Sucrose protects cell wall invertase but not vacuolar invertase against proteinaceous inhibitors

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Abstract Vacuolar (VI) and cell wall invertases (CWI) of higher plants can be inactivated in vitro and, possibly, in vivo by proteinaceous inhibitors. The respective mechanisms have not yet been compared. Therefore, partially purified CWI from transformed tobacco cells and VI from tomato fruit were pre-incubated with invertase-inhibitor fractions isolated from the same tissues. Both inhibitors were able to inhibit both invertases. However, VI was fully inhibited within less than 1 min by both inhibitors, whereas inactivation of CWI was much slower. Furthermore, CWI, but not VI, was strongly protected against inhibition by sucrose. A polyclonal antiserum directed against the tobacco inhibitor (I_{NT}) cross-reacted with a 19 kDa polypeptide in the partially purified tomato inhibitor (I_{LE}) fraction. The results indicate that I_{NT} and I_{LE} have similar structural properties, whereas the mechanism of inactivation is clearly different for CWI and VI.

Key words: Cell wall invertase; Vacuolar invertase; Invertase inhibitor; Substrate protection; Solanaceae

1. Introduction

Cell wall (CWI) and vacuolar (VI) invertases play central roles in the sucrose metabolism of higher plants [1,2]. Both invertase types exist in different isoforms [3-5]. Sequence comparisons indicate a common evolutionary origin [4]. However, VI and CWI can be clearly distinguished based on structural and kinetic properties. Purified VI have K_m values ranging from 5 to 10 mM, or even higher [3], whereas CWI exhibit K_m values of 0.5–1.0 mM [5-7]. Furthermore, the pI of VI is around 5, whereas CWI are basic proteins with a pI around 9.5 [3,7,8].

Developmental control of gene expression has been observed for different isoforms of VI and CWI [9,10]. Furthermore, CWI has been implicated in the wound response and may be induced by pathogen attack [10]. Apart from control of gene expression, enzyme activity may be affected by modulation of vacuolar (VI) or apoplasmic (CWI) pH. In addition, plant invertases may be regulated by proteinaceous inhibitors found in different species [11-18]. However, it has been questioned whether inhibitors and invertases are localized in vivo in the same cellular compartment [19]. We have recently demonstrated that the invertase inhibitor expressed in tobacco cells transformed with *Agrobacterium tumefaciens* (I_{NT}) shows several characteristics which strongly indicate a role for an in-vivo regulation of CWI [5,18]: (i) I_{NT} is co-localized with CWI in the apoplasmic space, (ii) inactivation of CWI is modulated by divalent cations at millimolar concentrations and exhibits a pronounced pH-dependence between 4 and 6, (iii) CWI may be protected against inhibition by sucrose (half maximum protection at 1.2 mM), and (iv) CWI and I_{NT} form tight complexes in sucrose-starved cells. I_{NT} has been purified to homogeneity and a polyclonal antiserum has been raised [5,18]. In view of (i) the inconsistencies of previous studies on the localization of invertases and inhibitors, and (ii) the significant structural differences between CWI and VI, we have addressed the following question: do invertase inhibitors inactivate CWI and VI by the same mechanism? We have partially purified inhibitors from tomato fruit [20], I_{LE}, and transformed tobacco cells [18], I_{NT}, and characterized the inactivation of two plant invertases, i.e. VI from tomato fruit (in this tissue 3 highly similar VI isoforms are co-expressed [3]) with a K_m of 7.4–7.9 mM and a pI of about 5, and CWI from tobacco cells with a K_m of 0.6 mM and a pI of 9.5 [7]. The results indicate conspicuous differences in the mechanisms of inactivation.

2. Materials and methods

2.1. Plant material

Cell-wall proteins including CWI and I_{NT} were extracted from *Agrobacterium tumefaciens*-transformed cells (*Nicotiana tabacum* L. cv. Petit Havana, clone SR1-3845 [6,7]). The culture conditions have been described elsewhere [6]. Briefly, cells were grown for 3 weeks in the dark and, 24 h before harvesting, transferred to fresh medium without carbon and nitrogen sources. For isolation of tomato VI and I_{LE} ripe fruits of *Lycopersicon esculentum* L. cv. Bonny from the local market were used.

