ing a~~ metabolic cross-talk between male and female tissues and supported the requirement for carbohydrate supply in different tissues during pollination. ~~Reduction of extracellular invertase activity by t~~Tissue-specific expression of an invertase inhibitor ~~or~~ and addition of the chemical invertase inhibitor miglitol strongly reduced extracellular invertase activity and impair~~s~~ed pollen germination~~, highlighting the requirement of extracellular sucrose cleavage by invertases~~. ~~Sugar uptake m~~Measurements of (competitive) uptake of labelled sugars identified two import pathways for exogenously available sucrose into the germinating pollen operating in parallel: direct sucrose uptake and via the hexoses after cleavage by extracellular invertase~~ and direct sucrose uptake~~. Reduction of extracellular invertase activity in pollen decreases sucrose uptake and severely compromises pollen germination. We further demonstrate that glucose as sole carbon source is sufficient for pollen germination, whereas sucrose is ~~required~~ supporting~~as metabolic signal to sustain~~ tube growth, revealing an important regulatory role of both the invertase substrate and products contributing to ~~the~~ a potential metabolic and signaling-based multilayer regulation of pollination by carbohydrates.

MAIN TEXT

INTRODUCTION

Fertilization in flowering plants is the result of a series of complex and stringently regulated events that are initiated when the pollen grain is received by the stigma of a pistil. In the case of a compatible interaction, the pollen grain hydrates and germinates to initiate growth of a pollen tube. Pollen tubes consist of a single, large, vegetative cell carrying two sperm cells in ~~its tip~~the leading segment of the pollen tube. The growing pollen tube penetrates the stigmatic cell layers and elongates through the transmitting tissue of the style towards the ovary. Upon reaching an ovule, each pollen tube releases its two reproductive cells. One sperm cell fuses with the egg cell to form the embryo while the second sperm cell fuses with the central cell to form the endosperm (Heslop-Harrison, 1987; Taylor and Hepler, 1997; Lord and Russell, 2002).

Pollen germination ~~(PG)~~ and pollen tube growth are critical processes during plant reproduction. The initial pollen germination ~~PG~~ is under complex control with the major regulator being Rop, a GTPase from the Rho family (Fu and Yang, 2001) that also regulates the Ca^{2+}-dependent pollen tube growth and actin cytoskeleton organization (Zheng and Yang, 2000; Fu et al., 2001). Pollen tube growth, as a critical step in fertilization underlies a highly complex multilayer regulation (Qu et al., 2015). The speed of pollen hydration and pollen tube germination is variable and can occur within minutes or may take up to 1 hour, depending on the degree of pollen desiccation. The pollen tube is the fastest growing plant cell with growth rates of up to 1 cm h^{-1} (Barnabas and Fridvalszky, 1984; Jahnen et al., 1989). Growth of pollen tubes occurs via tip growth, ~~where~~ with new membranes and cell wall materials ~~are produced~~deposited at the ~~front~~ tip of the tubes. Pollen tube growth is an extremely energy-~~-~~consuming process~~,~~ that is initially fueled by mobilization of storage material of the pollen grain followed by the use of components present in the transmitting tissue of the style (Heslop-Harrison, 1987; Mascarenhas, 1993). The major energy- and carbohydrate-~~-~~consuming processes during pollen tube growth are the synthesis of cell wall material and their transport to the pollen tube tip (Schlüpmann et al., 1994; Derksen et al., 1995). The main components of the pollen tube wall are callose (Heslop-Harrison, 1987), cellulose (Engels, 1974; Engels and Kreger, 1974), pectic compounds (Nakamura and Yoshida, 1980) and monosaccharides, mainly glucose (Glu) (Van der Woude et al., 1971; Li and Linskens, 1983; Rae et al., 1985). Experiments using ^{14}C-sucrose (Suc) as carbohydrate source for growing pollen tubes resulted in the incorporation of labeled Glu, arabinose, galactose and minor amounts of other sugars into pollen tube walls of *Tradescantia paludosa*

(Mascarenhas, 1970). The transport of these carbohydrates to the growing tip region of the pollen tubes, where they are used for polysaccharide synthesis, is facilitated by cytoplasmic streaming, generated by an actinomyosin system (Mascarenhas, 1993). Additional material for pollen tube growth is supplied by cells of the transmitting tissue, that secret large amounts of free sugars, polysaccharides, glycoproteins, free amino acids and phenolic compounds into the extracellular matrix which are assumed to sustain these energy dependent processes (Konar and Linskens, 1966; Loewus and Labarca, 1973; Cheung, 1996). In particular, imported soluble sugars can be converted to cell wall material of the pollen tubes and used as an energy source (Mascarenhas, 1993; Derksen et al., 1995).

Pollen and pollen tubes are symplastically isolated cells. Thus, carbohydrates required to sustain germination and growth have to be imported from the surrounding apoplast (Reinders, 2016). In an apoplasmic unloading pathway, Suc is released from the sieve elements of the phloem into the apoplast via a Suc transporter. An extracellular invertase, ionically bound to the cell wall (cwINV), irreversibly hydrolyses the transport sugar Suc into hexose monomers that are taken up into the sink cell by high-affinity hexose transporters. These key reactions create a localized concentration gradient to promote phloem unloading via an apoplastic pathway and to increase the sink strength of the corresponding tissue (Roitsch and González, 2004). Therefore, cwINVs and hexose transporters are likely to be involved in the supply of growing pollen tubes with carbohydrates for metabolism (Roitsch and Tanner, 1996; Reinders, 2016). Invertases are encoded by small gene families that show a highly differential sink tissue-specific expression pattern (Lorenz et al., 1995; Godt and Roitsch, 1997; Tymowska-Lalanne and Kreis, 1998; Maddison et al., 1999; Roitsch and González, 2004). The importance of cwINVs for assimilate partitioning in plant cells has been shown ~~in recent years~~ by causal analytical approaches (Sturm, 1999; Goetz et al., 2001; Balibrea et al., 2004), and the functional coupling with hexose transporters is supported by a coordinated regulation (Ehness and Roitsch, 1997).

Tissue-specific overexpression of *NtcwINV* antisense-constructs or invertase inhibitors under control of the *NtcwINV2* (*Nin88*) promoter resulted in male sterile tobacco plants (Goetz et al., 2001; Hirsche et al., 2009; Engelke et al., 2010). Similar approaches to interfere with anther-specific cwINV function in Arabidopsis and rapeseed resulted also in male sterile plants (Hirsche et al., 2009; Engelke et al., 2011), which underlines the essential role of cwINV activity for functional pollen activity and fertilization. In tobacco, the regulation of carbohydrate metabolism during pollen germination~~PG~~ and tube growth has previously been specifically associated to the function of *NtcwINV2* (*Nin88*), which exhibits anther-specific

expression (Goetz et al., 2001). However, this specific NtcwINV isoform has recently been identified to be enzymatically inactive (Le Roy et al., 2013). Nevertheless, a regulatory function of NtcwINV2 (Nin88) in interaction with the very homologous active NtcwINV1 (Greiner et al., 1995) which potentially involves also proteinogenous invertase inhibitors such as NtCIF (Greiner et al., 1998) has been suggested (Le Roy et al., 2013). Additional regulatory NtcwINV2 (Nin88) function has been indicated in association with bZIP transcription factors (Iven et al., 2010). ~~The high homology between the different NtcwINV isoforms can explain the functionality of *NtcwINV2 (Nin88)* antisense constructs (Goetz et al., 2001), which can be assumed to target not only this specific, but also other (enzymatically active) cwINVs (Le Roy et al., 2013).~~

Summarized/In short, ~~These results clearly emphasize the~~ importance of carbohydrate supply and its complex regulation are indicated as essential parameters during fertilization. Since the symplastically isolated growing pollen tube also depends on the import of carbohydrates, we have addressed the role of pollen-specific cwINVs and monosaccharide transporters as well as pistil-specific invertases for pollen germination~~PG~~ and tube growth. The present study demonstrates a critical function for extracellular cleavage of Suc by invertase during pollination and establishes a vital role of an apoplastic carbohydrate supply via cwINVs for pollination and the metabolic control of the underlying processes.

RESULTS

Tobacco pollen germination and tube growth is inhibited by the chemical invertase inhibitor miglitol

Invertase activity has previously been demonstrated to be increased during pollen tube growth (Hirsche et al., 2009), which supports the hypothesis that pollen switches from an autotrophic metabolism during germination to a heterotrophic growth of the pollen tube. To further substantiate the critical role of invertases during this process *in vitro* pollen germination ~~(PG)~~ assays were performed in the presence of the chemical ~~glucosidase (~~invertase~~)~~ inhibitor miglitol, which has been demonstrated to inhibit tobacco invertases (Schäfer, 2012). Indeed, miglitol showed a strong inhibitory effect on pollen germination ~~PG~~ and pollen tube growth (Fig. 1). ~~PG~~ Pollen germination rates reached more than 50% in the controls lacking~~absence of~~ miglitol after 40 min of incubation, and 70% to 80% after 80 min. In contrast, when the glycosidase inhibitor miglitol was added, only 6% of pollen germinated after 80 min and 35% after 120 min in medium without Suc. The addition of Suc to outcompete the miglitol impact increased pollen germination~~PG~~ to 14% and 55%, respectively, but also clearly could not

fully restore the inhibitory effect of miglitol (Fig. 1A). These findings emphasize the expected competition between miglitol and Suc and thus the specific and efficient inhibitory effect of miglitol on invertase activity and subsequently on pollen germination~~PG~~.

Pollen tube growth was~~behaved~~ affected similarly, but after 100 min, the growth retards when Suc is omitted in the medium (Fig. 1B). The negative effect of miglitol is even more pronounced than on pollen germination~~PG~~: After 120 min of incubation in the presence of miglitol, pollen tubes reach lengths of only about one fifth ~~in the presence of miglitol~~ compared to the length in control medium without the invertase inhibitor (Fig. 1B). This clearly indicates the necessity of both the presence of Suc and of invertase activity to maintain pollen tube growth and supports the hypothesis that the initial autotrophic process of pollen germination switches (rapidly) to a heterotrophic process.

Extracellular invertase activity essentially regulates carbohydrate uptake during pollen germination

Hexose transporters are characterized by K_m values for the invertase reaction products in the micromolar range (Büttner and Sauer, 2000), whereas cwINVs are characterized by much higher K_m values in the millimolar range (Sturm, 1999). Thus cwINV activity is expected to limit the uptake of Suc. Therefore we analyzed pollen from plants overexpressing the invertase inhibitor *NtCIF* (Greiner et al., 1998; accession Y12805) under control of the *NtcwINV2* (*Nin88*) promotor. The *NtcwInv2*:*NtCIF* transgenic lines NT49 and NT51 used for the present study were generated independently of previously reported plants transformed with the same construct (Hirsche et al., 2009; Engelke et al., 2010), which showed strongly reduced cwINV activity in pollen and line-dependent (qualitative) impact on pollen germination~~PG~~. *NtcwINV2*:*NtCIF* pollen should exhibit a reduced uptake of the disaccharide Suc via the hexose monomers after cleavage by invertase. This assumption was verified by the measurement of Glu and Suc uptake into germinating pollen.

