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Research article

Interaction proteins of invertase and invertase inhibitor in cold-stored potato tubers suggested a protein complex underlying post-translational regulation of invertase

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ABSTRACT

The activity of vacuolar invertase (VI) is vital to potato cold-induced sweetening (CIS). A post-translational regulation of VI activity has been proposed which involves invertase inhibitor (VIH), but the mechanism for the interaction between VI and VIH has not been fully understood. To identify the potential partners of VI and VIH, two cDNA libraries were respectively constructed from CIS-resistant wild potato species *Solanum berthaultii* and CIS-sensitive potato cultivar AC035-01 for the yeast two-hybrid analysis. The StvacINV1 (one of the potato VIs) and StInvInh2B (one of the potato VIHs), previously identified to be associated with potato CIS, were used as baits to screen the two libraries. Through positive selection and sequencing, 27 potential target proteins of StvacINV1 and eight of StInvInh2B were clarified. The Kunitz-type protein inhibitors were captured by StvacINV1 in both libraries and the interaction between them was confirmed by bimolecular fluorescence complementation assay in tobacco cells, reinforcing a fundamental interaction between VI and VIH. Notably, a sucrose non-fermenting-1-related protein kinase 1 was captured by both the baits, suggesting that a protein complex could be necessary for fine turning of the invertase activity. The target proteins clarified in present research provide a route to elucidate the mechanism by which the VI activity can be subtly modulated.

1. Introduction

Potato cold-induced sweetening (CIS) is a well-known phenomenon which is a response to low temperature by producing reducing sugars (RS) in tubers [1]. It has long been known that the CIS is detrimental to quality of potato frying products, a higher RS content will result in a darker chip color [2]. Under low temperature, the RS accumulation in potato tubers has been proved to be positively correlated to the acid invertase activity [3], and the vacuolar invertase (VI) is considered critical in this process [4]. When silencing the potato vacuolar invertase gene *VInv* in potato, the reducing sugar accumulation in cold-stored tubers was almost absolutely prevented in RNAi lines with more than 90% reduction of *VInv* transcript and relatively small increases were detected in RNAi lines with intermediate levels of *VInv* silencing [5]. Although a low VI activity level was observed in the *VI* gene suppressed lines [6–8], there were no significant relationships established between *VI* gene transcript and VI activity in either potato cultivars distinct in CIS resistance [6], or the transgenic potatoes over-expressing or RNAi-silencing the *VI* gene [7–9]. These results are in accordance with the sequences of the *VI* promoters cloned from CIS-resistant and CIS-sensitive potato genotypes, which contain almost the same *cis*-elements while they are identical with negligible difference of the bases [10], speculating that the difference in CIS-resistance level between potato genotypes may be resulted from the mechanism of post-transcriptional and/or post-translational regulation.

Schwimmer et al. first concluded that there was endogenous inhibitor of invertase in potato tubers [11]. Pressey et al. convincingly demonstrated that it was a 17KD inhibitor protein that might have inhibitory function of invertase [12,13]. It has been evidenced since two invertase inhibitors (VIHs) from *Nicotiana tabacum* were functionally characterized [14,15], and the post-translational regulation of invertase activity has attracted extensive attentions.

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Table 1
Related quality data of *S. berthaultii* and AC035-01 libraries.

	S. berthaultii	AC035-01
Transformation efficiency[a] (3 μg pGADT7-Rec)	1×10^7	8×10^6
Insert size (bp)		
Minimum	300	300
Maximum	2500	2000
Average	1000	700
Percentage of very short fragment (<300 bp)	≈1%	≈1%
Percentage of positive recombinant clones	96%	94%
Cell density of frozen library (cells/mL)	7×10^7	4×10^6

[a] The expected efficiency ≥ 1×10^6/3 μg pGADT7-Rec.

Table 2
Y2H screening of CW2-1 and AC035-01 libraries with StvacINV1 and StInvInh2B.