2.2. Partial purification and separation of invertases and invertase inhibitors

CWI and I_{NT} were extracted by salt-elution from intact cells and partially purified by $(NH_4)_2SO_4$-precipitation and ConA-affinity chromatography as previously described [7,18]. A protein fraction of tomato fruit containing VI and I_{LE} was isolated according to Pressey [20]. Briefly, after extraction of tomato fruits the crude homogenate was adjusted to pH 3 and centrifuged at $11\,000 \times g$ for 15 min. The pellet was washed in deionized H_2O and resuspended in 0.25 M NaCl. After acid precipitation at pH 1.6 and centrifugation the supernatant was adjusted to pH 6.5. The resulting protein fraction contained VI and I_{LE} which were separated by ConA-chromatography according to Weil et al. [18]. VI and I_{LE} were recovered from the ConA-bound and ConA-nonbound fraction, respectively.

2.3. Assay for invertase activity and in-vitro inactivation

The assay for invertase inactivation has been described elsewhere [18]. For dose–response experiments increasing amounts of inhibitor were added at 15 min (I_{LE}) or 60 min (I_{NT}) before starting the enzyme assay. To study the time-dependence of invertase inactivation constant amounts of inhibtor fractions were pre-incubated with CWI or VI for different time intervals. For substrate protection assays, invertases were pre-incubated with the respective inhibitor in the presence or absence of 20 mM sucrose. The glucose released during the subsequent invertase assay was corrected for the glucose released during the pre-incubation.

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2.4. In-vivo inactivation of CWI by I_{NT}

Cells were first starved for 24 h as described above (section 2.1.). Thereafter, cell clusters (diameter approx. 2 mm) were washed with distilled water and twice with 10 mM MES/TRIS buffer, pH 5.0, and briefly blotted dry on filter paper. MES/TRIS buffer and partially purified I_{NT} (1.5 ml final volume) were added to 0.5 g fresh weight of cells. Cells were pre-incubated at 25°C for 60 min. The CWI in-vivo assay was started by addition of sucrose at a final concentration of 20 mM. Cells without sucrose served as controls.

2.5. Preparation of polyclonal antiserum directed against I_{NT} and Western blot analysis

I_{NT} was purified to homogeneity as described by Weil et al. [18]. A rabbit polyclonal antiserum directed against I_{NT} was obtained which specifically cross-reacted with the inhibitor [5]. Crude and partially purified I_{LE} fractions were analyzed by Western blot according to Weil and Rausch [7].

3. Results and discussion

3.1. The invertase inhibitors of tomato, I_{LE}, and tobacco, I_{NT}, are structurally related

The purification and N-terminal sequences of I_{NT} (M_r 17 kDa) and I_{LE} (M_r 20 kDa) have been previously described [18,20]. An alignment of the N-terminal sequences reveals a strong similarity with 11 identical amino acids out of 16 residues. In order to further compare the two inhibitors we have analyzed I_{LE} from tomato fruit in the crude extract, the ConA-bound and the ConA-nonbound fraction, respectively, by Western blot (Fig. 1) using the polyclonal antiserum raised against I_{NT} [5,18]. In the crude extract and the ConA-nonbound fraction a polypeptide of approx. 19 kDa specifically cross-reacted with the tobacco antiserum, and the 19 kDa protein was clearly enriched in the latter fraction. The result indicates that the 19 kDa polypeptide is identical with I_{LE} [20]. We conclude that, based on their conserved N-terminus and their immunological properties, the invertase inhibitors expressed in tomato fruit and transformed tobacco cells are likely to be homologous proteins.