Pollen of the *NtcwINV2*:*NtCIF* lines NT49 and NT51 exhibited a trend of lower vitality and in few lines negative impact on development (Table I). Furthermore, pollen of all tested lines revealed a significantly reduced (except NT51-6; $p = 0.0503$) germination efficiency (Table I) as indicated in lines used for previous studies (Hirsche et al., 2009; Engelke et al., 2010). Interestingly, the individual lines covered a wide range of pollen germination~~PG~~ efficiency compared to the more uniformly distributed vitality and development levels. To determine the role of cwINV activity in the regulation of carbohydrate uptake during pollen germination~~PG~~, we compared wild-type pollen to pollen from three

165 selected *NtcwINV2*:*NtCIF* lines representing low (NT51-17), medium (NT51-1) and relatively
166 high (NT51-6) pollen germination~~PG~~ efficiency, respectively.
167 The determination of uptake rates for ^{14}C-Suc showed clear differences between pollen
168 from wild-type and *NtcwINV2*:*NtCIF* plants. While the uptake rate was 5.63 nmol min^{-1}
169 million pollen^{-1} for the wild-type, the rates were significantly lower in pollen from
170 *NtcwINV2*:*NtCIF* plants. The uptake rates ranged from 3.24 nmol min^{-1} million pollen^{-1} for
171 NT51-6 and 1.28 nmol min^{-1} million pollen^{-1} for NT51-1 to 1.17 nmol min^{-1} million pollen^{-1}
172 for NT51-17 (Fig. 2), demonstrating that the Suc uptake rates strongly correlated (correlation
173 coefficient of 0.94) with the germination efficiencies (Table I) indicating the importance of
174 functional invertase activity for pollen germination~~PG~~. Since asymmetrically labeled Suc was
175 used, competition experiments were carried out to verify the assumed cleavage of Suc prior to
176 uptake via monosaccharide transporters. The uptake of ^{14}C-Suc was competitively inhibited
177 only in part by an excess of unlabeled ^{12}C-Glu (Fig. 2), indicating that only a fraction of the
178 disaccharide is cleaved by cwINV prior to the uptake of the cleavage products via
179 monosaccharide transporters, whereas a certain amount of Suc is directly imported . In wild-
180 type pollen only 40% of the uptake was competitively inhibited by an excess of unlabelled
181 Glu, representing the proportion of Suc cleaved by cwINV and sugar uptake via
182 monosaccharide transporters. Thus, 60% of the exogenously available Suc is imported
183 directly by Suc transporters without prior cleavage, indicating that two types of uptake
184 pathways occur simultaneously. This is in agreement with the identification of a Suc
185 transporter that is expressed in anthers and growing pollen tubes of tobacco (Lemoine et al.,
186 1999). In contrast to wild-type pollen, the degree of competition of ^{14}C-Suc uptake by
187 unlabelled Glu was lower in pollen from *NtcwINV2*:*NtCIF* plants. The degree of competitive
188 inhibition of ^{14}C-Suc uptake varied between 37% in NT51-6, 29.9% in NT51-1 and 16.5% in
189 NT51-17. The degree of competitive inhibition of sugar uptake by unlabeled Glu was
190 inversely correlated to the reduction in cwINV activities (Suppl. Fig. S1) and the germination
191 efficiencies (Table I). In pollen with the highest reduction in cwINV activity the fraction of
192 direct uptake of sucrose without cleavage is maximal. Thus the analysis of the degree of
193 competition of the Suc uptake by Glu revealed the relative contribution of a pathway
194 involving extracellular cleavage of Suc in supplying carbohydrates to the germinating pollen
195 versus the direct uptake of Suc.
196 The measurement of the uptake of ^{14}C-Glu into pollen showed that the reduced cwINV
197 activity in pollen from *NtcwINV2*:*NtCIF* plants had no effect on hexose transporter activity in
198 two (NT51-1 and NT51-6) out of the three lines (Fig. 2). The mean uptake rate into pollen

both from wild-type and *NtcwINV2*:*NtCIF* plants was about 3 nmol million pollen^{-1} min^{-1}. In contrast, even Glu uptake was severely impaired in pollen of line NT51-17 (1.11 nmol million pollen^{-1} min^{-1}). This general and extreme limitation of carbohydrate supply could be the explanation for the poor pollen germination~~PG~~ observed for this line, which could be associated with a signaling role of the metabolic invertase activity itself (Albacete et al., 2011) as particularly suggested for a cwINV in maize seed development (Cheng and Chourey, 1999; Ruan, 2014).

The sugar uptake measurements demonstrate that two pathways for import of exogenously supplied Suc operate in parallel: uptake of Suc via the hexose monomers after cleavage by cwINV and direct uptake of Suc. When cwINV is inhibited the potential to import sugars is reduced, causing reduced germination rates, thus demonstrating the requirement for extracellular Suc cleavage despite the presence of a second pathway for Suc uptake.

~~Cloning of anther specific hexose transporters from tobacco~~ Hexose transporters *NtMST2* and *NtMST3* are expressed in germinating pollen and growing pollen tubes

The uptake of extracellular sugars as an important process within pollen germination~~PG~~ and tube growth as described above depends on the function of diverse transporter proteins, which in several plant species have been described to be essential for pollen related processes (Hirose et al., 2010; Cheng et al., 2015; Eom et al., 2015). CwINVs and hexose transporters are functionally linked to successively cleave the transport sugar Suc and import the resulting hexoses into the cells. So far only one hexose transporter from tobacco, NtMST1, has been characterized, which was shown to be predominantly expressed in roots (Sauer and Stadler, 1993). To further verify the critical role of an apoplasmic pathway involving extracellular Suc cleavage and sugar uptake via monosaccharide transporters for pollen tube growth, the hexose transporters that function in this process, NtMST2 and NtMST3, were identified and partial sequences cloned from tobacco anthers. ~~Fragments were amplified by RT-PCR with degenerated primers directed against conserved regions of known hexose transporters (Roitsch and Tanner, 1994). Sequence analysis of the cloned PCR fragments showed that two different hexose transporters, NtMST2 and NtMST3, were cloned from tobacco anthers.~~ The deduced amino acid sequences of both transporters showed typical monosaccharide-transporter sequence motifs (Williams et al., 2000). ~~Nucleotide sequence alignment of *NtMST2* and *NtMST3* revealed 62.1% identity between the two sequences, while the cloned~~

233 region of *NtMST2* shows 60.18% and *NtMST3* 66% identity with *NtMST1*, respectively.
234 Comparison of the obtained *NtMST2* and *NtMST3* sequences to database sequences (NCBI
235 BLAST) identified the highest similarity of *NtMST2* to the predicted *Nicotiana*
236 *tomentosiformis* sugar transport protein 8-like (accession XM_009622914) and of *NtMST3* to
237 the predicted *N. tomentosiformis* sugar transport protein 10-like (accession XM_009616810).
238 Sequence comparison to characterized monosaccharide transporters revealed that *NtMST2*
239 shows the highest similarity to the sugar transporter STP11 from *Solanum lycopersicum*
240 which specifically clustered together with pollen specific Arabidopsis STPs (Reuscher et al.,
241 2014). The *NtMST3* sequence showed the highest similarity to the hexose transporter *Pmt1*
242 from *Petunia hybrida* that is strongly expressed in pollen and is thought to play a role during
243 pollen germinationPG in *P. hybrida* (Ylstra et al., 1998). Thus both transporters show a
244 particular high sequence identity to monosaccharide transporters from other species which are
245 potentially related to pollen function.
246
247 ***NtMST2* and *NtMST3* are co-expressed in germinating pollen and growing pollen tubes**
248 To analyze the tissue specificity of the partially cloned hexose transporters, RNA gel
249 blot analysis was performed using specific cDNA probes for *NtMST2* and *NtMST3*. The RNA
250 gel blots revealed a highly specific expression pattern of both monosaccharide transporters in
251 flower organs (Suppl. Fig. S2A). Whereas *NtMST2* was exclusively expressed in anthers,
252 *NtMST3* was expressed both in anthers and petals. Thus, the two cloned hexose transporters
253 are appropriate candidate monosaccharide transporters to be functionally linked with cwINVs
254 for supplying carbohydrates during pollen development and germination via an apoplasmic
255 pathway.
256 To gain insight into the physiological role of the two cloned anther specific hexose
257 transporters *NtMST2* and *NtMST3* in supplying carbohydrates to germinating pollen tubes, an
258 analysis of their expression levels during *in vitro* germination of wild-type tobacco pollen and
259 *in vitro* pollen tube growth was performed. The analysis by RNA gel blots demonstrated the
260 expression of *NtMST2* and *NtMST3* throughout pollen germinationPG from its initiation at 0 h
261 to 8 h (Suppl. Fig. S2B) indicating their important function to import monosaccharides into
262 the pollen and pollen tube. Additional RNA gel blots revealed concomitant expression of
263 *NtcwINV2* (*Nin88*) in the germinating pollen and growing pollen tubes. Due to the high
264 similarity between *NtcwINV2* (*Nin88*) and other *NtcwINVs*, it can be assumed that this probe
265 not only detects transcripts of this enzymatically inactive, potentially regulatory invertase, but
266 also other (enzymatically active) isoforms (Le Roy et al., 2013). The identified simultaneous

267 ~~co-~~expression of *NtcwINV*s and the two identified *NtMST*s during pollen germination~~PG~~
268 strongly indicate a coordinated process based on the cleavage of Suc, including its regulation,
269 and the import of hexoses.
270
271 **Verification of the expression patterns of tobacco invertases and sugar transporters *in***
272 ***vivo***
273 Since the *in vitro* experiments supported a role of cwINVs for pollen tube growth, the
274 localization of the *cwINV* mRNA and protein was analyzed after pollination *in vivo* by
275 immunocytochemistry and *in situ* hybridization.
276 Stigmas were pollinated with wild-type pollen and a time course of the pollen
277 germination~~PG~~ and ~~pollen~~ tube growth was established by staining the cell walls with aniline
278 blue. This callose staining revealed that the germination of the pollen on the stigma begins 2
279 hours after pollination (Fig. 3A). After 4 hours the pollen tubes were observed in the upper
280 part of the pistil, in the stigma, and they began to enter the transmitting tissue of the style (Fig.
281 3B). 24 hours after pollination, the pollen tubes had reached the basal part of the style by
282 progressing through the peripheral part of the transmitting tissue (Fig. 3C and D).
283 To determine the localization of cwINVs by immunocytochemistry a polyclonal
284 antibody raised against a conserved region of tobacco cwINVs was used (Goetz et al., 2001).
285 Prior to pollination, no immunosignal was detected (Fig. 3E and F), neither in the stigma (St)
286 nor in the transmitting tissue (Tt). 2 hours after pollination, i.e. when the first pollen grains
287 began to germinate, a weak immunosignal was found to be present both in the cells of the
288 stigma and in the pollen grains, whereas no signal was detected in any other part of the style.
289 Thereafter, 4 hours after pollination, a strong invertase immunosignal was observed in the
290 stigma and in the upper part of the style where the pollen tubes are growing (Fig. 3G). No
291 invertase signal was observed at that time in the basal part of the style. After 24 hours,
292 incubation with the NtcwINV2 (Nin88) antibody resulted in a signal in the cells of the female
293 tissue, the cortex and transmitting tissue (Fig. 3H), and in the growing pollen tubes (Fig. 3I,
294 arrowhead), but not any longer in the stigma. In the transmitting tissue (Tt), the signal was
295 only localized in the transversal cell walls (Fig. 3H, arrowheads).
296 To complement the immunohistochemical data, whole-mount *in situ* hybridizations
297 were carried out. The mRNA of *NtcwINV2* (*Nin88*) was only found to be localized in the
298 pollen grains and pollen tubes (Fig. 3J and K). No signal was detected in any cells of the pistil
299 at any time, which is in contrast to the immunolocali~~s~~zation data. In order to also elucidate the
300 *in vivo* function of the two novel hexose transporters *NtMST2* and *NtMST3* after pollination,

additional whole-mount *in situ* hybridizations were carried out. In non-pollinated pistils, no expression of any of the two genes was detected (Fig. 3L). 4 hours after pollination only the presence of the mRNA for *NtMST3* (Fig. 3O) was detected in the growing pollen tubes while no signal was detected for *NtMST2* (Fig. 3M), and 24 hours after pollination, signals for both hexose transporter genes were detected in growing pollen tubes (Fig. 3N and P), with stronger expression of *NtMST3* (Fig. 3P). No signal for the mRNAs for either of the two monosaccharide transporters genes was detected in any cells of the style. The highly specific expression of these two hexose transporter genes in growing pollen tubes shows that these pollen tubes import monosaccharides present in the transmitting tissue of the style.

Identification of additional tobacco invertases involved in pollination

As the *in vivo* analyses of cwINVs using *NtcwINV2* (*Nin88*)-derived probes described above did not identify the invertases present in the maternal tissue, we addressed the function of other known invertases in this tissue. Initially, blot analyses were performed with RNA derived from whole flowers and anthers of different stages, pollen tubes as well as pollinated and non-pollinated styles and probes for the known *NtcwINV1* to *6* and the vacuolar invertases (vacINVs) *NtvacINV1* and *2*, respectively (Fig. 4). The examination of *NtcwINV2 (Nin88)* by RNA gel blot revealed the increase of the steady state level in the growing anther with a maximum level in matured anthers and pollen tubes as was previously described (Goetz et al., 2001; Hirsche et al., 2009). Transcripts in non-pollinated styles are not evident, while in pollinated styles a moderate transcript level was detected. A comparable expression pattern was found for *NtcwINV3* and, with a lower intensity for *NtcwINV1*. Due to the high sequence similarities of these three *NtcwINV*s, unspecific detection of their transcripts by the individual probes has however to be considered. In contrast to the expression patterns of *NtcwINV1* to *3*, no transcripts of *NtcwINV4* and *NtcwINV6* have been detected in the tissues investigated. Interestingly, a highly specific expression of *NtcwINV5* in the style has been identified. Additionally, both the *NtvacINV*s showed expression in non-pollinated and pollinated styles, while particularly *NtvacINV1* transcripts have also been detected in anthers and *NtvacINV2* transcripts in pollen tubes (Fig. 4).

The expression patterns of *NtcwINV5* as well as *NtvacINV1* and *2* thus indicated them as promising candidates for important *in vivo* function during pollination processes which is in agreement with the recently identified role of vacINV in cotton floral organ development and fertility (Wang and Ruan, 2016). To address this in more detail, we performed additional *in situ* hybridizations of pollinated and non-pollinated pistils for these specific invertases (Fig.