	Hybrid colonies	X-α-Gal and sequencing	Potential positives
CW2-1 Y2H library			
Bait: StvacINV1	360	173	24
Bait: StInvInh2B	108	28	8
AC035-01 Y2H library			
Bait: StvacINV1	222	30	3
Bait: StInvInh2B	50	0	0

The VIH proteins can be categorized into two families. One is the pectin methylesterase-related proteins (PMEI-RP), they have similar key structural scaffolds (an α-helical hairpin and a four-helix bundle) but independent function with pectin methylesterase inhibitor [16], such as Nt-inh1 and Nt-inh2 of *N. tabacum* [14,15]. Another is the Kunitz-type proteinase inhibitors (KPI) [17], most of which contain the subcellular localization related consensus sequence NPIxLP [18,19], such as StInh from potato [20]. Brummell et al. reported that vacuolar invertase inhibitor in potato tubers contributes to CIS resistance [21]. At the same time, Liu et al. demonstrated that among three acid invertase genes detected in potato tubers, the vacuolar invertase gene *StvacINV1* had the highest transcript level and its expression was strongly elevated by low temperature [7]. Their further research indicated that StInvInh2A and StInvInh2B could function similarly as inhibitor of StvacINV1 [22,23].

Previous studies also showed that both PMEI-RP [23] and KPI inhibitors [24] played roles in potato CIS even if they exhibited different levels of the impact. However, the information available so far is still insufficient to draw a conclusion for how the VI activity could be subtly regulated at post-translational level without understanding the interacting proteins of both VI and VIH.

In present research, for globally exploring the potential target proteins that may be involved in VI activity modulation in the process of potato CIS, two cDNA prey libraries for yeast two-hybrid were constructed and screened individually with the vacuolar invertase (StvacINV1) and the invertase inhibitor (StInvInh2B) genes. The captured proteins suggested a potential protein complex underlying the regulation of VI activity.

2. Results

2.1. Generation of cDNA prey libraries for Y2H

Two cDNA prey libraries were constructed separately from potato CIS-resistant wild species *Solanum berthaultii* (accession CW2-1) and CIS-sensitive cultivar AC035-01 [22] for Gal4-based yeast two-hybrid (Y2H) (Table 1). The transformation efficiencies were 1×10^7/3 μg pGADT7-Rec for CW2-1 and 8×10^6/3 μg pGADT7-Rec for AC035-01. The insert fragments ranged from 200 to 2500 bp with a normal distribution, averagely 1000 bp for CW2-1 and 700 bp for AC035-01. The ratios of short insert fragments (<300 bp) of the two libraries were less than 1%, and the percentages of positive recombinant clones were between 94 and 96%. Therefore, these two libraries were qualified for the following screening.

2.2. Screen of prey libraries and sequence analysis

The *StvacINV1*-BD and *StInvInh2B*-BD vectors were constructed as baits for screening above Y2H libraries. Because intact StvacINV1 did not have any hybrid colony in a primary testing, a truncated version of StvacINV1 without the predicted anchor region (1–56 aa) was developed to adapt to present Y2H system specially for nuclear localized protein interaction. The function test showed that the truncated StvacINV1 protein had almost full function of intact StvacINV1 to catalyze the sucrose cleavage and exhibited similar response to its specific inhibitor StInvInh2 (Fig. 1). Therefore, the truncated StvacINV1 was used in present research for screening the prey libraries.

From millions of clones of each library there were 582 and 158 diploids obtained by StvacINV1 and StInvInh2B, respectively (Table 2). To select positive colonies, the diploids were further assayed by X-α-Gal (5-Bromo-4-chloro-3-indoxyl-α-D-galactopyranoside) to preclude positive false results. More than 200