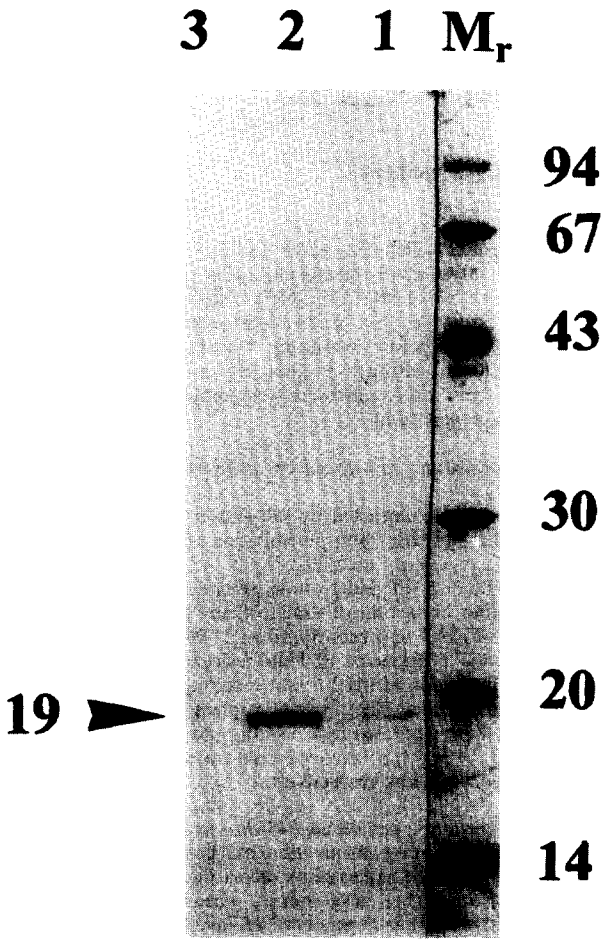

Fig. 1. Western blot of inhibitor-containing fractions from tomato fruit developed with a polyclonal antiserum directed against I_{NT} [5]. 1, supernatant after pH 1.6 precipitation; 2, ConA-nonbinding fraction; 3, ConA-binding fraction. Proteins (15 μg/lane) were separated by SDS-PAGE on a 13% gel.

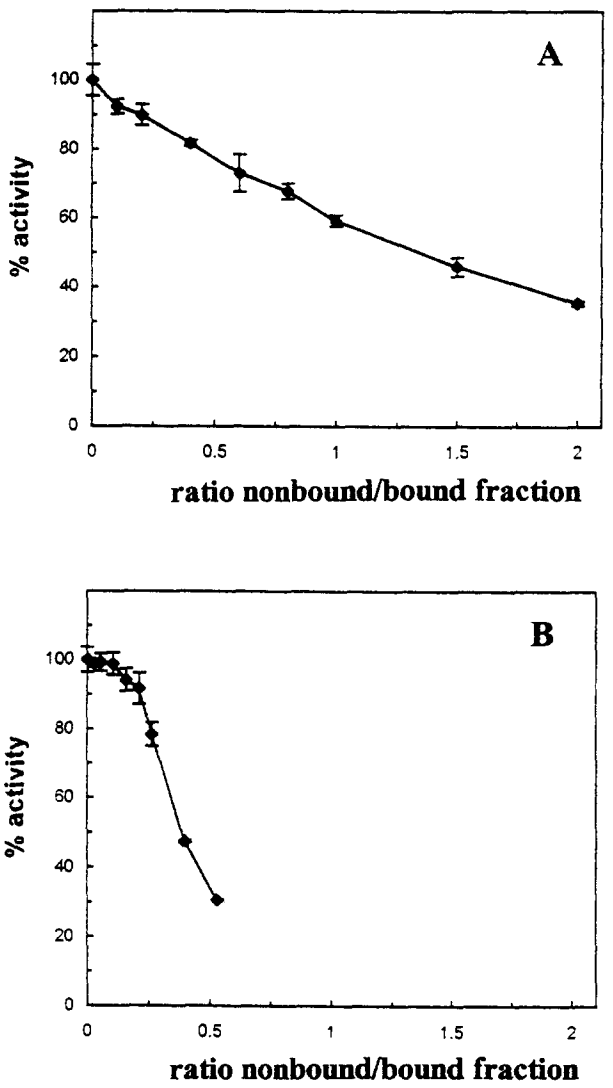

Fig. 2. Dose-dependent inactivation of partially purified tobacco CWI (ConA-bound fraction) by partially purified inhibitor-containing fractions (ConA-nonbound fraction) from transformed tobacco cells (A) and tomato fruit (B). A nonbound/bound fraction ratio of 1 corresponds to inhibitor and CWI extracted from 1 g fresh weight equivalent each. Inhibitor and CWI were pre-incubated for 60 min.

3.2. I_{NT} and I_{LE} fully inactivate both tobacco CWI and tomato VI

To determine whether I_{NT} and I_{LE} may equally inhibit CWI and VI, the dose-dependence of inactivation was analyzed (Figs. 2 and 3). The data are normalized based on the ratio

of fresh weight equivalents of inhibitor (ConA-nonbound fraction) and invertase (ConA-bound fraction). Assuming the formation of binary complexes [18] the results indicate that the tissue concentrations of invertases and inhibitors are of the same order of magnitude. However, it should be noted that the total recoveries of CWI, VI, I_{NT} and I_{LE} can not be assessed with certainty. Figs. 2 and 3 show that CWI and VI are inhibited by 70–80%. By further increasing the amount of inhibitors both invertases could be inhibited by >90% (data not shown), indicating that CWI and VI did not exist in sensitive and resistent conformers.