5). In agreement with the expression data, *NtcwINV5* was detected in pollinated (24 h post pollination) and non-pollinated pistils with a specific localization to the transmitting tissue (Fig. 5A and B). The presence particularly in the transmitting tissue (Tt) could indicate an important role of this invertase for the external supply of hexoses to the growing pollen tube. The presence of *NtcwINV5* in non-pollinated pistils however indicates a general function in the carbon metabolism of this tissue. In contrast, mRNAs of both *NtvacINV*s were only detected in pollinated (Fig. 5D and F), but not in non-pollinated pistils (Fig. 5C and E) indicating their specific function during pollination. While *NtvacINV1* was specifically localized in the transmitting tissue (Fig. 5D), *NtvacINV2* was detected in the transmitting tissue as well as in the cortex (Fig. 5F). These differences in the localization of the two *NtvacINV*s indicate specific functions of the two isoforms during pollination.

As invertases are also tightly regulated by proteinogenous inhibitors, we complemented the *in situ* analyses by hybridizations for the tobacco invertase inhibitor *NtCIF* (Greiner et al., 1998) to identify potential posttranslational regulation of the detected invertases (Fig. 5G and H). *NtCIF* transcripts were not detected in either non-pollinated or pollinated pistils, indicating that the regulation of invertases during tobacco pollination is not mediated by inhibition of their specific enzymatic activities.

Pollen germination and pollen tube growth are differentially regulated by sugars

There is increasing experimental evidence for a regulatory role of sugars for gene regulation and developmental processes in higher plants. The substrate and products of invertases seem to be of particular importance as metabolic signals in the plant kingdom (Borisjuk et al., 2004; Gibson, 2005; Ruan, 2014). To gain insight into the role of sugars in the metabolic regulation of pollination, *in vitro* tobacco pollen germination~~PG~~ was compared between germination medium containing Suc and Glu, respectively. The germination efficiency in the presence of 10% Glu or 10% Suc was 53.8% and 53.3%, respectively, demonstrating that Glu alone is sufficient as carbon source to induce pollen germination~~PG~~. When Glu was added to the Suc-containing medium, an increase in germination efficiency was observed in a dose-dependent manner. The germination efficiency increased to 72% in the presence of 0.01% Glu and to 81.9% with 0.14% Glu, revealing that pollen germination ~~PG~~ was stimulated by Glu. Thus, when invertase activity was inhibited, the low amount of Glu available to support germination led to a reduced pollen germination~~PG~~, which is in agreement with the analysis of pollen from *NtcwINV2*:*NtCIF* plants (Table I).

Although Glu was sufficient for initial pollen germination~~PG~~, it did not support subsequent pollen tube growth. Pollen were germinated in the presence of Glu, Suc and other sugars individually or in combinations, and the length of individual pollen tubes were determined after 2.5 hours (Fig. 6; Suppl. Fig. S3). The size distribution of pollen tubes in the presence of 10% Glu showed that about 80% and thus a vast majority of the pollen tubes did not exceed 100 µm (Fig. 6A). In contrast, in medium containing 10% Suc, 80% of pollen tubes were longer than 100 µm and even reached maximal lengths of more than 1000 µm (Fig. 6B), resulting in a significantly increased average pollen tube length (Fig. 6E). Furthermore the addition of Suc in the range of 0.05% to 2% to the 10% Glu-containing medium was sufficient to cause a shift to longer pollen tubes. The effect of such small amounts of ~~This suggests that~~ Suc could indicate a potential function ~~is required~~ as developmental signal in addition to its function as nutrient during pollen tube growth (Suppl. Fig. S3A and B). The potential regulatory role of disaccharides was further verified by analyzing the effect of turanose (Tur) and palatinose (Pal). Tur (3-O-d-glucopyranosyl-Fru) and Pal (isomaltulose, 6-O-d-glucopyranosyl-Fru) are structural isomers of Suc composed of Glu and fructose with a different glycosidic linkage. They are not synthesized in higher plants and cannot be cleaved or transported by plant enzymes. Evidence for extracellular, disaccharide-specific sugar sensing has been obtained using these non-metabolizable Suc-isomers (Loreti et al., 2000; Fernie et al., 2001; Sinha et al., 2002). Supplementing the 10% Glu-containing medium with 0.1% Pal was sufficient to promote elongation of the majority of the pollen tubes (Fig. 6C) and thus causing a significantly increased average pollen tube length (Fig. 6E). Pal concentrations between 2% and 0.05% were sufficient to elicit the shift in size distribution compared to the medium only containing 10% Glu (Suppl. Fig. S3C). Also by supplementing the 10% Glu-containing medium with the alternative Suc-isomer Tur a shift in pollen tube length was observed, which however was less pronounced as compared to the addition of Suc or Pal (addition of 2% of the individual disaccharide; Suppl. Fig. S3A). These data resulting from the addition of Pal, Suc and Tur strongly support the ~~requirement for~~function/presence a disaccharide signal to promote the growth of the pollen tubes. ~~To further substantiate that a disaccharide-specific sensing is required for pollen tube growth, the~~This was further substantiated by the effect of mannoheptulose (Mhl) ~~has been tested that~~ which specifically inhibits hexokinase that was shown to be involved in certain hexose-based (particularly Glu) signaling pathways (Moore et al., 2003). The addition of 2.1% (100 mM) Mhl had no significant impact on the stimulatory effect of Pal on pollen tube growth (Fig. 6D and E), supporting that the underlying signaling mechanism is hexokinase-independent and is

403 rather based on disaccharides.

404

405 **DISCUSSION**

406 CwINVs have been shown to play an important role in different aspects of plant growth and
407 development as well as in stress responses by controlling sugar composition, metabolic fluxes
408 and generating metabolic signals (Sturm, 1999; Wobus and Weber, 1999; Roitsch et al., 2003;
409 Roitsch and González, 2004; Koch, 2004; Albacete et al., 2011; Albacete et al., 2014).
410 Previously, an essential role of cwINV activity in male gametophyte development has been
411 functionally demonstrated for tobacco (Goetz et al., 2001), tomato (Proels et al., 1996),
412 Arabidopsis (Hirsche et al., 2009) and oilseed rape (Engelke et al., 2010). Tissue-specific
413 antisense repression of *cwINV*s resulted in an early arrest of pollen development causing male
414 sterility. So far, the role of carbohydrates in relation to invertase function during pollen
415 germination~~PG~~ and tube growth has not been elucidated, although pollen tubes are
416 symplastically isolated and thus need to be supplied with carbohydrates by an apoplasmic
417 pathway. To gain insight into the role of sugars in the spatiotemporal regulation of these
418 linked processes, modulation of invertase activity by different approaches was combined with
419 the analyses of *in vitro* growing pollen tubes, and the *in situ* characterization of pollen
420 germination~~PG~~ on the stigma and pollen tube growth through the style.

421

422 **Extracellular invertase function is essential for pollen germination and pollen tube**
423 **growth**

424 A crucial function of cwINVs in providing carbohydrates for the male gametophyte
425 development was supported by the identification of anther-specific isoenzymes in *Vicia faba*
426 (Weber et al., 1996), lily (Clément et al., 1996), maize (Xu et al., 1996; Kim et al., 2000),
427 tomato (Godt and Roitsch, 1997), potato (Maddison et al., 1999), and tobacco (Goetz et al.,
428 2001). All these *cwINV* genes were induced during anther development, but their expression
429 during pollination has not been studied so far. Pollination is initiated by germination of the
430 pollen on the stigma followed by a rapid growth of the symplastically isolated pollen tube
431 through the transmitting tissue of the style. The present study demonstrates the spatiotemporal
432 and tissue-specific regulation of invertases during pollination.

433 *Various* ~~C~~c*wINV*s were shown to be expressed during *in vivo* pollination as well as
434 during *in vitro* pollen germination~~PG~~ as evidenced by cwINV2 expression. Since ~~the~~
435 *NtcwINV*2 *is* ~~genes are~~ expressed in pollen tubes grown *in vitro* the regulation is solely
436 determined by the growing pollen tube and thus independent from signals derived from the

maternal tissue. Immunolocalization and whole-mount *in situ* hybridization revealed that *NtcwINV*s were detected only at low levels in germinating pollen 2 hours after pollination, whereas after 4 hours a strong signal was observed (Fig. 7). The presence of a 'lag phase' is consistent with the hypothesis that the initial events of pollen germination~~PG~~ are sustained by internal reserves, stored during pollen development, as initial pollen tube growth is autotrophic followed by a switch to heterotrophic growth (Read et al., 1993). When endogenous resources are exhausted, carbohydrates required to sustain pollen tube growth need then to be imported, which can be mediated by cwINVs cleaving Suc into hexoses, which are imported.

The requirement of extracellular cleavage of the transport sugar Suc for pollen germination~~PG~~ is further substantiated by the finding of strongly impaired pollen germination~~PG~~ when cwINV activity was specifically reduced either by the tissue-specific over-expression of a tobacco invertase inhibitor (Weil et al., 1994; Greiner et al., 1998) or alternatively by addition of the chemical invertase inhibitor miglitol (Schäfer, 2012). Expression of the proteinogenous invertase inhibitor *NtCIF* under control of the *NtcwINV2* (*Nin88*) promoter resulted in the generation of fully developed pollen that were characterized by a low cwINV activity and low germination efficiency. These results are distinctly different from the effect of *NtcwINV2* (*Nin88*) antisense repression (Goetz et al., 2001), despite the use of the same promoter. Whereas pollen from Nin88 antisense plants were arrested very early in development before pollen mitosis II, *NtcwINV2*:*NtCIF* pollen development was apparently not as strongly affected and the reduced invertase activity particularly affected pollen germination~~PG~~. The fact that pollen development is not severely affected by *Nt*CIF may be due to high local Suc concentrations protecting cwINVs against the inhibition. This substrate protection has been described for the tobacco invertase inhibitor protein before (Sander et al., 1996; Greiner et al., 1998), thus cwINV activity seems to be less~~may only be slightly~~ affected during *NtcwINV2*:*NtCIF* early pollen development. At later stages of pollen development the depletion of carbohydrates possibly results in a reduced protection of the invertases from the inhibitor resulting in a stronger perturbation of pollen development. Such developmentally disturbed pollen are then also characterized by a low germination rate. ~~The remaining cwINV activity is apparently high enough to support normal development of a large fraction of the pollen. However, during *in vitro* germination assays, the local Suc concentration is lower and substrate protection is not effective any more.~~ The results from *NtcwINV2*:*NtCIF* pollen were complemented and extended~~supported~~ by *in vitro* pollen germination~~PG~~ in the presence of the chemical invertase inhibitor miglitol (Schäfer, 2012), which also resulted in lower

471 germination rates of normally developed pollen. Furthermore, germinated *NtcwINV2*:*NtCIF*
472 pollen as well as wild-type pollen germinated in the presence of miglitol developed
473 significantly shorter pollen tubes, indicating that pollen tube growth is also affected in
474 response to the reduced invertase activity.

475 Sugar uptake measurements demonstrated that two pathways for import of
476 exogenously supplied Suc into the germinating pollen operate in parallel: uptake of Suc via
477 the hexose monomers after cleavage by cwINV and direct uptake of Suc. The reduced cwINV
478 activity in *NtcwINV2*:*NtCIF* pollen was shown to result in a reduced uptake of Suc and
479 subsequently in a severely compromised pollen germination~~PG~~ which demonstrates that
480 cwINV function is critical for pollen germination ~~PG~~ despite the still operational direct Suc
481 uptake. The reduced invertase activity affects the supply of carbohydrates as energy source
482 for pollen germination ~~PG~~ and tube growth, but may also affect the generation of a metabolic
483 signal as it is apparently the case during pollen development (Goetz et al., 2001).

484 The *in vivo* immunolocalization and *in situ* hybridization data of pollinated stigmas
485 revealed additional invertases expressed in the maternal tissue, namely *NtcwINV5* (also in
486 non-pollinated stigmas), *NtvacINV1* and *2* (pistil-INVs), which seem to be involved in
487 supplying carbohydrates to the growing pollen tube. VacINV activities are probably involved
488 in the general carbon supply and movement in the pistil as shown for maternal reproductive
489 tissue in maize (Neumann Andersen et al., 2002). Functional vacINV has also been shown to
490 be crucial for proper and synchronized development of style and other flower organs in cotton
491 to maintain male and female fertilities (Wang and Ruan, 2016). Already very early after the
492 pollen interacts with the stigma, an invertase immunosignal was detected beneath the stigma
493 which was also observed in lower parts of the style as pollen tube growth progresses and *in*
494 *situ* hybridizations for the three indicated pistil-INVs revealed their specific localization in
495 transmitting and/or cortical tissue (Fig. 7). Thus a set of maternal invertase genes participate
496 to sustain the highly ~~-~~energy ~~-~~consuming growth of the pollen tube, which represent a
497 metabolic cooperation between male and female tissues.