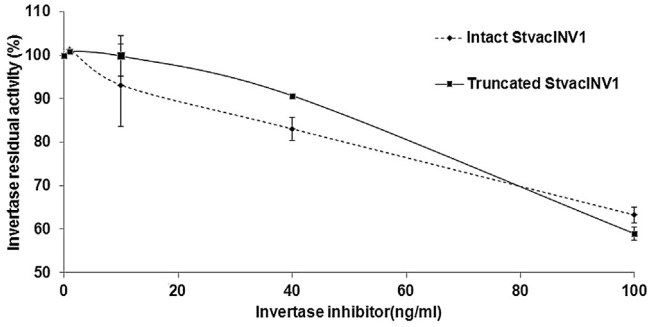

Fig. 1. Inhibitory effects of StInvInh2 on eukaryotic expressed StvacINV1 and truncated StvacINV1 (without anchor peptide 1–56 aa) proteins. Similar dose-effects of StInvInh2B recombinant protein on StvacIINV1 and truncated StvacINV1 are shown; The StvacIINV1 protein and truncated StvacINV1 protein (both are 107 ng/mL) were mixed with recombinant StInvInh2B protein separately; Residual invertase activity was measured at pH 4.6 and 37 °C after 1 h pre-incubation. Biological tri-replicates were used for each measurement, and data were presented as mean ± SD.

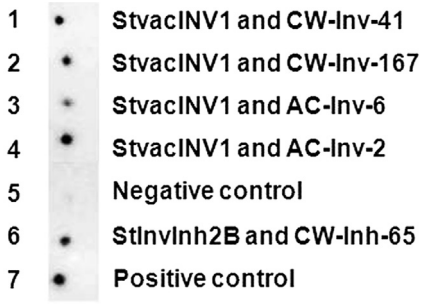

Fig. 2. Selected results of X-α-Gal assay. The survival and color of target yeast clones on QDO/X-α-Gal (SD/-Leu/-Trp/-His/-Ade/X-α-Gal) selecting plate represent the interaction between bait and target as addressed in the handbook of the BD Matchmaker™ Screening Kit (Clontech). 1–4, interactions between StvacINV1 and the four KPI inhibitors captured by StvacINV1; 5, negative control; 6, interaction between StInvInh2B and a putative invertase; 7, positive control. The positive and negative controls are presented by pGBK-53/pGAD-RecT[b] and pGBK-Lam/pGAD-RecT[b], respectively.

positive clones (Table 2) were selected by X-α-Gal assay and the interactions between StvacINV1 and the four KPIs as well as the interaction between StInvInh2B and a putative invertase were shown in Fig. 2 as an example. All the positive colonies were proved non-auto-activation and then sequenced. After BLAST searching, 35 potential interacting proteins (function-known proteins) were identified (Table 2). Of which, 27 were captured by StvacINV1 (24 from the CW2-1 library and three from the AC035-01 library) and eight by StInvInh2B (all from CW2-1 library).

All potential interaction proteins from each screening case were tabulated in Table 3 for further analysis. As expected, invertase inhibitors were captured by StvacINV1 from both the libraries by using StvacINV1 as bait, and a putative invertase from CW2-1 library, CW-Inh-65, was identified by StInvInh2B. However, no interaction protein of AC035-01 library could be selected by

Table 3
List of putative interaction proteins identified from the two Y2H libraries.

