

Understanding the Role of Defective Invertases in Plants: Tobacco Nin88 Fails to Degrade Sucrose^{1[W]}

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Cell wall invertases (cwINVs), with a high affinity for the cell wall, are fundamental enzymes in the control of plant growth, development, and carbon partitioning. Most interestingly, defective cwINVs have been described in several plant species. Their highly attenuated sucrose (Suc)-hydrolyzing capacity is due to the absence of aspartate-239 (Asp-239) and tryptophan-47 (Trp-47) homologs, crucial players for stable binding in the active site and subsequent hydrolysis. However, so far, the precise roles of such defective cwINVs remain unclear. In this paper, we report on the functional characterization of tobacco (*Nicotiana tabacum*) Nin88, a presumed fully active cwINV playing a crucial role during pollen development. It is demonstrated here that Nin88, lacking both Asp-239 and Trp-47 homologs, has no invertase activity. This was further supported by modeling studies and site-directed mutagenesis experiments, introducing both Asp-239 and Trp-47 homologs, leading to an enzyme with a distinct Suc-hydrolyzing capacity. In vitro experiments suggest that the addition of Nin88 counteracts the unproductive and rather aspecific binding of tobacco cwINV1 to the wall, leading to higher activities in the presence of Suc and a more efficient interaction with its cell wall inhibitor. A working model is presented based on these findings, allowing speculation on the putative role of Nin88 in muro. The results presented in this work are an important first step toward unraveling the specific roles of plant defective cwINVs.

The nonreducing disaccharide Suc, the primary end product of photosynthesis, plays a central role in plant metabolism and development (Salerno and Curatti, 2003) and is used as the major transport sugar in plants (Koch, 2004). Besides its central position as a nutrient, this key sugar has a major role in carbohydrate partitioning and acts, together with Glc and Fru, as an important signal molecule regulating different processes throughout the plant life cycle (Bolouri-Moghaddam et al., 2010; Smeekens et al., 2010; Ruan, 2012). Metabolic use of Suc as a carbon or energy source through respiration requires the cleavage of its glycosidic bond. In plants, this reaction can be catalyzed by two types of enzymes: Suc synthase (EC 2.4.1.13) reversibly hydrolyzes Suc into UDP-Glc and Fru, whereas the irreversible hydrolysis into Glc and Fru is catalyzed by invertase (EC 3.2.1.26). Since both the substrate and reaction products of invertases are not only important nutrients but also are recognized as pivotal regulatory molecules controlling gene expression related to plant

metabolism, stress resistance, growth, and development, invertases are fundamental enzymes in the control of plant cell differentiation and plant development (Roitsch and González, 2004; Ruan et al., 2010).

Invertases are classified into two major groups with different biochemical properties and localized in different subcellular compartments (Tymowska-Lananne and Kreis, 1998; Koch, 2004). Neutral/alkaline invertases (A/N-Invs) are nonglycosylated proteins characterized by a neutral to alkaline pH optimum classified in glycoside hydrolase family 100 (GH100; Sturm et al., 1999; Lammens et al., 2009). These enzymes have long remained poorly studied, and for decades, A/N-Invs were believed to occur exclusively in the cytosol. However, recent studies clearly demonstrated the presence of A/N-Invs in various organelles in addition to the cytosol (Vargas et al., 2008; Xiang et al., 2011). It is now clear that A/N-Invs play an important role in plant development and stress tolerance (Barratt et al., 2009; Vargas and Salerno, 2010; Xiang et al., 2011). Acid invertases are glycosylated proteins localized in the vacuole (vacuole invertases [vINVs]) or in the apoplast (cell wall invertases [cwINVs]) and belong to the family GH32, together with plant fructan-metabolizing enzymes. Close relationships between vINVs and fructan-biosynthesizing enzymes, on the one hand, and cwINVs and fructan-breakdown enzymes, on the other hand, were described (Ritsema et al., 2006; Lammens et al., 2009). vINVs play important roles in cell expansion (Wang et al., 2010), and they determine the sugar composition in sugar-storing sink organs (Tang et al., 1999; Bhaskar

¹ This work was supported by the Fund for Scientific Research Flanders (grant no. 1209709N00 to K.L.R.) and the Research Fund of the Katholieke Universiteit Leuven (grant to W.L.).

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^[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.112.209460

et al., 2010). cwINVs are typically characterized by a high pI, which allows them to bind ionically to the cell wall. cwINVs often play a crucial role in phloem unloading in sink tissues by creating a Suc gradient, enhancing sink strength-mediated long-distance transport of Suc (Sturm, 1999). Their function is most prominent in sinks with no plasmodesmatal connections between cells, such as developing seeds and pollen (Proels et al., 2006; Hirsche et al., 2009; Kang et al., 2009). The expression and activity of cwINVs are regulated by a number of stimuli that are known to affect carbohydrate requirements, such as phytohormones (Balibrea-Lara et al., 2004; Hayes et al., 2010) and pathogen infection (Benhamou et al., 1991; Siemens et al., 2011). The activity of cwINVs is not only tightly regulated at both transcriptional and posttranscription levels; at the posttranslational level as well, cwINVs can be targeted by endogenous compartment-specific inhibitor proteins (Rausch and Greiner, 2003; Ruan et al., 2009).

Recently, deeper insights into the targeting mechanism of cwINVs by specific inhibitor proteins were provided by structural data on the cwINV/inhibitor complex (Hothorn et al., 2010, and refs. therein). A Glu in the complex interface was previously identified as an important quantitative trait locus affecting fruit quality (Fridman et al., 2004), leading to the hypothesis that not cwINVs as such but cwINV/inhibitor complexes should be considered as the main regulators of carbon partitioning in plants, also fulfilling crucial roles during leaf senescence, fruit and seed development, and the initial stages of host-pathogen interactions (Jin et al., 2009; Bonfig et al., 2010).

Plant cwINVs and fructan-breakdown enzymes (fructan exohydrolases [FEHs]) are closely related at both the molecular and structural levels, but they are functionally different. FEHs are unfunctional enzymes hydrolyzing terminal Fru-Fru linkages in fructans and are unable to hydrolyze the Glc-Fru linkage of Suc (Van den Ende et al., 2000). By contrast, cwINVs can be considered as β -fructosidases not exclusively hydrolyzing Suc but also, although to a limited extent, releasing terminal Fru residues from other oligosaccharides such as raffinose, stachyose, and 1-kestotriose (De Coninck et al., 2005). It is generally postulated that FEHs evolved from cwINVs by a few mutational changes. Indeed, it could be clearly demonstrated by site-directed mutagenesis experiments on *Arabidopsis thaliana* Cell Wall Invertase1 (AtcwINV1) that the ability to hydrolyze Suc is determined by the presence of the crucial amino acids Asp-239 and Trp-47, allowing Suc to bind in a stable way in the active site and to be subsequently hydrolyzed (Le Roy et al., 2007a). It was postulated that the presence/absence of an Asp-239 and Trp-47 homolog can be considered as a reliable tool to predict the functionality of (new) members within the cwINV/FEH group.

Intriguingly, three of the six cwINV-encoding complementary DNAs (cDNAs) of *Arabidopsis* turned out to encode FEHs (later renamed as defective cwINVs

with remaining FEH side activities), which highlights the necessity for a thorough functional analysis within the cwINV/FEH group (De Coninck et al., 2005). Most likely, such defective cwINVs are ubiquitous in higher plants, since homologs are found in (the genomes of) sugar beet (*Beta vulgaris*; Van den Ende et al., 2003), rice (*Oryza sativa*), maize (*Zea mays*), potato (*Solanum tuberosum*), poplar (*Populus* spp.), and chicory (*Cichorium intybus*). Although functions in defense and signaling were proposed (Van den Ende et al., 2004), and despite extensive research efforts, no hard evidence could be generated to uncover the precise roles of defective cwINVs in planta.