498

499 **Functional coupling of extracellular invertases and hexose transporters is reflected by**
500 **tissue- and development-specific co-regulation**

501 CwINVs are functionally linked with hexose transporters for supplying carbohydrates via an
502 apoplasmic pathway into sink cells. Two hexose transporters, *NtMST2* and *NtMST3*, have
503 been identified and found to be highly tissue specifically expressed in flower organs. *In vitro*
504 and *in vivo* expression analysis revealed that both hexose transporters are ~~co-~~expressed

together with *NtcwINVs* in growing pollen tubes in a spatiotemporally similar/coordinated manner, supporting their functional coupling (Fig. 7). Their activity was demonstrated by the measurement of Glu uptake into germinating pollen. Also the pistil-INVs expressed in the maternal tissue may contribute to the generation of hexoses that are subsequently imported by NtMST2 and NtMST3 into the growing pollen tubes indicating their important function in maintaining pollen tube growth similar to STP10 in *Arabidopsis* (Rottmann et al., 2016).

Suc uptake measurements demonstrated that in addition to cleavage and uptake of hexoses Suc is also imported directly into germinating pollen (Fig. 7) which is in agreement with Suc uptake identified in growing pollen tubes of various species such as lily (Deshusses et al., 1981), petunia (Ylstra et al., 1998), *Arabidopsis* (Stadler et al., 1999) and tobacco (Lemoine et al., 1999). The Suc-transporter *NtSUT3* has been shown to be exclusively expressed in anthers, and during pollen germination~~PG~~ and tube growth. In contrast to the hypothesis that Suc is the preferred substrate during initial pollen germination~~PG~~, the present study demonstrates a critical function of invertase cleavage and uptake of Glu via hexose transporters in this phase. Despite the presence of direct Suc uptake, the inhibition of Suc cleavage strongly impairs pollen germination ~~PG~~ and tube growth. Likewise, it has been shown for *Arabidopsis* that both, a Suc-H^+ symporter (Stadler et al., 1999) and monosaccharide-H^+ symporters (Truernit et al., 1999; Schneidereit et al., 2003; Rottmann et al., 2016) are expressed in growing pollen tubes supporting the requirement of both pathways for functional sugar supply.

Differential metabolic regulation of tobacco pollen germination and pollen tube growth by glucose and sucrose

Glu and Suc exhibited distinctly different functions in regulating pollen germination~~PG~~. *In vitro* germination experiments showed that Glu is sufficient as a carbon source for the process of pollen germination ~~PG~~ but Suc was required as metabolic signal for pollen tube growth (Fig. 7). The importance of Glu for pollen germination ~~PG~~ is supported by lower germination rates when cwINV activity and subsequently the generation of hexoses is reduced. Pollen germination ~~PG~~ is a highly energy-consuming process that begins with pollen hydration (Franklin-Tong, 2002). Pollen hydration induces metabolic activities in the pollen grain which become highly polarized after cytoskeleton and cytoplasmic rearrangements, prior to the emergence of the pollen tube. CwINV activity is required to provide Glu as carbon source for these processes. Glu could potentially also acts as signal to initiate pollen germination~~PG~~, since Glu has been identified as a signaling molecule implicated in the control of various plant

developmental processes (Gibson, 2005; Granot et al., 2013; Ruan, 2014; Sheen, 2014).

While Glu ~~alone~~ as sole carbon source is sufficient for pollen germination~~PG~~, tube elongation requires Suc as metabolic signal. Pollen tube growth was already supported by the addition of low concentrations of Suc (0.05 to 2%) to Glu-containing medium, which indicates that Suc may also act as metabolic signal rather than ~~(~~only~~)~~ as carbon source, a function which is indicated for other processes of plant growth and development (Ruan, 2012; Lastdrager et al., 2014). The signaling function of disaccharides in this aspect is supported by similar results of longer pollen tubes after addition of the Suc-isomers Pal or Tur to Glu-containing medium. Both isomers are neither cleaved by invertases nor imported into plant cells (Loreti et al., 2000; Fernie et al., 2001; Sinha et al., 2002) indicating extracellular sensing of this metabolic signal. A disaccharide signaling pathway is further supported by experiments using Mhl as inhibitor of hexokinase-dependent Glu-sensing pathways which did not affect the Pal-supported pollen tube growth. A hexokinase-independent Suc-signaling pathway has also been identified in the regulation of radicle elongation in carrot embryos (Yang et al., 2004). Also *Arabidopsis* pollen tubes were only able to elongate in Suc-containing medium indicating that Suc is required as signal for pollen tube elongation beyond the function as carbon source in other species as well (Stadler et al., 1999). The regulatory role of Suc is further supported by the finding that *Camellia* pollen tube growth is stimulated by oligosaccharides susceptible to invertases (Nakamura et al., 1991).

Metabolic regulation of tobacco pollination by carbohydrates

Our data reveal a highly spatiotemporal and tissue-specific regulation of different *Nt*cw and *Nt*vacINVs as well as monosaccharide transporters *Nt*MST2 and *Nt*MST3 which in combination with results on the Suc transporter *Nt*SUT3 (Lemoine et al., 1999) underpin the complexity to maintain carbohydrate supply and metabolic control of tobacco pollen germination ~~PG~~ and tube growth summarized in Figure 7. After interaction of pollen with a stigma, *Nt*cwINV activity and the cleavage product Glu are critical for germination. When cwINVs are inhibited, pollen germination ~~PG~~ is strongly impaired. The additional invertases expressed in the maternal tissue beneath the stigma (pistil-INVs) are also involved in supplying carbohydrates and/or the generation of the Glu-signal to initiate germination. Pollen tube growth then requires extracellular Suc-sensing as a metabolic signal. The coordinated ~~tightly linked co-~~expression of *NtcwINV*s and the hexose transporters *NtMST2* and *NtMST3 in vitro* and *in vivo* support the importance of Suc cleavage and Glu uptake via an apoplasmic pathway for supplying growing pollen tubes with carbohydrates despite the simultaneously

573 occurring Suc uptake. The deduced regulatory mechanism indicates a highly fine-tuned
574 multilayer regulation of metabolic and transport processes during the interaction of the male
575 gametophyte and female sporophyte during the events preceding fertilization. Although pollen
576 tubes are able to grow *in vitro*, a metabolic interaction exists between pollen tube and the style
577 *in vivo* as demonstrated by the presence of pistil-INVs in the cortical as well as the
578 transmitting tissue of the style. Strikingly, the presence of the invertases in the maternal tissue
579 was not constant during pollination and pollen tube progression but was tightly coordinated
580 with the tip zone where pollen tube growth occurs. The pistil-INVs can generate hexoses from
581 Suc present in the female tissue which serve as carbon source for the growing pollen tube, but
582 also as carbohydrate signal molecules potentially involved in pollen tube guidance similar to
583 Glu function in pearl millet (Reger et al., 1992). This suggests a metabolic crosstalk between
584 pollen tubes and the cells of the transmitting tissue. Alternatively the generated
585 monosaccharides could serve as substrates for the generation of glycoproteins and extensions
586 that have been implicated to be involved in pollen tube guidance (Higashiyama et al., 2003).
587 An additional role of maternally expressed invertases could be related to sporophyte self-
588 incompatibility by regulating the availability of sugars both as nutrients, metabolic signals and
589 substrates for the glycoproteins that were shown to be involved (Cruz et al., 2005).
590 The present study provides further evidence for the close relation between
591 carbohydrate metabolism and sugar signaling as basis for a multilayer regulation of
592 developmental and growth processes in general and pollination in particular.
593
594 **MATERIAL AND METHODS**
595 **Plant growth conditions**
596 *Nicotiana tabacum* Samsun NN wild-type plants and *NtcwINV2:NtCIF* lines, which have
597 been generated according to Goetz et al. (2001), were grown on standard potting soil under
598 greenhouse conditions at 20°C to 24°C and a 16-h/8-h day/night cycle by supplemented
599 lighting (approx. 100 µmol m^{-2} s^{-1}). Plants were fertilized weekly (20+5+10+(2) composition)
600 with 'Hakaphos® grün' (COMPO Expert GmbH, Münster, Germany). Soil was treated with
601 Agritox® (Kwizda Agro GmbH, Austria) prior to sowing according to the supplier's
602 instructions.
603
604 **RNA extraction and RNA gel blots**
605 Total RNA was isolated from pollen, germinating pollen tubes and anthers essentially
606 according to the method of Chomczynski and Sacchi (1987). For isolation of RNA from

pollen tubes, the samples were resuspended in 550 µL of RNA-denaturing solution and, after adding 700 µL of glass pearls, vigorously shaken for approximately 25 min. The supernatant was collected by centrifugation, and subsequently used for RNA extraction.

RNA gel blot was performed as described (Godt and Roitsch, 1997) by using radioactively labelled specific probes. The cloned cDNA fragments of *NtcwINV1* to *6, NtcavINV1* and*2*, as well as *NtMST2* and *NtMST3* were labelled by using a random primer DNA-labelling kit (MBI Fermentas, St. Leon-Rot, Germany).

Partial cloning of *NtMST2* and *NtMST3*

RNA from anthers was reverse-transcribed to cDNA using random hexamer primers. The degenerated primers NtMST-F 5'-GGWTTYGGWRTWGGWTTYGCWAAYCA-3' and NtMST-R 5'-WGGDATWCCYTTDGTYTCWGG-3' which bind to conserved regions of known monosaccharide transporters were used to obtain amplicons of approx. 1 kb by PCR. Sequencing identified two different amplicons designated *NtMST2* (1002 bp; accession KT240187) and *NtMST3* (1000 bp; accession KT240188) following the nomenclature for tobacco monosaccharide transporters by Sauer and Stadler (1993).

RNA *in situ* hybridization

For *NtcwINV2* (*Nin88*), Nt*MST2* and Nt*MST3* mRNA localizations a whole-mount adapted technique from de Almeida Engler et al. (1998) was used. Briefly, fixed materials were sectioned (70 µm) with a vibratome, washed twice in PBS then in methanol and finally incubated for 2 days at -20°C in absolute ethanol. After prehybridization, hybridization was carried out with a digoxigenin-labeled cRNA probe (500 µg x mL^{-1}) at 57°C for *NtMST2* and *NtMST3* mRNA and at 42°C for *NtcwINV2* (*Nin88*) mRNA. After washes and RNase A incubation, immunodetection was done with antidigoxigenin antibody coupled to alkaline phosphatase (Roche) diluted to 1 unit x mL^{-1}. The alkaline phosphatase reaction was carried out by 1 h incubation in the presence of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, BioRad) and nitroblue tetrazolium (NBT, BioRad). Control hybridizations with a sense probe and without a probe were conducted; representative controls are shown in Suppl. Fig. S4A to G.

***In vivo* visualization of pollen tubes**

Pollen tubes were visualized by fluorescence microscopy after staining of pistil sections (60-70 µm) by 0.05 % aniline blue in 50 mM KPO_4 buffer for 5 min.

Immunolocalization of invertases

Immunolocalization for cwINV proteins was done as described previou~~l~~sly (Goetz et al., 2001), using an antibody raised against a conserved region of cwINVs based on *Nt*cwINV2 (Nin88). Sections incubated without the primary antibody were used as controls as represented in Suppl. Fig. S4H.

Sugar uptake measurements

The sugar uptake measurements were basically performed as described by Roitsch and Tanner (1994). Pollen were resuspended in the premix to a concentration of about 100,000 to 200,000 pollen x 100 μL^{-1}. For Glu measurements, the premix used was composed of 50 mM Glc and 0.8 µC ^{14}C-Glc in 50 mM MES; for Suc measurements, the premix used was composed of 500 mM Suc and 0.8 µC ^{14}C-Suc in 50 mM MES buffer. For the competition experiment, a premix of 500 mM Suc, 0.8 µC ^{14}C-Suc, and 1 M Glc in 50 mM MES buffer was used.

For each measurement 200,000 to 400,000 pollen were resuspended in the appropriate premix and 100 µL were immediately sampled as time point 0. For sampling, the pollen were pipetted into 1 mL of water, filtered and washed with two volumes of water. The filter with the pollen was then transferred into a scintillation-glass, which contained 5 mL of scintillation cocktail. The remaining pollen were incubated for 30 min at 25°C. After this time 100 µL were sampled as described above. To determine the over-all activity, 100 µL of the premixes was directly added to 5 mL of scintillation cocktail and counted in a scintillation counter.