To demonstrate inhibition of CWI by I_{NT} in vivo we have incubated transformed tobacco cells with partially purified inhibitor (Table 1). The incubation medium was buffered at pH 5.0 with 10 mM MES/TRIS. After a pre-incubation for 60 min in the absence or presence of I_{NT} apoplasmic sucrose hydrolysis was estimated by the release of glucose. The cells had to be starved for 24 h before the experiment (see section 2 to reduce the release of endogenous glucose to a negligible amount. After this pre-treatment glucose release by CWI activity showed a linear time-dependence for more than 120 min (data not shown). The observed inhibition of CWI by I_{NT} did not exceed 30%. However, as the in-vitro inhibition obtained after pre-incubation of CWI and I_{NT} at 25°C was only 50% of the inhibition found at 37°C (normally used for in-vitro experiments) the in-vivo inhibition is certainly significant.

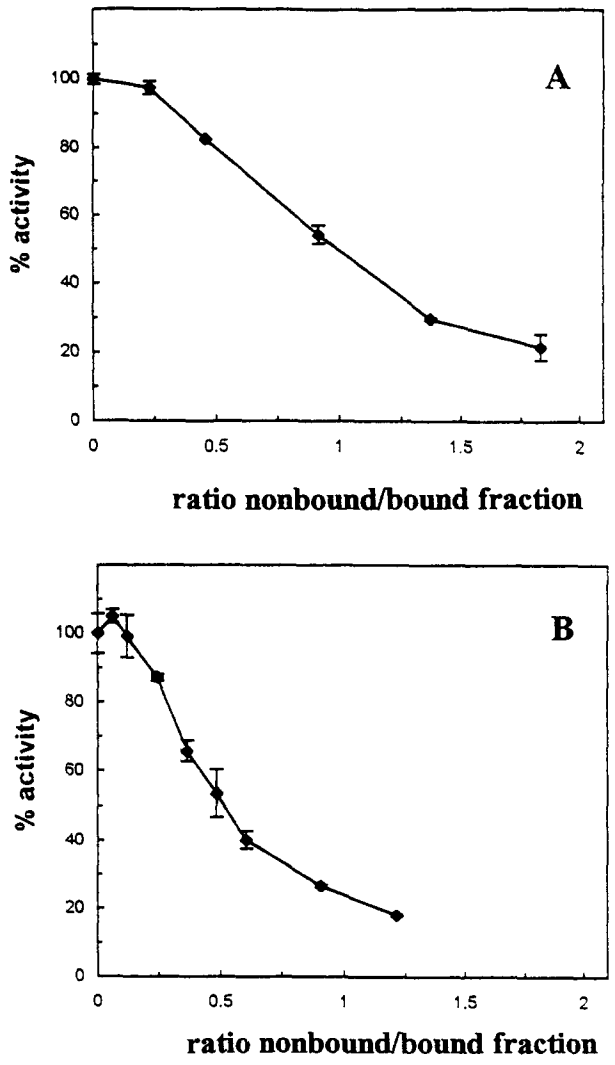

Fig. 3. Dose-dependent inactivation of partially purified tomato fruit VI (ConA-bound fraction) by partially purified inhibitor-containing fractions (ConA-nonbound fraction) from transformed tobacco cells (A) and tomato fruit (B). A nonbound/bound fraction ratio of 1 corresponds to inhibitor and vacuolar invertase extracted from 1 g fresh weight equivalent each. Inhibitor and VI were pre-incubated for 15 min.