Most interestingly, it has been demonstrated that Nin88, previously designated as a cwINV of tobacco (*Nicotiana tabacum*), plays a crucial role in the early stages of pollen development in tobacco. Tissue-specific suppression of Nin88 resulted in male sterility (Goetz et al., 2001). In this paper, we report on the functional characterization of Nin88, lacking both crucial Asp-239 and Trp-47 homologs. Together with site-directed mutagenesis and modeling studies, it was demonstrated that the *Nin88* cDNA does not encode a catalytically active cwINV. The data presented in this work should be considered as an important step toward understanding the role of defective cwINVs in plants.

RESULTS

Cloning of the Full-Length *Nin88* cDNA

Although it is widely accepted that the *Nin88* gene of tobacco encodes a fully active cwINV with a crucial role during pollen development, only a partial cDNA was obtained (Goetz et al., 2001; GenBank accession no. AF376773), and the corresponding enzyme was never fully characterized to confirm its assumed functionality. Therefore, the full-length *Nin88* cDNA was generated by a PCR-based 3' RACE strategy using tobacco pollen mRNA. The deduced amino acid sequence shows a very high homology (75%) to tobacco Cell Wall Invertase1 (NtcwINV1; GenBank accession no. X81834), which has been fully characterized as an active cwINV residing in the apoplast (Greiner et al., 1995; Fig. 1). Both NtcwINV1 and Nin88 contain very similar N-terminal sequences (Fig. 1), and both proteins show high pI values (9.1 and 8.5, respectively) for interaction with the cell wall. An apoplastic localization for Nin88 is also predicted by the Plant mPLOC prediction tool (www.csbio.sjtu.edu.cn/bioinf/plant-multi). So far, not a single high-pI cwINV is known to reside in the vacuole. Although some low-pI vacuolar FEHs contain an extra vacuolar targeting motif, no such motif is present in Nin88 (L. Xiang and W. Van den Ende, unpublished data). A detailed comparison of both cDNA-derived amino acid sequences reveals a striking difference in putative N-glycosylation sites (N-X-T/S). In Nin88, three putative N-glycosylation sites seem to be selectively mutated when compared with NtcwINV1 (Fig. 1).

Figure 1. Sequence alignment of the full-length cDNA deduced amino acid sequences of tobacco NtcwINV1 (GenBank accession no. X81834), tobacco Nin88 (GenBank accession no. KC788220), and Arabidopsis AtcwINV1 (GenBank accession no. AY079422). For the Nin88 sequence, the newly cloned region is presented in italics. The putative N-terminal amino acids of the mature proteins are indicated with the arrow. The three catalytic residues of the active site are in italics and magnified, and the amino acids that are crucial for stable Suc binding and hydrolysis (Le Roy et al., 2007a) and/or subjected to site-directed mutagenesis are magnified and underlined. Putative N-glycosylation sites are in italics and underlined.

NtcwINV1	--MEFLRNSLSLW--LQILLLVGFVFSNNGVDASHKVYMHQLQSTS-SNVKNVHRTGY 54
NtNin88	--MELFRKSSFHCALPVFILLVCLFIIILSNYVVFAPNYDVFTCFQSSKDA NI TTSNYRTGY 58
AtcwINV1	MTKEVCSNIGLWL---LLTLLIGNYVFN--LEASHHVYKRLTQSTNTKSPSNVQPYRTGF 55
NtcwINV1	HFQPPKN W IN D PNGPMYNGVYHLFYQYNPKGAV W GNIVWAHVSVDLNLINWIPLEPAIYP 114
NtNin88	HFQPPKN C MN D PNAPMYNGVYHLFYQYNPKGST M NNIVWAHVSVDKDLINWINLEPAIYP 118
AtcwINV1	HFQPPKN W MN D PNGPMIYKGIYHLFYQYNPKGAV W GNIVWAHSTVDLNLINWDPPHAIYP 115
	Trp20 Trp47
NtcwINV1	SKIFDKFGT W SGSATILPGNKPIILYTG I I D ANRTQVQNYAIPAN LS DPYLRKWKIPDNN 174
NtNin88	SKPFDKYGT W SGSATILPGNKPIILYTG V V D ANMTQVQNYAVPAN LS DPYLRWKNKPDNN 178
AtcwINV1	SAPFDINGC W SGSATILPGNKPVILYTG-IDPKNQVQNI A EPK NL SDPYLRWKKSPLN 174
	Trp82
NtcwINV1	PLIVADMS-- I N K TQ F R D PTTAWMGRDGHWRILVGSVKNHRGKVIYRSKDFMKWTKAKH 232
NtNin88	PLIVPDIS--ITKTQ F R D PTTAWMGKDGHWIRVVGSSRNRRGLLAIYRSRNFMKWIKAEH 236
AtcwINV1	PLMAPDAVNGI N AS S F R DPTTAWLGQDKKRWVIGSKIHRRGLAIYTSKDFLKWEKSPE 234
NtcwINV1	PLHTATNTGN E CPDFFPVSLKHTNGLDTSYRG---EYTKHVLKVS L DVTRFEYYTVGTY 289
NtNin88	PLHSSAKTGN E CPDFFPVSLQGSNGLDASYNG---KYVKYVLKNS L PVAAFEYYTIGTY 293
AtcwINV1	PLHYDDGSGM E CPDFFPVTRFGSNGVETSSFGPENEILKHVLIK S LD D TKHDYTTIGTY 294
	Asp239
NtcwINV1	DTRKDRYIPD N TSV D GWKGRLRLDYGNYASKSFFDPSKNRRIMLGWA N ESD-TVDDDVVK 348
NtNin88	DAKQDRYIPD N TSV D GWKGRLRLDYGIFYASKSFYDPSKDRRIVGWGSYELDGLPNNENNK 353
AtcwINV1	DRVKDKFVPDNGFKMDGTAPRYDYGKYASKTFDPSAKNRRILGW T N E SS-SVEDDVVK 353
NtcwINV1	GWAGVHPIPRKLWLDPSGKQLVQWPVEELETLRKKKVQ-LSNHKLYKGEMIEVKGITVAQ 407
NtNin88	GWAGIQAI PRKVWLDPSGKQLVQWPIEELKTLRQKNVR-LSNKRLDNGEKIEVKGITASQ 412
AtcwINV1	GWSGIQTI PRKIWLDRSGKQLIQWPVEVERLRTKQVKNLRNKLKSGSRLEVYGVTAQ 413
NtcwINV1	ADVEVTFSFASLDKAEPPDPSWADLYAQDVCAIKGSTVQGGRRPFLITLASKNLEEYTL 467
NtNin88	ADVEVTFSSSLDKAEPPDPSWADLYAQDVCAIKGSTVPGGLGPPGLATLASQNLEEYTP 472
AtcwINV1	ADVEVLFKVRDLEKADVIEPSWTDPP--QLICSMNVSVKSGLGPPGLMVLASKNLEEYTS 471
NtcwINV1	VFRVFKAQDK---YKVLMSDASRSTL K N ET M YKPSFGGYVDVLDADKLSLRS L IDN 524
NtNin88	VFRVFKAQEN---FKVLMSDATR S T T Q H YNAMCKPSFAGYVDVLDV D KLSLRS L IDN 529
AtcwINV1	VYFRIFKARQNSNKYVVLMSDQSRSS L KEDN--DKTTYGAFVDIN-PHQPLSLRALIDH 528
NtcwINV1	SVVESFGAGGKTCITSRVYPTLAIFDKAHLFAFN NG TEAITIETLN A WSM A DAKLH 580
NtNin88	SVVESFGAGGK C ITSRAYPTLA I HDKAHLFAFN NG TE M IT I ETL D AWS M D T PKIN 585
AtcwINV1	SVVESFGGKGRACITSRVYPKLAIGKSSHLFAFN Y GYQ S VDV L NLN A WS M NS A Q T S 584

Sequence and Structural Modeling Data of Nin88 Predict an Inactive Invertase

Despite the high homology between the amino acid sequences of Nin88 and NtcwINV1, it is incorrect to presume and ascribe the invertase functionality to Nin88 based only on these criteria. Indeed, a detailed inspection of the Nin88 sequence reveals the absence of some amino acids known to be crucial for invertase functionality. Earlier site-directed mutagenesis studies on AtcwINV1 demonstrated that the highly conserved Asp-239 is crucial for the hydrolysis of Suc (Le Roy et al., 2007a). Moreover, the presence of some conserved Trp residues (Trp-20, Trp-47, and Trp-82) was shown to be essential for further stabilizing Suc binding in the active site (Le Roy et al., 2007a). Strikingly, no Asp-239 homolog is present in Nin88 (Fig. 1), already predicting that Nin88 cannot be a typical invertase with a distinct Suc-hydrolyzing capacity. Intriguingly, both Trp-20 and Trp-47 homologs are absent in Nin88 (Fig. 1), further supporting this hypothesis.