***In vitro* pollen germination and pollen tube length measurement**

Pollen from single flowers were collected in a reaction tube and frozen in liquid nitrogen. For *in vitro* germination, pollen grains f~~o~~rom 5 to 10 flowers were mixed and incubated for the indicated times at 26°C in 1 mL of standard germination medium (Schmülling et al., 1993) containing 10% Suc. In order to study the effect of sugars on pollen germination~~PG~~, the standard concentration of Suc in the medium was substituted by different concentrations of Glu, Suc, Pal, and Mhl as indicated. An aliquot of 50 µL was counted for the determination of pollen germination ~~PG~~ efficiency. To determine the effect of different sugars on pollen tube length, an aliquot of 50 µL was analyzed by light microscopy (100x magnification). Photographs were taken of 9 randomly selected microscope fields with more than 50 pollen grains. Pollen tube length was determined for an average number of 100 pollen tubes per individual experiment. To assess the inhibitory effect of miglitol on pollen germination ~~PG~~

675 and tube growth, 20 mM miglitol was added to germination medium with or without 1% Suc.
676 Photographs of 3 randomly selected microscope fields from 3 independent aliquots with more
677 than 100 pollen were determined per individual experiment.
678
679
680 **Invertase activity measurements**
681 Non-germinated and germinating pollen were prepared for invertase extraction as described
682 before (Hirsche et al., 2009) and invertase activity was determined in enzymatic assays
683 according to Jammer et al. (2015).
684
685 **Statistical analysis**
686 Statistical analyses were performed based on unpaired Student's t-test on datasets derived
687 from in minimum three independent biological replicates. P values ≤ 0.05 were considered
688 significant and used as basis for significance groups indicated by individual letters; *, **, and
689 *** indicate significant differences at the 0.05, 0.01, and 0.001 levels of confidence,
690 respectively.
691
692 Sequence data from this article can be found in the GenBank database accession numbers
693 KT240187 (*NtMST2*) and KT240188 (*NtMST3*).
694
695 **SUPPLEMENTAL DATA**
696 The following supplemental materials are available.
697 **Supplemental Figure S1.** Extracellular invertase activity during germination of wild-type
698 and *NtcwINV2*:*NtCIF* pollen.
699 **Supplemental Figure S2.** Spatiotemporal regulation of *NtMST2* and *NtMST3* mRNAs.
700 **Supplemental Figure S3.** Sugar dependence of pollen tube growth.
701 **Supplemental Figure S4.** Representative controls of *in situ* hybridizations and
702 immunocytochemical analyses.
703
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713
714

715 **REFERENCES**

716 **Albacete A, Cantero-Navarro E, Balibrea ME, Großkinsky DK, González MC,**
717 **Martínez-Andújar C, Smigocki AC, Roitsch T, Pérez-Alfocea F** (2014) Hormonal and
718 metabolic regulation of tomato fruit sink activity and yield under salinity. J Exp Bot **65:**
719 6081-6095

720 **Albacete A, Grosskinsky DK, Roitsch T** (2011) Trick and treat: a review on the function
721 and regulation of plant invertases in the abiotic stress response. Phyton – Ann Rei Bot A
722 **50:** 181-204

723 **Andersen MN, Asch F, Wu Y, Jensen CR, Næsted H, Mogensen VO, Koch KE** (2002)
724 Soluble invertase expression is an early target of drought stress during the critical,
725 abortion-sensitive phase of young ovary development in maize. Plant Physiol **130:** 591-604

726 **Balibrea Lara ME, Gonzalez Garcia M-C, Fatima T, Ehneß R, Kyun Lee T, Proels R,**
727 **Tanner W, Roitsch T** (2004) Extracellular invertase is an essential component of
728 cytokinin-mediated delay of senescence. Plant Cell **16:** 1276-1287

729 **Barnabas B, Fridvalszky L** (1984) Adhesion and germination of differently treated maize
730 pollen grains on the stigma. Acta Bot Hungar **30:** 329-332

731 **Borisjuk J, Rolletschek H, Radchuk R, Weschke W, Wobus U, Weber H** (2004) Seed
732 development and differentiation: a role for metabolic regulation. Plant Biol **6:** 375-386

733 **Büttner M, Sauer N** (2000) Monosaccharide transporters in plants: structure, function and
734 physiology. Biochim Biophys Acta **1465:** 263-274

735 **Cheng J, Wang Z, Yao F, Gao L, Ma S, Sui X, Zhang Z** (2015) Down-regulating CsHT1, a
736 cucumber pollen-specific hexose transporter, inhibits pollen germination, tube growth, and
737 seed development. Plant Physiol **168:** 635-647

738 **Cheng WH, Chourey PS** (1999) Genetic evidence that invertase-mediated release of hexoses
739 is critical for appropriate and normal seed development in maize. Theor Appl Genet **98:**
740 485-495

741 **Cheung AY** (1996) The pollen tube growth pathway: its molecular and biochemical
742 contributions and responses to pollination. Sex Plant Reprod **9:** 330-336

743 **Chomczynski P, Sachi N** (1987) Single-step method of RNA isolation by acid guanidium
744 thiocyanate-phenol-chloroform extraction. Anal Biochem **162:** 156-159

745 **Clément C, Burrus M, Audran J-C** (1996) Floral organ growth and carbohydrate content
746 during pollen development in *Lilium*. Am J Bot **83:** 459-469

747 **Cruz GF, Hancock CN, Kim D, McClure B** (2005) Stylar glycoproteins bind to S-RNase in
748 vitro. Plant J **42:** 295-304

749 **De Almeida Engler J, Van Montagu M, Engler G** (1998) Whole-mount *in situ*
750 hybridization in plants. Methods Mol Biol **82:** 373-384
751 **Derksen J, Rutten T, van Amstel T, de Win A, Doris F, Steer M** (1995) Regulation of
752 pollen tube growth. Acta Bot Neerl **44:** 93-119
753 **Deshusses J, Gumber SC, Loewus FA** (1981) Sugar uptake in lily pollen: A proton symport.
754 Plant Physiol **67:** 793-796
755 **Ehness R, Roitsch T** (1997) Co-ordinated induction of mRNAs for extracellular invertase
756 and a glucose transporter in *Chenopodium rubrum* by cytokinins. Plant J **11:** 539-548
757 **Engelke T, Hirsche J, Roitsch T** (2010) Anther-specific carbohydrate supply and restoration
758 of metabolically engineered male sterility. J Exp Bot **61:** 2693-2706
759 **Engelke T, Hirsche J, Roitsch T** (2011) Metabolically engineered male sterility in rapeseed
760 (*Brassica napus* L.). Theor Appl Genet **122:** 163-174
761 **Engels FM** (1974) Function of Golgi vesicles in relation to cell wall synthesis in germinating
762 *Petunia* pollen. II. Chemical composition of Golgi vesicles and pollen tube wall. Acta Bot
763 Neerl **23:** 81-89
764 **Engels FM, Kreger DR** (1974) Cellulose in the walls and Golgi vesicles of pollen tubes of
765 *Petunia hybrida*. Naturwissenschaften **61:** 273
766 **Eom J-S, Chen L-Q, Sosso D, Julius BT, Lin IW, Qu X-Q, Braun DM, Frommer WB**
767 (2015) SWEETs, transporters for intracellular and intercellular sugar translocation. Curr
768 Opin Plant Biol **25:** 53-62
769 **Fernie AR, Rossner U, Geigenberger P** (2001) The sucrose analog palatinose leads to a
770 stimulation of sucrose degradation and starch synthesis when supplied to discs of growing
771 potato tubers. Plant Physiol **125:** 1967–1977
772 **Franklin-Tong VE** (2002) The difficult question of sex: the mating game. Curr Opin Plant
773 Biol **5:** 14-18
774 **Fu Y, Yang Z** (2001) Rop GTPase: a master switch of cell polarity development in plants.
775 Trends Plant Sci **6:** 545-547
776 **Fu Y, Wu G, Yang Z** (2001) Rop GTPase-dependent dynamics of tip-localized F-actin
777 controls tip growth in pollen tubes. J Cell Biol **152:** 1019-1032
778 **Gibson SI** (2005) Control of plant development and gene expression by sugar signaling. Curr
779 Opin Plant Biol **8:** 93-102
780 **Godt DE, Roitsch T** (1997) Regulation and tissue-specific distribution of mRNAs for three
781 extracellular invertase isoenzymes of tomato suggests an important function in establishing
782 and maintaining sink metabolism. Plant Physiol **115:** 273-282

783 **Goetz M, Godt DE, Guivarc'h A, Kahmann U, Chriqui D, Roitsch T** (2001) Induction of
784 male sterility in plants by metabolic engineering of the carbohydrate supply. Proc Natl
785 Acad Sci USA **98:** 6522-6527
786 **Granot D, David-Schwartz R, Kelly G** (2013) Hexose kinases and their role in sugar-
787 sensing and plant development. Front Plant Sci **4:** 44
788 **Greiner S, Krausgrill S, Rausch T** (1998) Cloning of a tobacco apoplasmic invertase
789 inhibitor. Plant Physiol **116:** 733-742
790 **Greiner S, Weil M, Krausgrill S, Rausch T** (1995) A tobacco cDNA coding for cell-wall
791 invertase. Plant Physiol **108:** 825-826
792 **Heslop-Harrison J** (1987) Pollen germination and pollen tube growth. Int Rev Cytol **107:** 1-
793 78
794 **Higashiyama T, Kuroiwa H, Kuroiwa T** (2003) Pollen-tube guidance: Beacons from the
795 female gametophyte. Curr Opin Plant Biol **6:** 36-41
796 **Hirose T, Zhang Z, Miyao A, Hirochika H, Ohsugi R, Terao T** (2010) Disruption of a
797 gene for rice sucrose transporter, *OsSUT1*, impairs pollen function but pollen maturation is
798 unaffected. J Exp Bot **61:** 3639-3646
799 **Hirsche J, Engelke T, Völler D, Götz M, Roitsch T** (2009) Interspecies compatibility of the
800 anther specific cell wall invertase promoters from *Arabidopsis* and tobacco for generating
801 male sterile plants. Theor Appl Genet **118:** 235-245
802 **Iven T, Strathmann A, Böttner S, Zwafink T, Heinekamp T, Guivarc'h A, Roitsch T,**
803 **Dröge-Laser W** (2010) Homo- and heterodimers of tobacco bZIP proteins counteract as
804 positive or negative regulators of transcription during pollen development. Plant J **63:** 155-
805 166
806 **Jahnen W, Lush WM, Clarke AE** (1989) Inhibition of *in vitro* pollen tube growth by
807 isolated S-glycoproteins of *Nicotiana alata*. Plant Cell **1:** 501-510
808 **Jammer A, Gasperl A, Luschin-Ebengreuth N, Heyneke E, Chu H, Cantero-Navarro E,**
809 **Großkinsky DK, Albacete AA, Stabentheiner E, Franzaring J, Fangmeier A, van der**
810 **Graaff E, Roitsch T** (2015) Simple and robust determination of the activity signature of
811 key carbohydrate metabolism enzymes for physiological phenotyping in model and crop
812 plants. J Exp Bot **66:** 5531-5542
813 **Kim JY, Mahé A, Guy S, Brangeon J, Roche O, Chourey PS, Prioul JL** (2000)
814 Characterization of two members of the maize gene family, *Incw3* and *Incw4*, encoding
815 cell-wall invertases. Gene **245:** 89-102
816 **Koch K** (2004) Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar

817 sensing and plant development. Curr Opin Plant Biol **7:** 235-246
818 **Konar RN, Linskens HF** (1966) Physiology and biochemistry of the stigmatic fluid of
819 *Petunia hybrida*. Planta **71:** 372-387
820 **Lastdrager J, Hanson J, Smeekens S** (2014) Sugar signals and the control of plant growth
821 and development. J Exp Bot **65:** 799-807
822 **Le Roy K, Vergauwen R, Struyf T, Yuan S, Lammens W, Mátrai J, De Maeyer M, Van**
823 **den Ende W** (2013) Understanding the role of defective invertases in plants: tobacco
824 Nin88 fails to degrade sucrose. Plant Physiol **161:** 1670-1681
825 **Lemoine R, Bürkle L, Barker L, Sakr S, Kühn C, Regnacq M, Gaillard C, Delrot S,**
826 **Frommer WB** (1999) Identification of a pollen-specific sucrose transporter-like protein
827 NtSUT3 from tobacco. FEBS Lett **454:** 325-330
828 **Li Y-Q, Linskens HF** (1983) Neutral sugar composition of pollen tube walls of *Lilium*
829 *longiflorum*. Acta Bot Neerl **32:** 437-445
830 **Loreti E, Amedo A, Perata P** (2000) Glucose and disaccharide mechanisms modulate the
831 expression of a-amylase in barley embryos. Plant Physiol **123:** 939–948
832 **Loewus F, Labarca C** (1973) Pistil-secretion product and pollen tube wall formation. In
833 "Biogenesis of Plant Cell Wall Polysacharrides" (F. Loewus, ed.) Academic Press, New
834 York
835 **Lord EM, Russell SD** (2002) The mechanisms of pollination and fertilization in plants. Annu
836 Rev Cell Dev Biol **18:** 81-105
837 **Lorenz K, Lienhard S, Sturm A** (1995) Structural organization and differential expression
838 of carrot α-fructofuranosidase genes: identification of a gene coding for a flower bud-
839 specific isoenzyme. Plant Mol Biol **28:** 189-194
840 **Maddison AL, Hedley PE, Meyer RC, Aziz N, Davidson D, Machray GC** (1999)
841 Expression of tandem invertase genes associated with sexual and vegetative growth cycles
842 in potato. Plant Mol Biol **41:** 741-751
843 **Mascarenhas JP** (1970) A new intermediate in plant cell wall synthesis. Biochem Biophys
844 Res Commun **41:** 142-149
845 **Mascarenhas JP** (1993) Molecular mechanisms of pollen tube growth and differentiation.
846 Plant Cell **5:** 1303-1314
847 **Moore B, Zhou L, Rolland F, Hall Q, Cheng W-H, Liu Y-X, Hwang I, Jones T, Sheen J**
848 (2003) Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal
849 signaling. Science **300:** 332-336
850 **Nakamura N, Yoshida K** (1980) A pectic substance extracted from the pollen tube wall of