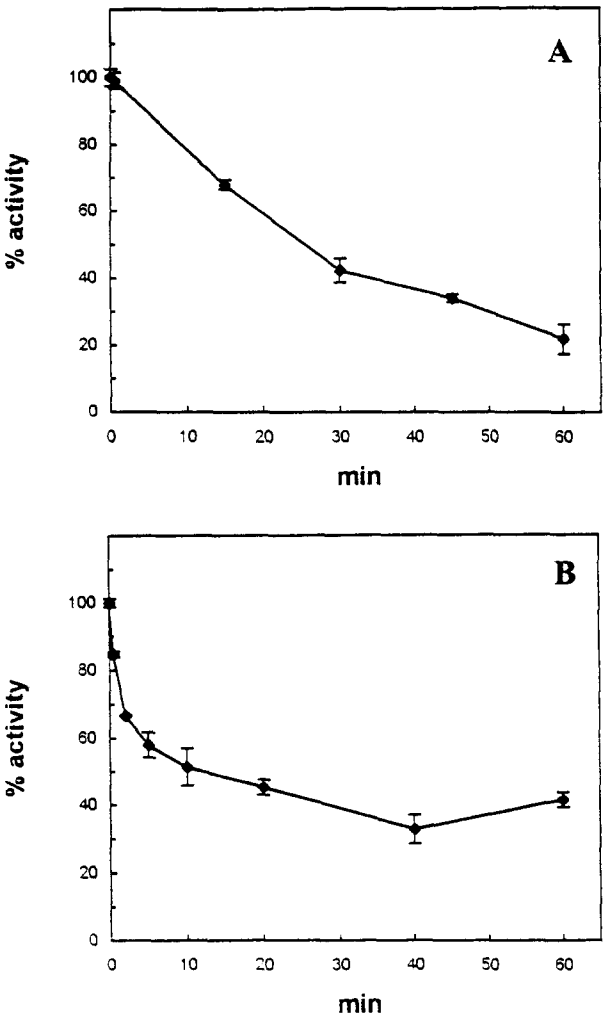

Fig. 4. Time-dependence of inactivation of partially purified tobacco CWI by inhibitor fractions from transformed tobacco cells (A) and tomato fruit (B). After pre-incubation for the indicated time intervals substrate was added and enzyme activity determined. As sucrose strongly protects CWI against both inhibitors (see Fig. 6) the additional inhibition during the enzyme assay could be neglected.

Table 1. In-vivo effect of partially purified inhibitor from transformed tobacco cells, I_{NT}, on apoplasmic sucrose hydrolysis in transformed cells

Experiment	Enzyme activity (μmol glucose/h per g fresh weight)	
	control	+ inhibitor
1	4.80 ± 0.16 (100)	4.02 ± 0.24 (83)
2	5.08 ± 0.17 (100)	3.56 ± 0.08 (70)

Cell clusters (diameter ca. 2 mm) were pre-incubated in the presence or absence of I_{NT} in sucrose-free MES/TRIS buffer (10 mM, pH 5.0) for 60 min. Thereafter, substrate hydrolysis in the medium was determined. Enzyme activity was linear with time for more than 120 min.

3.3. The kinetics of inactivation and the selective substrate protection of CWI distinguish the inhibition mechanisms for CWI and VI

A detailed time course of invertase inactivation (Figs. 4 and 5) revealed that the formation of complexes between inhibitors and VI was much faster than with CWI. For experimental reasons the rapid formation of I_{NT}–VI and I_{LE}–VI complexes could not yet be further resolved. The rather slow formation of I_{NT}–CWI and I_{LE}–CWI complexes indicates a different binding mechanism. It is noteworthy that due to the different pI values of CWI and VI, the former carries a much higher surface charge at pH 4.6 (pH during pre-incubation of inhibitor and invertase [18]). The most conspicuous difference between CWI and VI is the substrate-protection of CWI. It has been previously shown that sucrose protects CWI against I_{NT} (half-maximum protection at 1.2 mM [18]). This observation was now confirmed for the inhibition of CWI by I_{LE} (Fig. 6). In clear contrast, VI could not be substrate-protected, neither against I_{LE} nor against I_{NT}. As VI has an about 10-fold higher K_m for sucrose as compared to CWI we have tested sucrose concentrations up to 300 mM; however, no protection was observed. The results indicate that sucrose concentration might tightly control CWI inactivation but not VI inactivation by invertase inhibitors.