Structural modeling of both Nin88 and NtcwINV1 shows the intactness of the catalytic triad in both cases (Fig. 2). However, the absence of structural homologs of Asp-239, Trp-20, and Trp-47 in the active site of Nin88 is noticeable (Fig. 2). In addition, a putative unfavorable orientation of Trp-82 can be observed in Nin88 as compared with its homolog in NtcwINV1.

Heterologous Expression of Nin88 and NtcwINV1 in *Pichia pastoris*: Nin88 Encodes a Catalytically Inactive Invertase

Heterologous expression in the methylotrophic yeast *P. pastoris* is generally considered as a reliable method to study the functionality of GH32 enzymes (Van den Ende et al., 2009). Both full-length cDNAs of Nin88 and NtcwINV1, starting from the presumed N terminus of the protein (Fig. 1), were cloned into the pPicZαA vector and heterologously expressed in *P. pastoris* to derive their exact functionality. Based on the

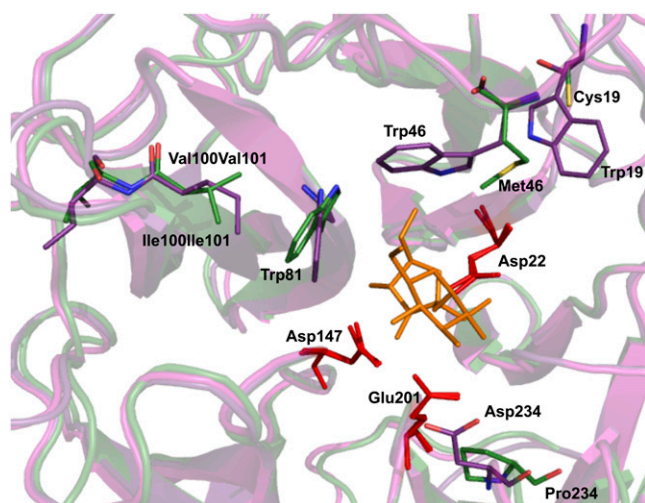


Figure 2. Superposition of the active-site regions of NtcwINV1 (purple) and Nin88 (green) shows the absence of three crucial amino acids, Trp-19, Trp-46, and Asp-234, and the unfavorable orientation of Trp-81 in Nin88. The three catalytic residues of the active site are shown in red. Modeling of both sequences was performed based on the known three-dimensional structures of AtcwINV1 (PDB code 1ST8) and chicory 1-FEH IIa (PDB code 2AC1) using modeller9v7. Suc, as present in AtcwINV1 (PDB code 2QQV), is shown in orange. The figure was prepared with PyMol (Delano, 2002).

amino acid sequence of Nin88 (Fig. 1), it was expected that the Nin88 gene would not encode an enzyme with “normal” invertase activity when compared with other well-characterized invertases. Incubation experiments with recombinant Nin88 at different Suc concentrations (up to 1 M final concentration) demonstrated that Suc is not a substrate for Nin88, at any concentration (Fig. 3A). Comparable to the empty-vector control (pPicZ α A), no invertase activity could be detected (Fig. 3B), not even during long-term incubations (up to 96 h; data not shown). Likewise, no hydrolytic activity could be detected after long-term incubations with other putative substrates such as 1-kestotriose, inulin, and levan (Fig. 4). Taken together, these results show that Nin88 is a catalytically dead enzyme, which was rather unexpected since other previously investigated “defective cwINVs” (e.g. from *Arabidopsis*) still contained residual invertase and FEH activities (De Coninck et al., 2005). Therefore, proper integration of the *Nin88* cDNA in the *P. pastoris* genome was ascertained and could be confirmed by PCR with Nin88-specific primers and sequencing of the resulting PCR products. Furthermore, western-blot analysis with NtcwINV1 antibodies on Nin88 protein fractions showed a band of around 70 kD (Supplemental Fig. S1), demonstrating the presence of the recombinant protein in the yeast supernatant. In further experiments investigating whether Nin88 might be characterized by some alternative substrate specificity among GH32 enzymes, the recombinant protein was incubated with arabinan and arabinosaccharides, and also putative

endohydrolase activity was tested on inulin, levan, and arabinan substrates. In all cases, no hydrolyzing activity could be detected (data not shown).

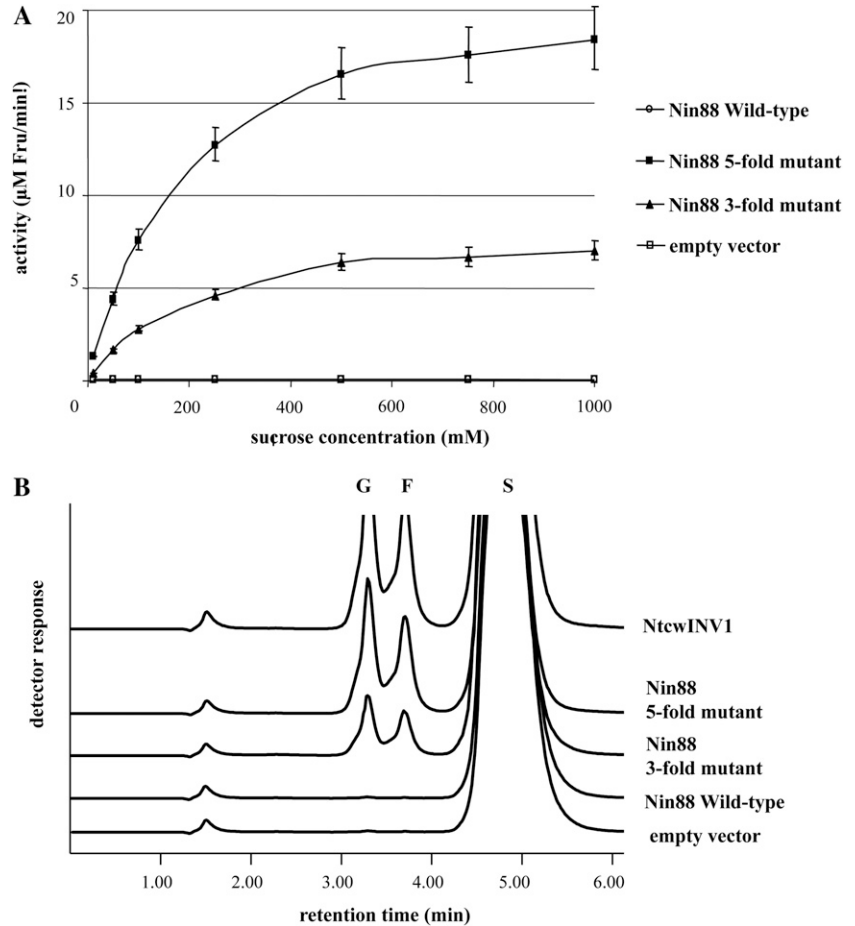
Introducing Invertase Activity in Nin88 by Site-Directed Mutagenesis