851 *Camellia japonica*. Japan J Palynol **25:** 11-16
852 **Nakamura N, Shimizu M, Suzuki H** (1991) Characterization of hexose kinases from
853 Camellia and Lily pollen grains. Physiol Plant **81:** 215-22
854 **Proels RK, González M-C, Roitsch T** (2006) Gibberellins dependent induction of tomato
855 extracellular invertase Lin7 is required for pollen development. Funct Plant Biol **33:** 547-
856 554
857 **Qu L-J, Li L, Lan Z, Dresselhaus T** (2015) Peptide signalling during the pollen tube
858 journey and double fertilization. J Exp Bot, DOI: 10.1093/jxb/erv275
859 **Rae AL, Harris PJ, Bacic A, Clarke AE** (1985) Composition of the cell walls of *Nicotiana*
860 *tabacum* Link et Otto pollen tubes. Planta **166:** 128-133
861 **Read SM, Clarke AE, Bacic A** (1993) Stimulation of growth of cultured *Nicotiana tabacum*
862 W38 pollen tubes by poly(ethylene glycol) and $Cu_{(II)}$ salts. Protoplasma **177:** 1-14
863 **Reger BJ, Pressey R, Chaubal R** (1992) *In vitro* chemotropism of pearl millet pollen tubes
864 to stigma tissue: a response to glucose produced in the medium by tissue-bound invertase.
865 Sex Plant Rep **5:** 201-205
866 **Reinders A** (2016) Fuel for the road – sugar transport and pollen tube growth. J Exp Bot **67:**
867 2121-2123
868 **Reuscher S, Akiyama M, Yasuda T, Makino H, Aoki K, Shibata D, Shiratake K** (2014)
869 The sugar transporter inventory of tomato: genome-wide identification and expression
870 analysis. Plant Cell Physiol **55:** 1123-1141
871 **Roitsch T, Tanner W** (1994) Expression of a sugar-transporter gene family in a
872 photoautotrophic suspension culture of *Chenopodium rubrum* L. Planta **193:** 365-371
873 **Roitsch T, Tanner W** (1996) Cell wall invertase: bridging the gap. Botanica Acta **109:** 90-93
874 **Roitsch T, Balibrea ME, Hofmann M, Proels R, Sinha AK** (2003) Extracellular invertase:
875 key metabolic enzyme and PR protein. J Exp Bot **54:** 513-524
876 **Roitsch T, González M-C** (2004) Function and regulation of plant invertases: sweet
877 sensations. Trends Plant Sci **9:** 606-613
878 **Rottmann T, Zierer W, Subert C, Sauer N, Stadler R** (2016) STP10 encodes a high-
879 affinity monosaccharide transporter and is induced under low-glucose conditions in pollen
880 tubes of Arabidopsis. J Exp Bot **67:** 2387-2399
881 **Ruan Y-L** (2012) Sucrose metabolism: gateway to diverse carbon use and sugar signaling.
882 Mol Plant **5:** 763-765
883 **Ruan Y-L** (2014) Sucrose metabolism: gateway to diverse carbon use and sugar signaling.
884 Annu Rev Plant Biol **65:** 33-67

885 **Sander A, Krausgrill S, Greiner S, Weil M, Rausch T** (1996) Sucrose protects cell wall
886 invertase but not vacuolar invertase against proteinaceous inhibitors. FEBS Lett **385:** 171-
887 175
888 **Sauer N, Stadler R** (1993) A sink-specific H^+/monosaccharide co-transporter from *Nicotiana*
889 *tabacum*: cloning and heterologous expression in baker's yeast. Plant J **4:** 601-610
890 **Schäfer B** (2012) Untersuchungen zur Regulation der Invertaseaktivität und zu
891 Invertaseinhibitoren aus Pflanzenextrakten. PhD thesis, University of Würzburg, Würzburg
892 **Schlüpmann H, Bacic A, Read SM** (1994) Uridine diphosphate glucose metabolism and
893 callose synthesis in cultured pollen tubes of *Nicotiana alata* Link et Otto. Plant Physiol
894 **105:** 659-670
895 **Schmülling T, Röhrig H, Pilz S, Walden R, Schell J** (1993) Restoration of fertility by
896 antisense RNA in genetically engineered male sterile tobacco plants. Mol Gen Genet **237:**
897 385–394
898 **Schneidereit A, Scholz-Starke J, Büttner M** (2003) Functional characterization and
899 expression analyses of the glucose-specific AtSTP9 monosaccharide transporter in pollen
900 of Arabidopsis. Plant Physiol **133:** 182-190
901 **Schneidereit A, Scholz-Starke J, Sauer N, Büttner M** (2005) *AtSTP11*, a pollen-specific
902 monosaccharide transporter in Arabidopsis. Planta **221:** 48-55
903 **Sheen J** (2014) Master regulators in plant glucose signaling networks. J Plant Biol **57:** 67-79
904 **Sinha AK, Hofman MG, Römer U, Köckenberger W, Elling L, Roitsch T** (2002)
905 Metabolizable and non-metabolizable sugars activate different signal transduction
906 pathways in tomato. Plant Physiol **128:** 1480-1489
907 **Stadler R, Truernit E, Gahrtz M, Sauer N** (1999) The AtSUC1 sucrose carrier may
908 represent the osmotic driving force for anther dehiscence and pollen tube growth in
909 *Arabidopsis*. Plant J **19:** 269-278
910 **Sturm A** (1999) Invertases. Primary structures, functions, and roles in plant development and
911 sucrose partitioning. Plant Physiol **121:** 1-7
912 **Taylor LP, Hepler PK** (1997) Pollen germination and tube growth. Annu Rev Plant Physiol
913 Plant Mol Biol **48:** 461-491
914 **Truernit E, Stadler R, Baier K, Sauer N** (1999) A male gametophyte-specific
915 monosaccharide transporter in *Arabidopsis*. Plant J **17:** 191-201
916 **Tymowska-Lalanne Z, Kreis M** (1998) Expression of the *Arabidopsis thaliana* invertase
917 gene family. Planta **207:** 259-265
918 **Van der Woude WJ, Morre DJ, Bracker CE** (1971) Isolation and characterization of

919 secretory vesicles in germinated pollen of *Lilium longiflorum*. J Cell Sci **8:** 331-351

920 **Wang L, Ruan Y-L** (2016) Critical roles of vacuolar invertase in floral organ development
921 and male and female fertilities are revealed through characterization of *GhVIN1*-RNAi
922 cotton plants. Plant Physiol **171:** 405-423

923 **Weber H, Borisjuk L, Wobus U** (1996) Controlling seed development and seed size in *Vicia*
924 *faba*: a role for seed coat-associated invertases and carbohydrate state. Plant J **10:** 823-834

925 **Weil M, Krausgrill S, Schuster A, Rausch T** (1994) A 17-kDa *Nicotiana tabacum* cell-wall
926 peptide acts as an in-vitro inhibitor of the cell-wall isoform of acid invertase. Planta **193:**
927 438-445

928 **Williams LE, Lemoine R, Sauer N** (2000) Sugar transporters in higher plants: A diversity of
929 roles and complex regulation. Trends Plant Sci **5:** 283-290

930 **Wobus U, Weber H** (1999) Sugars as signal molecules in plant seed development. Biol
931 Chem **390:** 937-944

932 **Xu J, Avigne WT, McCarty DR, Koch KE** (1996) A similar dichotomy of sugar modulation
933 and developmental expression affects both paths of sucrose metabolism: evidence from
934 maize invertase gene family. Plant Cell **8:** 1209-1220

935 **Yang Z, Zhang L, Diao F, Whang M, Wu N** (2004) Sucrose regulates elongation of carrot
936 somatic embryo radicles as a signal molecule. Plant Mol Biol **54:** 441-459

937 **Ylstra B, Garrido D, Busscher J, van Tunen AJ** (1998) Hexose transport in growing
938 petunia pollen tubes and characterization of a pollen-specific, putative monosaccharide
939 transporter. Plant Physiol **118:** 297-304

940 **Zheng Z-L, Yang Z** (2000) The Rop GTPase switch turns on polar growth in pollen. Trends
941 Plant Sci **5:** 298-303

942

943 **TABLES**

Table I. Vitality, *in vitro* germination and *in vivo* development of pollen from tobacco *NtcwINV2:NtCIF* plants

Line	Vitality [%]	Germination efficiency [%]	Development [%]
Wild-type (SNN)	85.1 (± 2.8)	54.8 (± 7.7)	78.3 (± 3.2)
NT49-6	63.4 (± 3.0) **	3.1 (± 0.5) *	54.4 (± 7.0) *
NT49-10	82.5 (± 2.6)	29.2 (± 8.0) *	79.0 (± 6.2)
NT51-1	74.9 (± 4.8)	23.9 (± 5.4) *	87.3 (± 12.5)
NT51-6	77.8 (± 1.1) *	34.5 (± 4.9) $^{p=0.0503}$	76.3 (± 7.3)
NT51-8	64.8 (± 4.6) **	10.8 (± 2.6) **	60.2 (± 12.9)
NT51-10	72.5 (± 3.0) *	24.1 (± 8.1) *	86.7 (± 8.3)
NT51-12	68.1 (± 2.9) **	23.4 (± 8.0) *	75.8 (± 11.9)
NT51-17	84.6 (± 1.5)	5.7 (± 3.6) **	36.8 (± 2.9) ***

Germination and development rates are given as percentage of vital pollen. The germination rate is the percentage of pollen that were able to form a pollen tube during an *in vitro* pollen germination ~~PG~~ assay. The development rate was determined under the light microscope by the phenotype of the pollen. Values represent the means (± SEM). *, **, *** indicate significantly difference to wild-type at the 0.05, 0.01, and 0.001 levels of confidence, respectively.

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947 **FIGURE LEGENDS**

948 **Figure 1.** The chemical invertase inhibitor miglitol negatively impacts *in vitro* pollen
949 germination (PG) and pollen tube growth of *N. tabacum*. A, PG [%] and B, pollen tube length
950 [µm] in medium with or without 30 mM sucrose (Suc) and 20 mM miglitol (Mig),
951 respectively, at indicated time-points post germination. Values represent means (± SEM);
952 letters indicate different significance groups based on unpaired Student's t-test ($p<0.05$)
953 comparing different treatments at the individual time-points.

954

955 **Figure 2.** Sugar uptake into pollen of wild-type and *NtcwINV2*:*NtCIF* tobacco. The uptake of
956 ^{14}C-glucose (Glu) and ^{14}C-sucrose (Suc) into pollen from wild-type (SNN) and
957 *NtcwINV2*:*NtCIF* plants was determined. In addition the uptake of ^{14}C-Suc was competed by
958 an excess of unlabelled ^{12}C-Glu. Values represent means (± SEM); letters indicate different
959 significance groups based on unpaired Student's t-test ($p<0.05$) between the plant lines within
960 the same treatment; a, b for ^{14}C-Glu; e, f, g for ^{14}C-Suc; i, j, k for ^{14}C-Suc + ^{12}C-Glu.

961

962 **Figure 3**. Spatiotemporal regulation of *NtcwINV*s, *NtMST2* and *NtMST3* after pollination of a
963 stigma. A to D, visualization of pollen tubes after staining by aniline blue, A, 2 hours B, 4
964 hours, C and D, 24 hours after pollination. In general, pollen grains germinated 2 hours after
965 pollination and 24 hours after pollination most of the pollen tubes have reached the basal part
966 of the style, the pollen tubes progressing in the peripheral zone of the transmitting tissue (D).
967 E to I, immunocytochemistry for *Nt*cwINV proteins, E and F, in non-pollinated pistil, G, in
968 pistil pollinated for 4 hours or H and I, 24 hours. F, Detail of E. H and I, detail of a region
969 shown in G in a pistil pollinated for 24 hours. Before pollination no signal was present in
970 pistil. After pollination a signal was observed in growing pollen tubes (I, arrowhead) and in
971 female tissues (G and H, arrowheads) as well. J to P, whole-mount *in situ* hybridization in
972 pollinated pistils with probes derived from *NtcwINV2* (*Nin88*) 24 h after pollination (J and K),
973 *NtMST2* 4 h (M) and 24 h after pollination (N), and *NtMST3* 4 h (O) and 24 h after pollination
974 (P). K, detail of J. L, Non-pollinated pistil hybridized with an *NtMST3* antisense probe.
975 Expression of the genes was restricted to the growing pollen tubes present at the periphery of
976 the transmitting tissue, no signal being detected in female tissues. Co, cortex; St, stigma; Tt,
977 transmitting tissue. Scale bar, 200 µm.