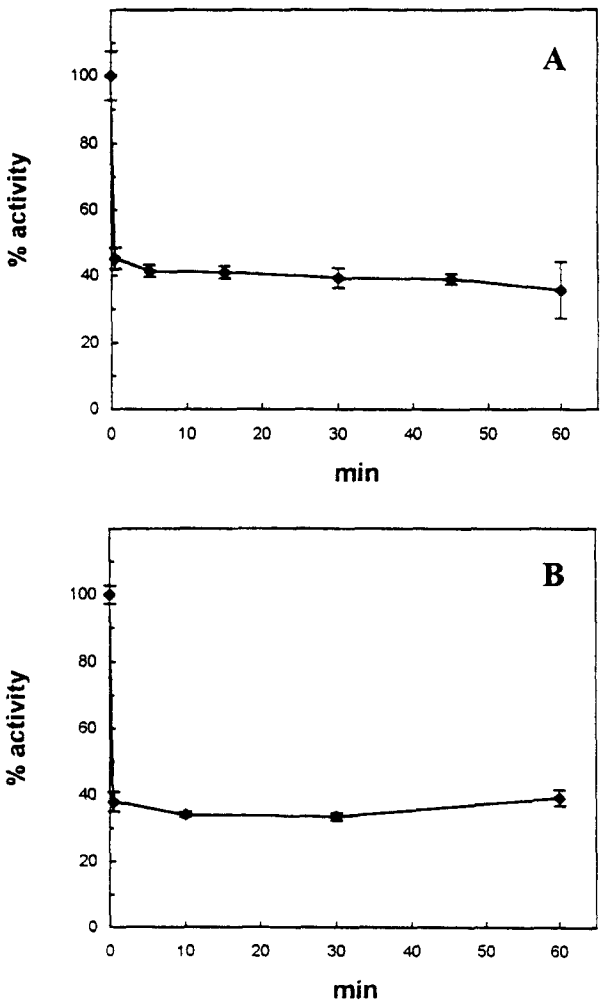

Fig. 5. Time-dependence of inactivation of partially purified tomato fruit VI by inhibitor fractions from transformed tobacco cells (A) and tomato fruit (B). After pre-incubation for the indicated time intervals substrate was added and enzyme activity determined. As sucrose does not protect VI against both inhibitors (see Fig. 6) an additional inactivation during the enzyme assay can not be excluded. However, the time course of sucrose hydrolysis was linear for up to 120 min after all pre-incubation times, indicating that inhibition was almost instantaneous.

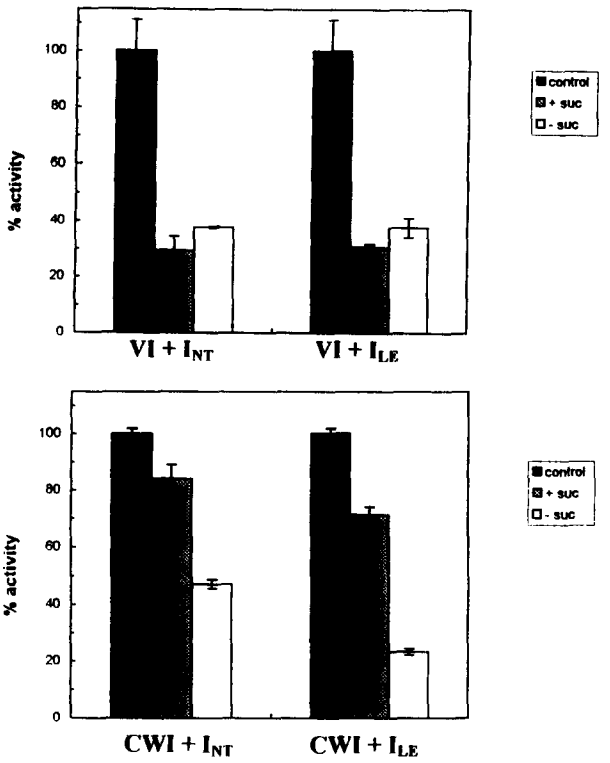

Fig. 6. Effect of 20 mM sucrose during pre-incubation of tomato fruit VI (A) or tobacco CWI (B) with inhibitors from tomato fruit (I_{LE}) and transformed tobacco cells (I_{NT}). Enzyme activities were determined after pre-incubation times of 10 min (A) and 60 min (B), respectively, in the absence or presence of inhibitors. Control, activity in the absence of inhibitor; +suc, activity after a pre-incubation with inhibitor in the presence of 20 mM sucrose; −suc, activity after pre-incubation with inhibitor in the absence of substrate.

In summary, we have compared for the first time the complex formation between defined VI and CWI preparations and two partially purified invertase inhibitors which have been shown to be structurally closely related. The observed in-vivo inhibition of CWI further supports the hypothesis that regulation of CWI by I_{NT} is not an in-vitro artefact [5,18], and may be physiologically relevant. The results indicate that the different inactivation mechanisms observed were due to the different properties of VI and CWI, respectively. Whether the inactivation of VI by invertase inhibitors is only an in-vitro artefact cannot be decided as long as co-localization has not been confirmed. Substrate-protection of CWI but not VI might indicate that both invertases are regulated differentially in their respective physiological context, e.g. different sucrose concentrations in the vacuole and the apoplasmic space. However, as differentially expressed isoforms have been described for both CWI and VI, it cannot yet be excluded that regulation by invertase inhibitors is confined to specific CWI (and, possibly, VI [13]) isoforms. The cDNA cloning of I_{NT} is under way in our lab to further analyze its function in transgenic plants.

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