The viewpoint that Nin88 should be considered as a true and fully defective cwINV was further confirmed by well-chosen site-directed mutagenesis experiments in the active-site region of Nin88. As mentioned before, three crucial amino acids for Suc binding and hydrolysis, Trp-19, Trp-46, and Asp-234, are absent in Nin88. First, the single mutations Cys-19Trp, Met-46Trp, and Pro-234Asp were introduced, but, not unexpectedly, on their own they did not lead to the creation of invertase activity in Nin88 (data not shown). Further mutagenesis experiments, constructing the combined 3-fold Nin88 mutant Cys-19Trp/Met-46Trp/Pro-234Asp, resulted in the introduction of a clear invertase activity (Fig. 3). Moreover, even higher invertase activities could be obtained in the 5-fold Nin88 mutant Cys-19Trp/Met-46Trp/Pro-234Asp/Val-100Ile/Val-101Ile (Fig. 3). Homology modeling suggests that Val-100 and Val-101 are possibly responsible for the unfavorable orientation of the Nin88 Trp-81 residue as compared with their Ile counterparts in NtcwINV1 (Figs. 1 and 2). It was previously described that the orientation of this Trp residue is also important for Suc catalysis (Yuan et al., 2012). By altering its orientation in chicory 1-FEH IIa, a small invertase activity could be introduced (Le Roy et al., 2008). Although the difference between Val and Ile is rather small, we postulated that they could be the reason for the more twisted position of Trp-81 toward the active site in NtcwINV1. This reasoning was further sustained by additional homology modeling efforts on 3-fold and 5-fold Nin88 mutants demonstrating that the introduction of two Ile residues results in an altered orientation of the Trp-81 residue that is identical to the orientation of Trp-81 in NtcwINV1 (Fig. 5). Moreover, docking experiments of Suc into the active site of the Nin88 model did not lead to a clear Suc-binding mode (data not shown), as was the case for AtcwINV1 (Lammens et al., 2009). By contrast, the same experiments on the Nin88 3-fold and 5-fold mutant models predicted the expected Suc-binding modes. Collectively, all the data provide enough evidence for the observed inactivity of the recombinant Nin88 protein, strongly suggesting that Suc can hardly bind to the Nin88 protein, and the fraction that might bind is not in the right conformation to allow Suc hydrolysis.

Nin88 Mutants Behave as Typical Invertases with a β -Fructosidase Side Activity

The 3-fold and 5-fold Nin88 mutants were also screened with other substrates besides Suc (Fig. 4). It is common that acid invertases (β -fructosidases) show

Figure 3. A, Comparison of the activities of recombinant Nin88 wild type, 3-fold mutant, and 5-fold mutant enzymes together with the empty vector pPicZαA control as a function of increasing Suc concentrations. B, Chromatographic pattern of recombinant NtcwINV1, Nin88 wild type, 3-fold mutant, and 5-fold mutant enzymatic activities as compared with the empty vector pPicZαA control. The reaction conditions were as follows: incubation of the recombinant protein with 100 mM Suc in 50 mM sodium acetate buffer, pH 5.0, during 60 min at 30°C. G, Glc; F, Fru; S, Suc. Results are means ± SE (n = 3).



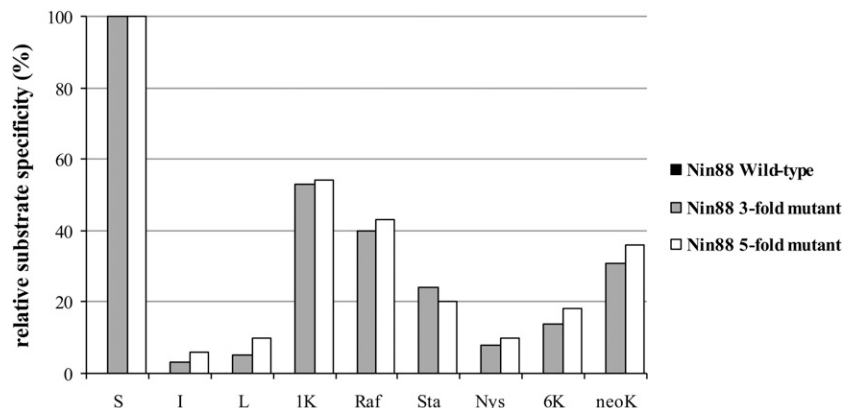
side activity toward several oligosaccharide substrates such as fructan trisaccharides, raffinose, and stachyose (Tymowska-Lananne and Kreis, 1998). Both Nin88 3-fold and 5-fold mutants behave as typical invertases, since their relative substrate specificities are fully comparable with other invertases (e.g. AtcwINV1; De Coninck et al., 2005). Generally, it can be concluded that the introduction of the three crucial amino acids Trp-19, Trp-46, and Asp-234 in Nin88 leads to mutant enzymes with a clear Suc-hydrolyzing activity that, in

addition, behave as typical acid invertases with distinct β-fructosidase side activities.

Toward Understanding the Putative Role of Catalytically Inactive Nin88

In an attempt to shed light on the putative function of Nin88, in vitro experiments were performed in which Nin88, NtcwINV1, and its inhibitor CIF (for cell

Figure 4. Comparison of the relative substrate specificities of recombinant Nin88 wild type, Nin88 3-fold mutant, and Nin88 5-fold mutant enzymes. The results are shown as values relative to the Suc-hydrolyzing activity. S, Suc; I, inulin; L, levan; 1K, 1-kestotriose; Raf, raffinose; Sta, stachyose; Nys, 1,1-nystose; 6K, 6-kestotriose; neoK, 6^G-kestotriose.



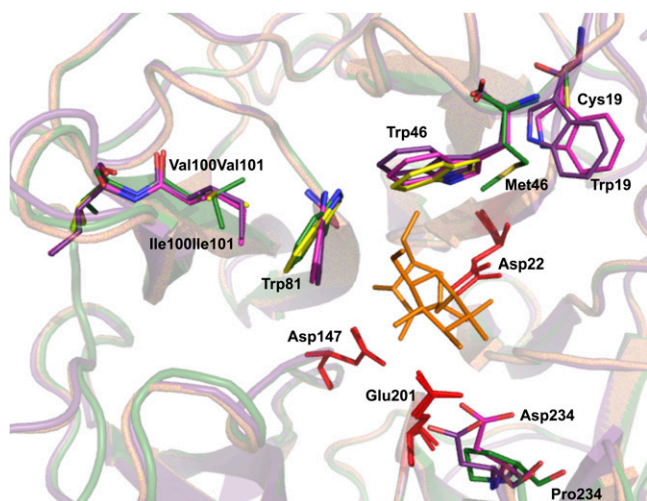


Figure 5. Superposition of the active-site regions of NtcwINV1 (purple), Nin88 wild type (green), Nin88 3-fold mutant (yellow), and Nin88 5-fold mutant (magenta) showing the difference in orientation of the Trp-81 homolog. The three catalytic residues of the active site are shown in red. Modeling of all sequences was performed based on the known three-dimensional structures of chicory 1-FEH IIa (PDB code 2AC1) and AtcwINV1 (PDB code 1ST8) using modeller9v7. Suc, as present in AtcwINV1 (PDB code 2QQV), is shown in orange. The figure was prepared with PyMol (Delano, 2002).