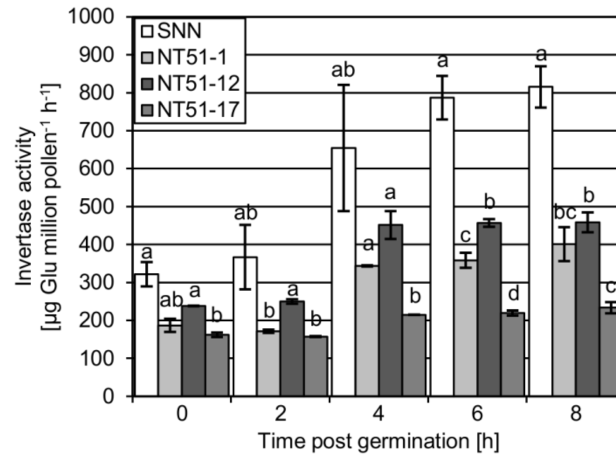
978

979 **Figure 4.** Blot analysis of *N. tabacum* invertases, probed against 15 µg RNA isolated from
980 different flower tissues reveals specific expression patterns of the individual invertase
981 isoforms. PT, pollen tube; np, non-pollinated; pp, post pollination.
982
983 **Figure 5**. Spatiotemporal regulation of tobacco invertase genes in non-pollinated and
984 pollinated stigma. Whole-mount *in situ* hybridization in non-pollinated pistils and 24 h post
985 pollination with probes for A and B, *NtcwINV5*, C and D, *NtvacINV1*, E and F, *NtvacINV2* as
986 well as G and H, *NtCIF*. While the different invertases show distinct expression patterns in
987 the different tissues of the pistil dependent on pollination, no expression of the invertase
988 inhibitor *NtCIF* was detected. Co, cortex; St, stigma; Tt, transmitting tissue. Scale bar, 200
989 µm.
990
991 **Figure 6.** Sugar dependence of pollen tube growth. Length distribution of pollen tube lengths
992 showing that a sucrose (Suc) signaling pathway independent of hexokinase is responsible for
993 pollen tube growth. Length distribution of pollen tubes grown in medium supplemented with
994 A, 10% glucose (Glu); B, 10% Suc; C, 10% Glu and 0.1 % palatinose (Pal); D, 10% Glu,
995 0.1% Pal and 2.1% (100 mM) mannoheptulose (Mhl). E, Average pollen tube length in
996 indicated medium of data shown in A to D, represented as means (± SEM); letters indicate
997 different significance groups based on unpaired Student's t-test ($p<0.05$).
998
999 **Figure 7.** Model illustrating the coordinated regulation of invertases and hexose transporters
1000 during germination and pollen tube growth and the distinct regulatory role of glucose (Glu)
1001 and sucrose (Suc). Extracellular invertase activities (black) from pollen and pistil and hexose
1002 transporter (red) activity from pollen are linked to supply Glu required for pollen germination
1003 and Glu and Suc are required as metabolic signals for germination and pollen tube growth,
1004 respectively. ~~The blue color corresponds to the expression of *NtcwINVs* as shown by *in situ*~~
1005 ~~hybridizations. The purple color corresponds to the expression of both *NtcwINV* and *NtMST*~~
1006 ~~genes as shown by *in situ* hybridization.~~ The green color corresponds to the expression of
1007 vacuolar invertase genes ~~(*cwINVs* and *vacINVs*)~~ specific to the female tissues (pistil-INVs).
1008 This expression is deduced from data obtained with immunocytochemistry and *in situ*
1009 hybridizations. The boxes with arrows correspond to the carbohydrate signal necessary for
1010 pollen germination and the square boxes symbolize the carbohydrates uptake to sustain
1011 growth of pollen tubes.

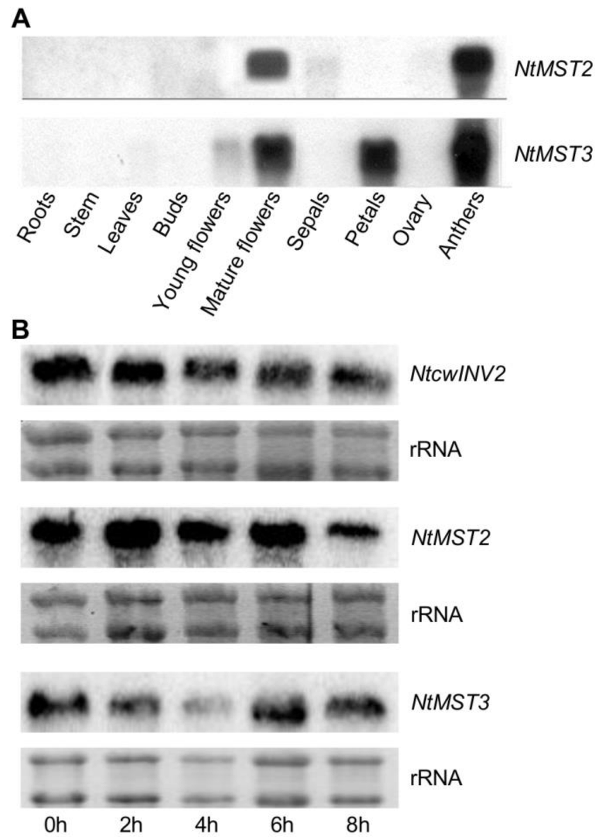
Supplemental Data

Metabolic control of tobacco pollination by sugars and invertases

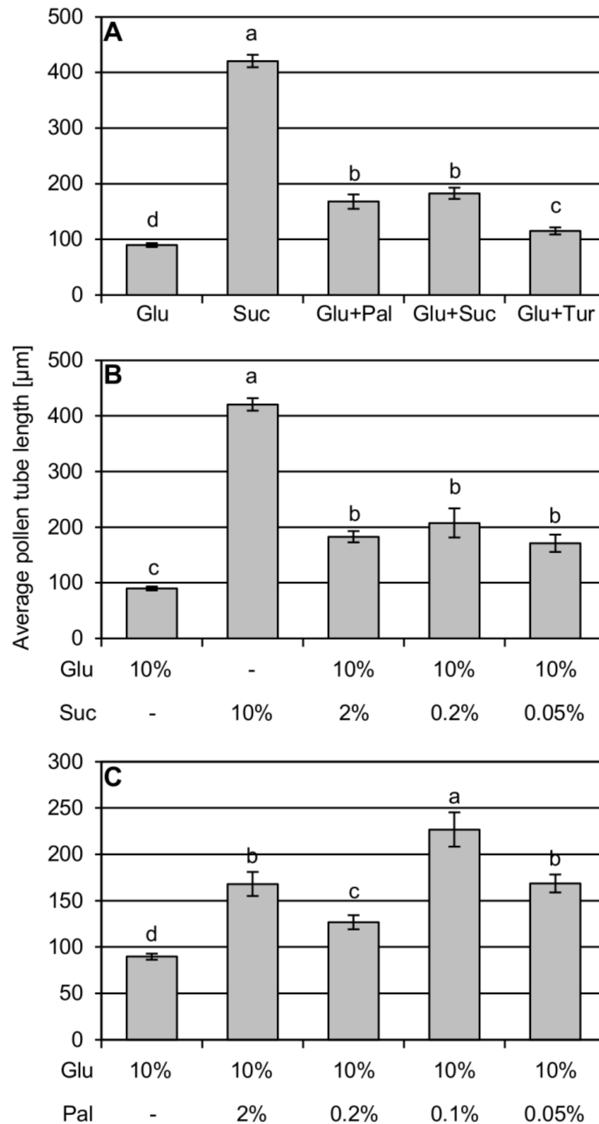
M Goetz, A Guivarc'h, J Hirsche, MA Bauerfeind, MC González, TK Hyun, SH Eom, D Chriqui, T Engelke, DK Großkinsky, T Roitsch



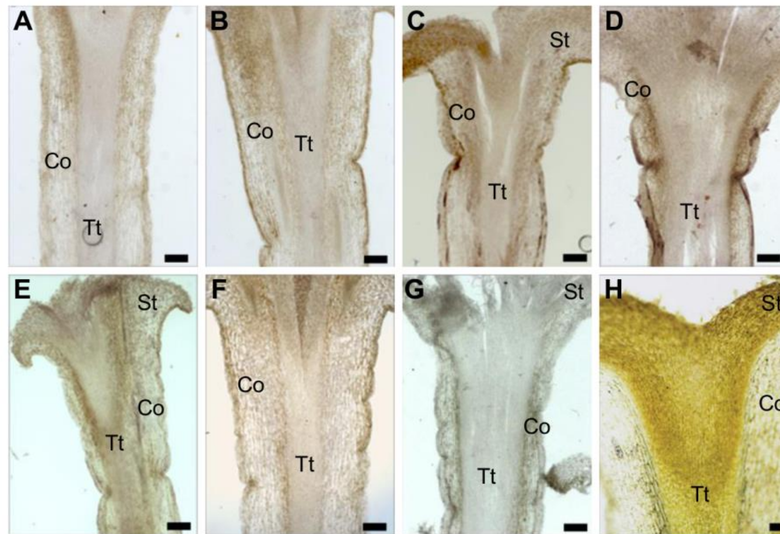
Supplemental Figure S1. Extracellular invertase activity during germination of wild-type and *NtcwINV2*:*NtCIF* pollen. Time course of extracellular invertase activity of pollen from wild-type plants and from *NtcwINV2*:*NtCIF* plants during an *in vitro* germination assay. The germination rates for the pollen of the lines used are 74.6% for wild-type, 31.9% for NT51-12, 20.5% for NT51-1, and 3.0% for NT51-17.



Supplemental Figure S2. Spatiotemporal regulation of *NtMST2* and *NtMST3* mRNAs. A, blot analysis of hexose transporters *NtMST2* and *NtMST3*, probed against 15 µg RNA isolated from different source tissue revealed flower-specific expression of the hexose transporters. *NtMST2* is exclusively expressed in anthers, whereas *NtMST3* shows expression in anthers and petals. B, blot analysis of *NtcwINV*s [*NtcwINV2* (*Nin88*) probe], *NtMST2* and *NtMST3* probed against RNA isolated from pollen after indicated time-points post germination revealed co-expression of these genes in germinating pollen. The rRNA labeled panels show the loading controls.



Supplemental Figure S3. Sugar dependence of pollen tube growth. Average pollen tube length of pollen tubes grown in medium supplemented with A, 10% glucose (Glu), 10% Suc, or 10% Glu in the presence of 2% of different disaccharides palatinose (Pal), Suc or turanose (Tur), B, 10% Glu, 10% Suc or 10% Glu in the presence of indicated concentrations of Suc, and C, 10% Glu in the presence of indicated concentrations of Pal. The presence of any disaccharide in Glu-containing medium resulted in a shift towards longer pollen tubes. Values are represented as means (± SEM); letters indicate different significance groups based on unpaired Student's t-test ($p<0.05$).



Supplemental Figure S4. Representative controls of *in situ* hybridizations and immunocytochemical analyses. Pistil pollinated for 24 hours and hybridized with a sense probe of A, *NtMST2*, B, *NtMST3*, C, *NtcwINV5*, D, *NtvacINV1*, E, *NtvacINV2* and F, *NtCIF* or G, without any probe but with the anti-digoxigenin antibody as representative controls for *in situ* hybridizations. H, pistil pollinated for 24 hours incubated without the primary antibody as control for immunocytochemistry. Co, cortex; St, stigma; Tt, transmitting tissue. Scale bar, 200 µm.

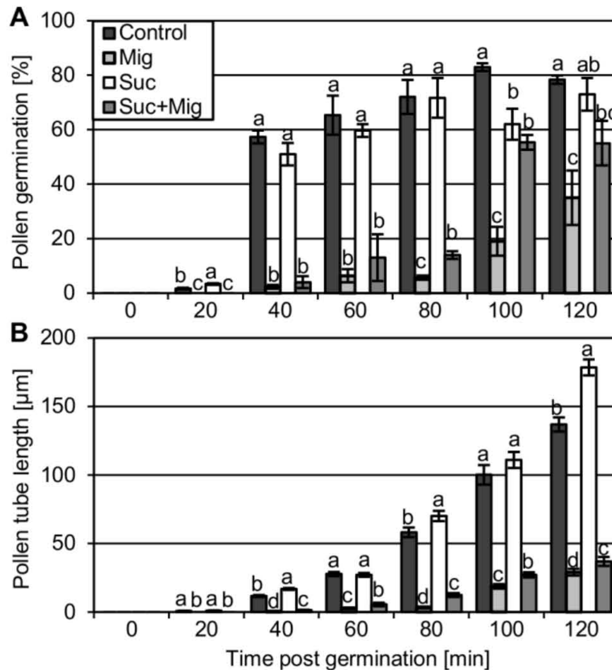


Figure 1. The chemical invertase inhibitor miglitol negatively impacts *in vitro* pollen germination (PG) and pollen tube growth of *N. tabacum*. A, PG [%] and B, pollen tube length [µm] in medium with or without 30 mM sucrose (Suc) and 20 mM miglitol (Mig), respectively, at indicated time-points post germination. Values represent means (± SEM); letters indicate different significance groups based on unpaired Student's t-test ($p<0.05$) comparing different treatments at the individual time-points.