wall inhibitor of β -fructosidase; Greiner et al., 1998) were combined to investigate the possible effect on the overall NtcwINV activity. Intriguingly, as shown in Figure 6A, Nin88 unambiguously enhances the activity of NtcwINV1 when they undergo a preincubation period before Suc is added to the enzyme mixture. This enhancement was not detected when Nin88 was excluded from the preincubation. When CIF was added to the preincubation mixture containing Nin88 and NtcwINV1, the stimulatory effect of Nin88 on NtcwINV1 vanished. Paradoxically, almost no invertase activity could be detected in this case (Fig. 6A). Next, it was examined whether Nin88 could be replaced by bovine serum albumin (BSA). NtcwINV1 activity also increased when BSA was added to the reaction mixture (Fig. 6B). Similarly, the strong inhibition of NtcwINV1 by CIF was also observed by adding BSA instead of Nin88 (Fig. 6C). Increasing the volume-surface ratio in the reaction tube resulted in an increase of the invertase activity (Fig. 6B) and a slightly stronger inhibition by CIF (Fig. 6C). Taken together, these data strongly suggested that NtcwINV1, in the absence of a cell wall fraction in the tube, interacts strongly with the wall of the reaction tube, thereby negatively influencing its overall activity. Indeed, if Suc was added directly to the reaction mixture without any preincubation step, a significantly higher activity was detected compared with the condition in which Suc was added after a preincubation period, allowing NtcwINV1 to bind in an aspecific way to the reaction tube wall rather than to its substrate (Fig. 6B). Since

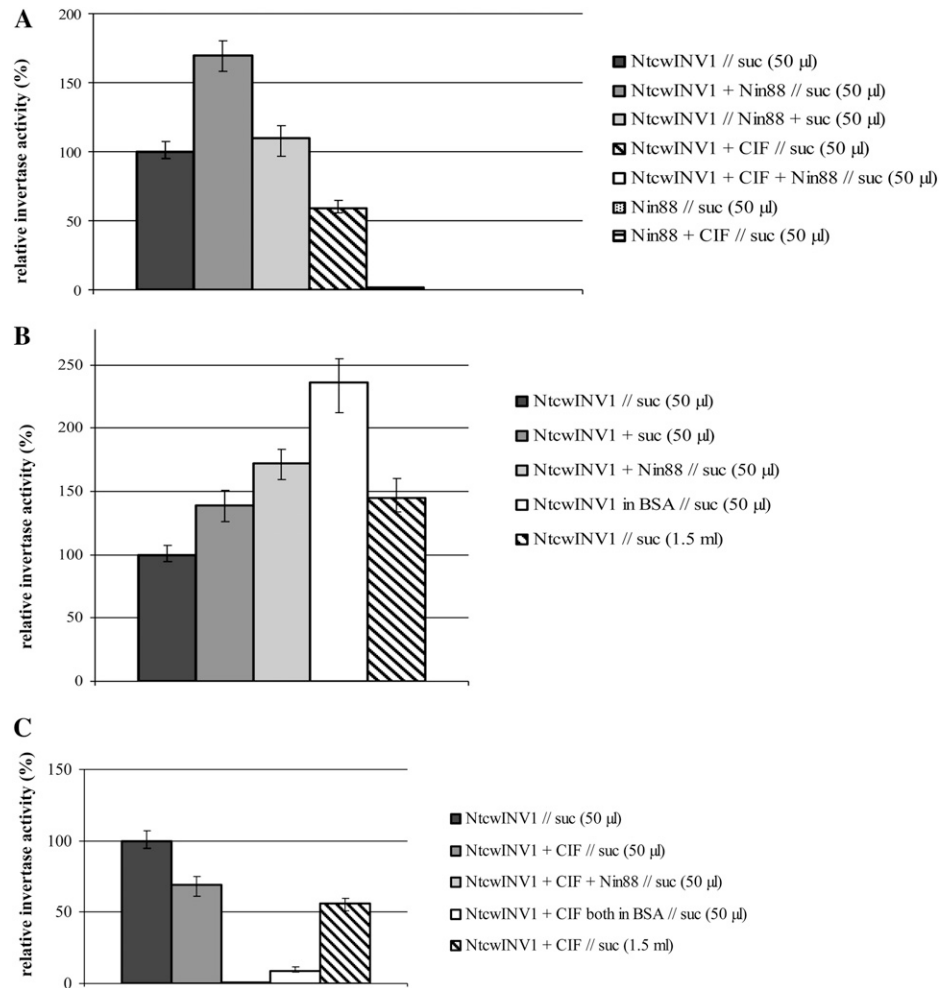
Nin88 and NtcwINV1 are known to occur together in tobacco pollen (Engelke et al., 2010), we isolated pollen cell walls and added them to the reaction mixtures (Fig. 7). Addition of the pollen cell wall resulted in increasing NtcwINV1 activities (Fig. 7). Moreover, a further increase was noted when the pollen cell wall and Nin88 were combined (Fig. 7). Furthermore, CIF addition was also more effective when pollen cell wall and Nin88 were combined (Fig. 7).

DISCUSSION

It is generally accepted that FEHs in fructan plants evolved from cwINVs and can be considered as defective cwINVs that lost their Suc-degrading capacity and further developed FEH capacity by additional mutational changes (Van den Ende et al., 2000, 2009; Le Roy et al., 2007a, 2007b). Probably, their counterparts in non-fructan-accumulating plants evolved from cwINVs too. Nowadays, the presence of such defective cwINVs seems ubiquitous in higher plants, but their precise roles remain puzzling.

It is demonstrated here that the tobacco *Nin88* cDNA does not encode an active cwINV, as generally presumed, but a protein with no Suc-degrading capacity, even toward very high Suc concentrations and after long-term incubations (Figs. 3 and 4). Unlike other previously described defective cwINVs, such as *Arabidopsis* 6-FEH (formerly AtcwINV3) and 6&1-FEH (formerly AtcwINV6; De Coninck et al., 2005) and sugar beet 6-FEH (Van den Ende et al., 2003), tobacco *Nin88* has no remaining FEH or β -fructosidase side activities. The absence of any hydrolytic activity prompted us to double check the insertion of *Nin88* in the *P. pastoris* genome and the presence of the recombinant protein in the yeast supernatant by western-blot analysis (Supplemental Fig. S1). It can be concluded that tobacco *Nin88* represents a catalytically deficient enzyme, since it tests completely negative to an array of (so far known) typical GH32 enzyme substrates (Fig. 4). Similar to other described defective cwINVs, the loss of a distinct Suc-hydrolyzing capacity in *Nin88* is caused by mutations in the active-site region (Figs. 1 and 2), more specifically the absence of Asp-239, Trp-21, and Trp-47 homologs (Le Roy et al., 2007a). Introduction of these three residues (Cys-19Trp/Met-46Trp/Pro-234Asp) resulted in an enzyme with a manifest Suc-hydrolyzing activity (Fig. 3). Moreover, a distinct β -fructosidase side activity was obtained (Fig. 4), clearly demonstrating that the *Nin88* triple mutant behaves as a typical cwINV. Overall, these results further confirm and complement the previously described mutagenesis experiments on AtcwINV1 (Le Roy et al., 2007a), since the reverse mutagenesis experiments on *Nin88* as a defective cwINV now result in a clear introduction of cwINV. Taken together, this demonstrates that Asp-239, Trp-47, and Trp-21 homologs are crucial determinants for cwINV functionality. As such, additional defective cwINVs can be

Figure 6. A, Relative invertase activities after in vitro combinations of tobacco Nin88, NtcwINV1, and CIF. The component names before the “//” underwent a preincubation step at 30°C during 15 min before Suc was added. All reactions were performed in a 50- μ L final reaction volume. B, Stimulation of NtcwINV1 activity by adding Nin88 or BSA to the reaction mixture (reactions in a 50- μ L final reaction volume) or by increasing the total reaction volume to 1.5 mL. C, Effect of adding Nin88, BSA (both in 50- μ L final reaction volumes), or an increased total reaction volume of 1.5 mL on the inhibition of NtcwINV1 by CIF. All results are shown as values relative to the activity of NtcwINV1 alone incubated with Suc. Results are means \pm SE ($n = 3$).



predicted in, for instance, carrot (*Daucus carota*), tobacco, cassava (*Manihot esculenta*), rapeseed (*Brassica napus*), maize, rice, wheat (*Triticum aestivum*), and asparagus (*Asparagus officinalis*).

Surprisingly, among all known cwINV sequences described to date, not a single example can be found in which one (or more) of the three highly conserved catalytic residues is mutated. Therefore, it seems reasonable to assume that during evolution, defective cwINVs were mainly generated by mutating the Asp-239 homolog (as is the case in all FEHs; Van den Ende et al., 2009), allowing the binding of Suc as an inhibitor but not as a substrate, without interfering with fructan degradation (e.g. Suc-inhibited FEHs; Verhaest et al., 2007). Further mutations in the surrounding Trp residues probably led to gradually more inactivation or, as is the case for Nin88, a completely inactivated form.

Although in the past, functions in defense and signaling were proposed for this class of defective cwINVs (Van den Ende et al., 2004), the results presented here and before (Goetz et al., 2001) sustain the idea that defective cwINVs most probably fulfill a regulatory role in plant developmental processes. Similarly, such defective enzyme isoforms have also

been reported in starch and trehalose metabolism. For instance, the catalytically inactive β -amylase BAM4 is demonstrated to be a starch-binding protein required for starch breakdown in Arabidopsis chloroplasts (Fulton et al., 2008; Li et al., 2009), although its exact role needs to be further unraveled. Furthermore, the trehalose-6-P synthase (TPS) gene family in Arabidopsis consists of 11 TPS-like genes, of which only one or, possibly, two (TPS1 and TPS11) were demonstrated to be catalytically active, strongly suggesting that the remaining inactive proteins fulfill a putative regulatory role (Vandesteene et al., 2010; Singh et al., 2011).

As noted before, tobacco Nin88, which from this point on needs to be considered as a catalytically inactive protein, is recognized to play a crucial role during pollen development. Tissue-specific antisense repression lines of *Nin88* in tobacco resulted in male-sterile plants in which pollen development is arrested at the early stages (Goetz et al., 2001). Given the high degree of homology between Nin88 and NtcwINV1 (Fig. 1), it cannot be excluded that the former antisense approach by Goetz et al. (2001) might not have been fully specific for Nin88, also (partially) attenuating the expression of some pollen cwINV genes encoding fully

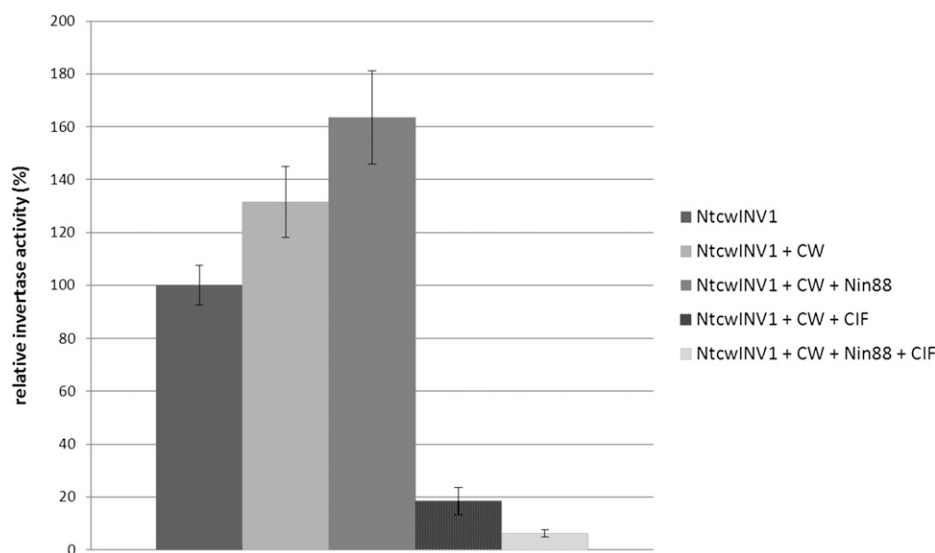


Figure 7. Relative invertase activities for the following combinations: NtcwINV1 (control), NtcwINV1 + pollen cell wall (CW), NtcwINV1 + CW + Nin88, NtcwINV1 + CW + CIF, and NtcwINV1 + CW + Nin88 + CIF. All combinations were preincubated for 20 min, after which the substrate Suc was added. Results are means \pm SE ($n = 3$).

active enzymes. From this point of view, the observed decreases in cwINV activities in these antisense lines might be explained both by repressing Nin88 as an activator and by directly repressing genes encoding fully active cwINVs. It can be concluded that the specific role of Nin88 in tobacco pollen development needs to be reconsidered. Furthermore, the previously supposed downstream Glc signaling effects cannot originate from Nin88 directly, since it is inactive. Instead, these sugar signals should arise from active cwINVs, indirectly controlled by Nin88 as a cwINV activator (see also below). In any case, the clear phenotype linked to Nin88 as a completely inactive defective invertase provides to our knowledge the first clue to unravel the specific and intriguing role of these proteins in plants.

Evidence is emerging that not cwINVs but rather cwINV/inhibitor complexes should be considered as the main regulators of carbon partitioning in plants (Hothorn et al., 2010). Therefore, CIF was also included in our *in vitro* experiments studying the effects of Nin88 on NtcwINV1 activity (Fig. 6). On the one hand, Nin88 was found to enhance the NtcwINV1 activity (Fig. 6A). On the other hand, Nin88 addition resulted in a stronger inhibition of the NtcwINV activity by CIF (Fig. 6A). These paradoxical results were difficult to explain in terms of changing NtcwINV1/CIF/Nin88 protein interactions. The fact that Nin88 could be replaced by BSA (Fig. 6B), a protein that is well known to interact with the walls of reaction tubes (Tarone et al., 1982), already suggested that the reaction tube walls themselves played a major role in explaining the observations. This was further confirmed by increasing the volume-surface ratio in the reaction tubes (Fig. 6). Aspecific binding to the reaction tube wall and associated cwINV activity losses could be counteracted by adding pollen cell wall to the reaction medium (Fig. 7), suggesting that NtcwINV1 prefers binding to the pollen cell wall rather than to the

reaction tube wall. Extra addition of Nin88 further enhanced this effect (Fig. 7), indicating that Nin88 should indeed operate in the extracellular environment, in line with previously obtained molecular data (Engelke et al., 2010). The biochemical data presented here suggest that both Nin88 and NtcwINV1 interact with the pollen cell wall through their high pIs. It can be speculated that Nin88 interacts with the pollen cell wall in such a way that the subsequent pollen cell wall/NtcwINV1 interaction becomes more optimal, resulting in a more productive (stable) binding modus and increased cwINV activities.

A working model is presented in Figure 8, explaining and summarizing all the observed data obtained during the performed *in vitro* experiments. Clearly, in the absence of pollen cell wall addition, the overall activity of NtcwINV1 is influenced by its interaction with the reaction tube wall, affecting the accessibility of the active site for Suc and CIF. Typically, GH32 enzymes consist of two domains, a β -propeller domain harboring the active site and a β -sandwich module that shows structural similarities to the concanavalin A-like lectins/glucanases superfamily (Alberto et al., 2004; Alvaro-Benito et al., 2010). Assuming a random interaction between NtcwINV1 and the wall of the reaction tube, part of the enzymes will interact with the wall via their β -propeller domain in such a way that the active site is not accessible for CIF or Suc (Fig. 8). Adding Nin88 or BSA (or increasing the volume-surface ratio) restricts the available wall-binding sites for NtcwINV1, resulting in more “free” and accessible NtcwINV1. Also, the inhibition of NtcwINV1 by CIF can be affected by this reaction tube wall-binding effect (Fig. 8). Since the effect of BSA and Nin88 on the inhibition is pronounced, it can be further hypothesized that also the CIF inhibitor shows an affinity for the reaction tube wall, which can be counteracted by adding Nin88. The results even indicate that Nin88 addition has a stronger impact on the wall binding of