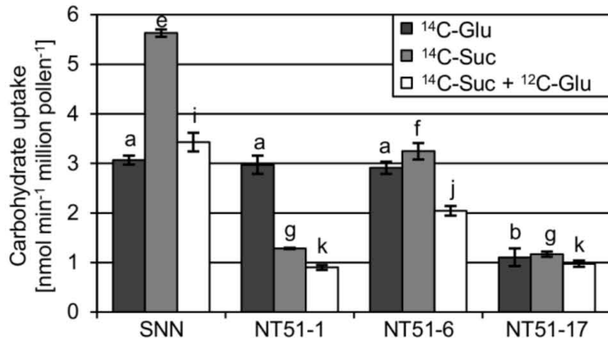


Figure 2. Sugar uptake into pollen of wild-type and *NtcwINV2:NtCIF* tobacco. The uptake of ^{14}C-glucose (Glu) and ^{14}C-sucrose (Suc) into pollen from wild-type (SNN) and *NtcwINV2:NtCIF* plants was determined. In addition the uptake of ^{14}C-Suc was competed by an excess of unlabelled ^{12}C-Glu. Values represent means (± SEM); letters indicate different significance groups based on unpaired Student's t-test ($p<0.05$) between the plant lines within the same treatment; a, b for ^{14}C-Glu; e, f, g for ^{14}C-Suc; i, j, k for ^{14}C-Suc + ^{12}C-Glu.

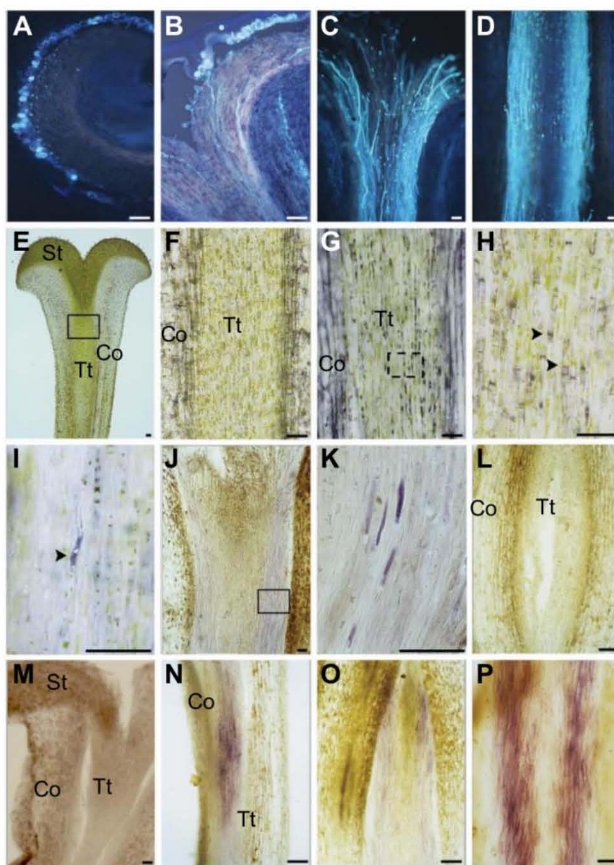


Figure 3. Spatiotemporal regulation of *NtcwINV*s, *NtMST2* and *NtMST3* after pollination of a stigma. A to D, visualization of pollen tubes after staining by aniline blue, A, 2 hours B, 4 hours, C and D, 24 hours after pollination. In general, pollen grains germinated 2 hours after pollination and 24 hours after pollination most of the pollen tubes have reached the basal part of the style, the pollen tubes progressing in the peripheral zone of the transmitting tissue (D).

E to I, immunocytochemistry for *Nt*cwINV proteins, E and F, in non-pollinated pistil, G, in pistil pollinated for 4 hours or H and I, 24 hours. F, Detail of E. H and I, detail of a region shown in G in a pistil pollinated for 24 hours. Before pollination no signal was present in pistil. After pollination a signal was observed in growing pollen tubes (I, arrowhead) and in female tissues (G and H, arrowheads) as well. J to P, whole-mount *in situ* hybridization in pollinated pistils with probes derived from *NtcwINV2* (*Nin88*) 24 h after pollination (J and K), *NtMST2* 4 h (M) and 24 h after pollination (N), and *NtMST3* 4 h (O) and 24 h after pollination (P). K, detail of J. L, Non-pollinated pistil hybridized with an *NtMST3* antisense probe. Expression of the genes was restricted to the growing pollen tubes present at the periphery of the transmitting tissue, no signal being detected in female tissues. Co, cortex; St, stigma; Tt, transmitting tissue. Scale bar, 200 µm.

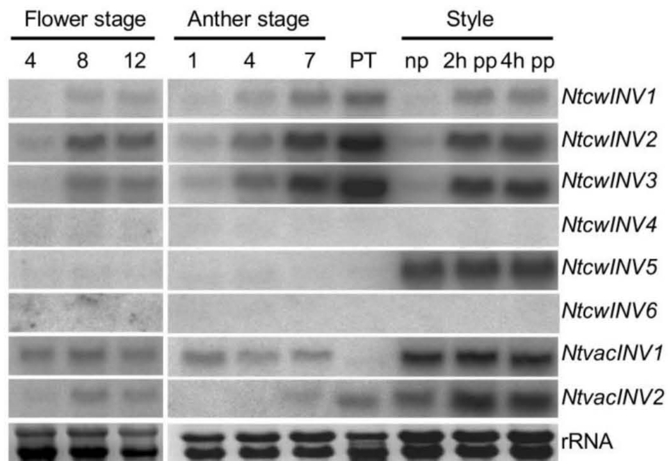


Figure 4. Blot analysis of *N. tabacum* invertases, probed against 15 μg RNA isolated from different flower tissues reveals specific expression patterns of the individual invertase isoforms. PT, pollen tube; np, non-pollinated; pp, post pollination.

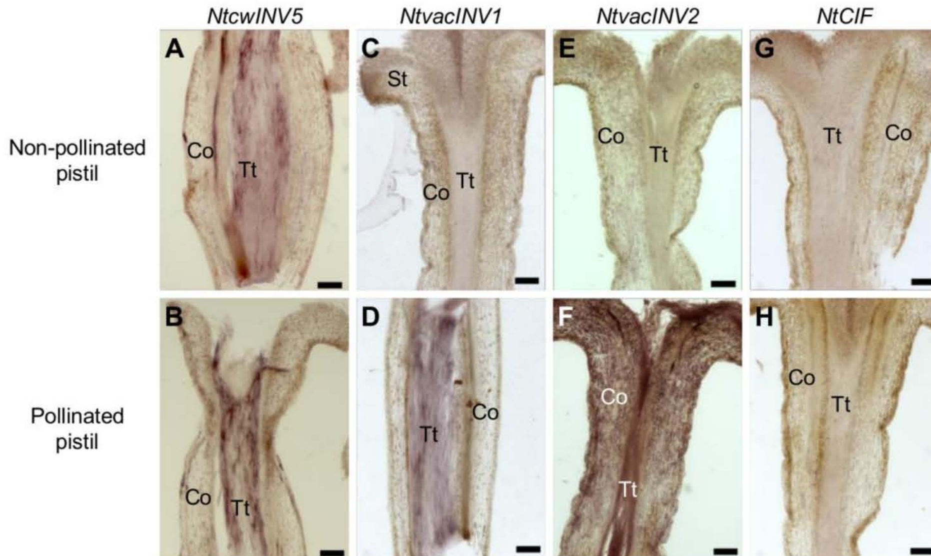


Figure 5. Spatiotemporal regulation of tobacco invertase genes in non-pollinated and pollinated stigma. Whole-mount *in situ* hybridization in non-pollinated pistils and 24 h post pollination with probes for A and B, *NtcwINV5*, C and D, *NtvacINV1*, E and F, *NtvacINV2* as well as G and H, *NtCIF*. While the different invertases show distinct expression patterns in the different tissues of the pistil dependent on pollination, no expression of the invertase inhibitor *NtCIF* was detected. Co, cortex; St, stigma; Tt, transmitting tissue. Scale bar, 200 μm.

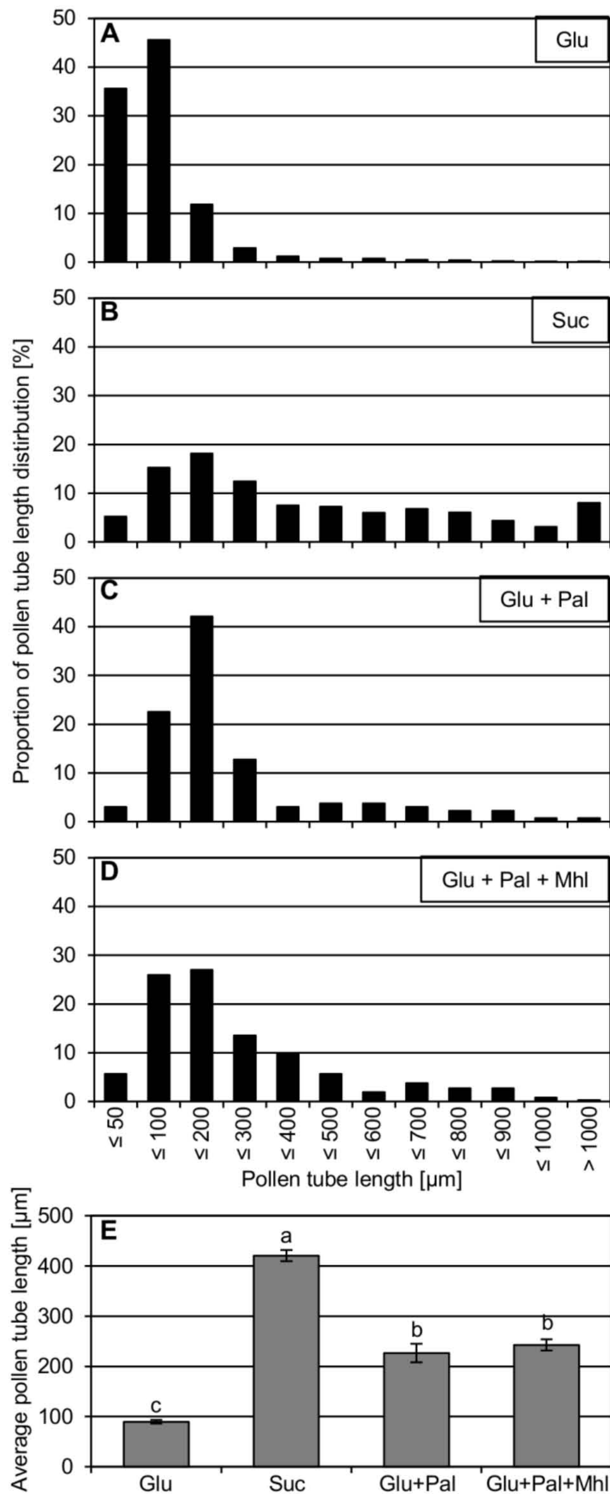


Figure 6. Sugar dependence of pollen tube growth. Length distribution of pollen tube lengths showing that a sucrose (Suc) signaling pathway independent of hexokinase is responsible for pollen tube growth. Length distribution of pollen tubes grown in medium supplemented with A, 10% glucose (Glu); B, 10% Suc; C, 10% Glu and 0.1 % palatinose (Pal); D, 10% Glu, 0.1% Pal and 2.1% (100 mM) mannoheptulose (Mhl). E, Average pollen tube length in indicated medium of data shown in A to D, represented as means (± SEM); letters indicate different significance groups based on unpaired Student's t-test ($p<0.05$).

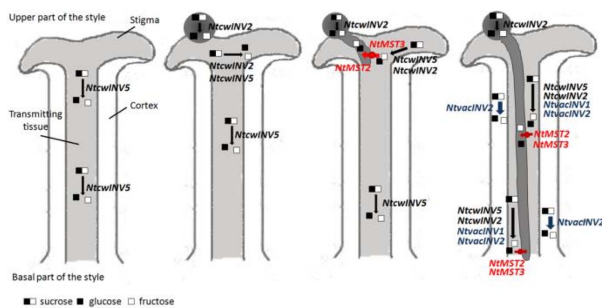


Figure 7. Model illustrating the coordinated regulation of invertases and hexose transporters during germination and pollen tube growth and the distinct regulatory role of glucose (Glu) and sucrose (Suc). Extracellular invertase activities (black) from pollen and pistil and hexose transporter (red) activity from pollen are linked to supply Glu required for pollen germination and Glu and Suc are required as metabolic signals for germination and pollen tube growth, respectively. The green color corresponds to the expression of vacuolar invertase genes specific to the female tissues (pistil-INVs). This expression is deduced from data obtained with immunocytochemistry and *in situ* hybridizations. The boxes with arrows correspond to the carbohydrate signal necessary for pollen germination and the square boxes symbolize the carbohydrates uptake to sustain growth of pollen tubes.