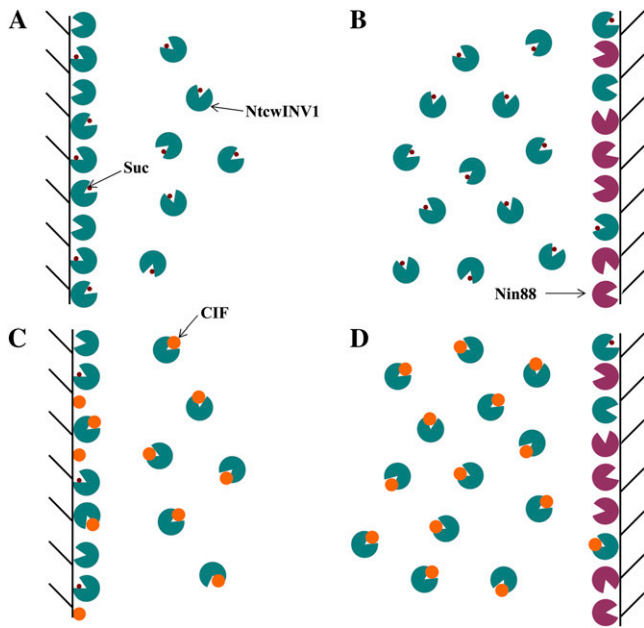


Figure 8. A model explaining the influence of Nin88 on the overall activity of NtcwINV1 in vitro. A, In the absence of added pollen cell wall, NtcwINV1 (shown in green) interacts randomly with the reaction vial wall, affecting the accessibility of Suc (shown in red) to its active site. B, The presence of Nin88 (shown in purple) results in more free NtcwINV1 (in a reaction tube without added pollen cell wall), resulting in an overall increase of the total invertase activity. C, The binding of NtcwINV1 to the reaction vial wall can affect both the accessibility of Suc and CIF (shown in orange) in a different way. Inhibition by free CIF is only possible on free NtcwINV1 or in the case of NtcwINV1 is bound to the reaction vial wall, still allowing CIF to reach its active site. D, The presence of Nin88 results in more free NtcwINV1, allowing a stronger inhibition of the overall invertase activity by CIF. When pollen cell wall is added in vitro, or when referring to the situation in muro, a rather similar model can be proposed, with the difference that the terms “random, aspecific reaction of NtcwINV1 with the reaction tube wall” and “free NtcwINV1” should be replaced by “aspecific interaction of NtcwINV1 with the pollen cell wall” and “specific, productive interaction with the pollen cell wall,” respectively. Indeed, it can be expected that the amount of soluble, free NtcwINV1 would be very low in muro or when pollen tube cell walls are added in the reaction tube.

CIF as compared with the wall binding of NtcwINV1 (Fig. 8, B and D).

To mimic the situation in muro, pollen cell walls were isolated and added in the presence and absence of extra Nin88 (Fig. 7). The data indicate, as expected, that NtcwINV shows a higher affinity for the pollen cell wall as compared with the polypropylene wall of the reaction tubes. Accordingly, apoplastic fluid isolations on tobacco leaves showed minimal cwINV activities, and repeated washing steps of isolated walls from tobacco did not substantially lower cwINV activities (data not shown). These observations confirmed the well-established view that cwINVs interact strongly with cell walls by ionic interactions (Kim et al., 2000). Hence, the level of “freely soluble” cwINV

in muro is probably very low, a situation that is clearly different from the one proposed in the in vitro model above (Fig. 8).

Referring to the situation in muro, it can be hypothesized that Nin88 might be involved in (so far unclear) interactions with the cell wall, leading to a more productive and stable binding of fully active cwINVs to the pollen cell wall. We speculate that the binding of cwINVs to the pollen cell wall should occur in such a way that their catalytic domains are fully exposed and available for interaction with substrate and/or inhibitor. It can be speculated that such efficient and unidirectional binding to the cell wall might be mediated by the β -sandwich module of the enzyme. This seems a reasonable hypothesis, since the carbohydrate-binding properties of the β -sandwich module were recently demonstrated (Le Roy et al., 2007b; Alvaro-Benito et al., 2010), showing that inulin-type fructans interact with the β -sandwich module. It would be interesting to further investigate whether the β -sandwich module also shows affinity for (different types of) cell wall polysaccharides. Similarly, the idea that specific interactions might occur between (defective) cwINVs and the plasma membrane seems attractive, since it was hypothesized that cwINVs might interact with sugar sensors in the plasma membrane (Ruan, 2012).

It can be further speculated that a duplication of a tobacco cwINV gene and its subsequent loss of catalytic activity resulted in the creation of the Nin88 defective isoform with less *N*-glycosylations (Fig. 1) and a slightly lower pI, perhaps making it somewhat more mobile in the apoplastic space as compared with fully active cwINVs, with their higher pIs and more extensive glycosylations. More detailed studies are required on the exact timing of the expression of Nin88 and active cwINVs in tobacco pollen and on their exact locations in the cell wall. However, it can be speculated that Nin88 and related defective invertases function as cell wall modifiers, interacting with cell walls in such a way that the more slowly diffusing catalytically active cwINVs can bind more efficiently later on. How Nin88 precisely interacts with cell wall constituents and how this could lead to a more productive cell wall binding of active cwINVs remain obscure, and this remains a challenging area for further research.

CONCLUSION

Tobacco Nin88 is not a functional cwINV, as previously thought. Instead, it is a defective cwINV that can be mutated back to a functional cwINV, indicating that its original function, linked to downstream Glc signaling, needs to be reconsidered. Our results led to a new working hypothesis in which Nin88 is proposed to conduct the interaction between functional cwINVs and the wall in such a way that the catalytic subunit of cwINV is more accessible for interaction with its substrate and/or inhibitor. This urges further research,

both in vitro and in muro, into the exact interactions between cell wall components and defective and fully active cwINVs or cwINV/inhibitor complexes.

MATERIALS AND METHODS

RNA Isolation and Cloning

RNA was isolated from tobacco (*Nicotiana tabacum*) anthers using the RNeasy Plant Mini Kit (Qiagen). A reverse transcription-PCR-based strategy was used for the cloning of the unknown 3' end of the *Nin88* cDNA using the specific forward primers Nin88F1 (5'-GATTTTTTCTGTTTCCTTGC-3') and Nin88F2 (5'-TAAGTACGTTCTCAAGAATAGCCTTCC-3'); both primer sequences were constructed based on the partial *Nin88* cDNA sequence [GenBank accession no. AF376773] combined with the reverse primers AWSM (conserved, 5'-GCYTTYTTCATRCTCCANGC-3') and an oligo(dT) primer. Subsequently, full length *Nin88* cDNA was obtained using the specific primers Ni-FOR (5'-CTATGTGTGGTTTGCCTTCAA-3') and Ni-REV (5'-TTAGTTATTTTTAGGTGTATCCATG-3') and cloned in the TopoXL vector (TopoXL PCR Cloning Kit; Invitrogen).

Heterologous Expression in *Pichia pastoris*

PCR was performed on the TopoXL-*Nin88* construct using the primers (with *EcoRI* and *SacII* restriction sites in boldface) Nin88Pic-F (5'-ACAGAATTCGCTAATACACTTCTAACTACAGAACT-3') and Nin88Pic-R (5'-GGTCCCCGGGTTAGTTATTTTAGGTGTAT-3') and on the pETM-NtcwINV1 plasmid (kindly provided by Dr. Steffen Greiner, Heidelberg University) using the primers NtlINV1-F (5'-ACAGAATTCCTAATGTCAAGAATGTTACAGAACT-3') and NtlINV1-R (5'-GGTCCCCGGGTTAGTGCAGCTTACATCGGC-3'). After restriction digest with *EcoRI* and *SacII*, the cDNA fragments were ligated in the pPicZa vector. The resulting expression plasmids *pNin88* and *pNtcwINV1* contain the mature protein part in frame behind the α -factor secretion signal sequence. Further handling and transformation of *P. pastoris* were as described by Le Roy et al. (2007b).

Genomic DNA Isolation and PCR Screening of *P. pastoris* Clones

After suspending *P. pastoris* cells in 200 μ L of a buffer of 10 mM Tris-HCl and 1 mM EDTA, pH 8.0, 200 μ L of a phenol, chloroform, isoamyl alcohol solution (25:24:1) was added. The cells were broken by adding glass beads and severe vortexing. After centrifugation (15 min, 16,000g, room temperature), 600 μ L of ice-cold 100% ethanol and 25 μ L of 3 M sodium acetate buffer, pH 4.8, were added to 150 μ L of the supernatant (top layer). The mixture was incubated for 20 min at -80°C and subsequently centrifuged (15 min, 16,000g, 4°C). A total of 100 μ L of sterile water was added to the completely dried pellet to dissolve the genomic DNA. PCR was performed using two internal *Nin88*-specific primers, Nin88int-F (5'-CTGGATGGGCAAGATGGT-3') and Nin88int-R (5'-GACCAACCCACACGATTTCT-3'), in order to check the proper integration of the *Nin88* cDNA in the *P. pastoris* genome.

Purification of the Recombinant Proteins

Purification of the proteins from the *P. pastoris* culture supernatant was achieved by 80% ammonium sulfate precipitation. Further purification of *Nin88* wild-type and mutant proteins was performed using the Fast Desalting Column HR 10/10 (Amersham Biosciences) as described by Le Roy et al. (2008). In the case of NtcwINV1, dialysis and subsequent loading on a Mono S column (Pharmacia Biotech HR 5/5) were performed as described by Verhaest et al. (2005). Concentration measurements of the purified enzymes were performed using the Bradford method with BSA as a standard.

Western-Blot Analysis

Nin88 protein was separated by SDS-PAGE using a loading buffer (2% SDS, 5% glycerol, 5% β -mercaptoethanol, and 0.01% bromophenol blue in Tris buffer, pH 6.8). After electrophoresis, the proteins were transferred from the gel to a membrane (Sequi-Blot PVDF Membrane; Bio-Rad) by electroblotting using a Tris-Gly buffer (25 mM Tris, 192 mM Gly, and 4 mL per 100 mL of methanol).

Subsequently, the membrane was incubated for 2 h at 37°C in blocking buffer (5% milk powder in 50 mM Tris-HCl, pH 7.5). After washing with Tris-saline (50 mM Tris-HCl, 0.09% NaCl, and 1 mL per 100 mL of Triton X-100, pH 7.5), the membrane was incubated overnight with NtcwINV1 antibody (kindly provided by Dr. Steffen Greiner) diluted in Tris-saline. Next, an incubation with anti-rabbit IgG conjugated to alkaline phosphatase (Sigma), 1:5,000 diluted in AP buffer (0.1 M Tris-HCl, 0.1 M NaCl, and 0.05 M MgCl_2 , pH 9.5), was performed during 45 min, and after washing with Tris-saline, the membrane was colored for a few minutes with 65 μ L of nitroblue tetrazolium (Sigma; 50 mg mL^{-1} 70% dimethylformamide) and 35 μ L of 5-bromo-4-chloro-3-indolyl phosphate (Sigma; 50 mg mL^{-1} 70% dimethylformamide) in 10 mL of AP buffer.

Site-Directed Mutagenesis

Single amino acid substitutions were generated following the Quick Change protocol (Stratagene) using the *pNin88* construct as a template. For site-directed mutagenesis, the following forward oligonucleotide primers (and complementary reverse primers; mutations are in boldface) were used: 5'-CCCCCAAGAACTGGATGAATGACCC-3' (Cys-19Trp), 5'-GGATCAACATG-GAACCAACATIGTTGGG-3' (Met-46Trp), 5'-GAATAGCCTTGATGTGCCGCG-3' (Pro-234Asp), and 5'-GTACTGGAATCATCGATGCCAACATG-3' (Val-100Ile/Val-101Ile). Further handling was as described by Le Roy et al. (2007a).

Enzyme Assays

For enzyme activity measurements, appropriate aliquots of purified enzyme were mixed with different concentrations of Suc (final concentrations, 10, 50, 100, 250, 500, and 750 mM and 1 M) in 50 mM sodium acetate buffer, pH 5.0. Relative substrate specificities were investigated by incubations with 5 mM Suc, 1-kestotriose, inulin, levan, 6-kestotriose, 6^G-kestotriose (neokestose), nystose, raffinose, and stachyose in 50 mM sodium acetate buffer, pH 5.0. All reaction mixtures were incubated at 30°C for different time periods. Sodium azide (0.02%, v/v) was added to prevent microbial growth. The interference of *Nin88* in the inhibition reaction of NtcwINV1 with its inhibitor CIF (Greiner et al., 1998) was tested through adding *Nin88* to a mixture of 12 nM purified NtcwINV1, 150 nM purified CIF (kindly provided by Dr. M. Hothorn), and 20 mM Suc. For *Nin88*, when compared with NtcwINV1, an equivalent amount of protein was taken from the fast desalting fractions. Appropriate dilutions of NtcwINV1 and CIF were made in 50 mM sodium acetate buffer, pH 5.0, or in 1% (v/v) BSA in the experiments investigating the role of BSA. The standard experiments were performed in a final reaction volume of 50 μ L. For the experiments performed in a 1.5-mL final reaction volume, equal protein and substrate concentrations were retained.

For all reactions, total enzyme activity was determined by measuring the amount of released Fru by high-performance anion-exchange chromatography with pulsed amperometric detection (Van den Ende and Van Laere, 1996). Different time points were analyzed, and only data from the linear range were used. All experiments were repeated three times with consistent results.

Preparation of the Tobacco Pollen Cell Wall

About 25 mg of closed and opened (with visible pollen) anthers from different developmental stages of opened flowers was boiled for 10 min in 1 mL of MilliQ water. After boiling, they were ground with mortar and pestle and washed five times with 1.5 mL of MilliQ water by subsequent centrifugation (5 min, 16,000g) and resuspension. Finally, the cell wall was resuspended in 300 μ L of 50 mM sodium acetate buffer, pH 5.0. The pollen cell wall concentration used in the reactions was 100 $\mu\text{g mL}^{-1}$.

Homology Modeling of *Nin88*

Homology models of the native *Nin88* were built in modeller9v7 (Eswar et al., 2006) with multiple template methods using the known protein crystal structures of AtcwINV1 (Protein Data Bank [PDB] code 2AC1; Verhaest et al., 2006) and 1-FEH Iia of chicory (*Cichorium intybus*; PDB code 1ST8; Verhaest et al., 2005) as model-building templates. The models were further optimized by subjecting them to the computer program Brugel (Delhaise et al., 1984). Finally, the optimized *Nin88* model was submitted to Molprobit (Chen et al., 2010) for a general model quality check. It was found that 94.4% of residues were in a Ramachandran-favored region and only 0.9% of residues were

within disallowed regions, which were all localized far from the active site. The models of two mutants (i.e. the 3-fold mutant Cys-19Trp/Met-46Trp/Pro-234Asp and the 5-fold mutant Cys-19Trp/Met-46Trp/Pro-234Asp/Val-100Ile/Val-101Ile) were built in modeller9v7 using its mutation protocol and subjected to Brugel for further refinements as described above. The optimized mutant models were submitted to Molprobit, and neither atom clashes nor Ramachandran outliers were found around the catalytic region.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers X81834 (NtcwINV1), KC788220 (NtNin88), and AY079422 (AtcwINV1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Western-blot analysis of Nin88.

ACKNOWLEDGMENTS

We thank Veerle Cammaer and Ingeborg Millet for their technical assistance.

Received October 19, 2012; accepted February 26, 2013; published February 27, 2013.

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