Clone no.	Bait	Library	Gene	GO description (http://www.blast2go.com/b2ghome)
CW-Inv-7	StvacINV1	*S. berthaultii*	Thylakoid membrane phosphoprotein 14 chloroplast	Plastoglobule, chloroplast envelope, chloroplast thylakoid membrane
CW-Inv-69	StvacINV1	*S. berthaultii*	Proteasome subunit alpha type-7	Ubiquitin-dependent protein catabolic process
CW-Inv-101	StvacINV1	*S. berthaultii*	Nuclear transport factor 2	Regulation of plant-type hypersensitive response, protein transporter activity, Ran GTPase binding
CW-Inv-102	StvacINV1	*S. berthaultii*	Zinc finger protein 7-like	Binding
CW-Inv-114	StvacINV1	*S. berthaultii*	Histone deacetylase complex subunit sap18	Fatty acid catabolic process
CW-Inv-117	StvacINV1	*S. berthaultii*	Patatin precursor	Defense response, hydrolase activity, nutrient reservoir activity, lipid catabolic process
CW-Inv-124	StvacINV1	*S. berthaultii*	Zinc finger CCCH domain-containing protein 32	Binding, response to stimulus, cytoplasmic membrane-bounded vesicle
CW-Inv-130	StvacINV1	*S. berthaultii*	Threonine synthase	Threonine biosynthetic process, pyridoxal phosphate binding
CW-Inv-134	StvacINV1	*S. berthaultii*	Proteinase inhibitor I	Response to wounding, negative regulation of peptidase activity, serine-type endopeptidase inhibitor activity
CW-Inv-138	StvacINV1	*S. berthaultii*	Cpi9_soltu ame: full = cysteine protease inhibitor 9 ame: full = pkixame: full = pt1 flags: precursor	Cysteine-type endopeptidase inhibitor activity, negative regulation of endopeptidase activity
CW-Inv-162	StvacINV1	*S. berthaultii*	2-dehydro-3-deoxyphosphoheptonate aldolase	Coumarin biosynthetic process, response to jasmonic acid stimulus
CW-Inv-167	StvacINV1	*S. berthaultii*	Kunitz-type protease inhibitor precursor	Negative regulation of endopeptidase activity, peptidase activity, aspartic-type endopeptidase inhibitor activity
CW-Inv-168	StvacINV1	*S. berthaultii*	Glutathione s-transferase zeta-class 1	Proteasomal ubiquitin-dependent protein catabolic process
CW-Inv-51	StvacINV1	*S. berthaultii*	Omega-3 fatty acid desaturase	Omega-3 fatty acid desaturase activity, oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water, response to cold
CW-Inv-69	StvacINV1	*S. berthaultii*	Cytochrome c oxidase subunit Vb	Cytochrome-c oxidase activity
CW-Inv-73	StvacINV1	*S. berthaultii*	Protein argonaute	Production of ta-siRNAs involved in RNA interference, methylation-dependent chromatin silencing, endoribonuclease activity
CW-Inv-91	StvacINV1	*S. berthaultii*	Snf1-related protein kinase catalytic subunit alpha kin10	Glucose catabolic process, protein phosphorylation, primary root development, protein serine/threonine kinase activity
CW-Inv-92	StvacINV1	*S. berthaultii*	Elongation factor 1-alpha-like protein	Translation elongation factor activity, sulfate adenylyltransferase (ATP) activity, translational elongation, GTP catabolic process
CW-Inv-41	StvacINV1	*S. berthaultii*	Kunitz-type protease inhibitor precursor	Negative regulation of endopeptidase activity, peptidase activity
CW-Inv-43	StvacINV1	*S. berthaultii*	Patatin precursor	Defense response
CW-Inv-62	StvacINV1	*S. berthaultii*	Lipase class 3 family protein	Phosphatidylcholine 1-acylhydrolase activity, monoacylglycerol catabolic process, negative regulation of seed germination
CW-Inv-16	StvacINV1	*S. berthaultii*	Aldehyde dehydrogenase family 7 member a1	Oxidation−reduction process, response to salt stress, response to desiccation, response to carbohydrate stimulus, 3-chloroallyl aldehyde dehydrogenase activity
CW-Inv-18	StvacINV1	*S. berthaultii*	Actin	Root hair elongation
CW-Inv-40	StvacINV1	*S. berthaultii*	DNA-directed RNA polymerase II subunit rpb11	M protein dimerization activity, ubiquitin-dependent protein catabolic process, ethionine biosynthetic process, RNA splicing
CW-Inh-18	StInvInh2B	*S. berthaultii*	Small uORF	Spermine biosynthetic process, adenosylmethionine decarboxylase activity
CW-Inh-22	StInvInh2B	*S. berthaultii*	DNA-directed RNA polymerase II subunit rpb11	M protein dimerization activity, ubiquitin-dependent protein catabolic process, ethionine biosynthetic process, RNA splicing
CW-Inh-27	StInvInh2B	*S. berthaultii*	NAC domain protein	Sequence-specific DNA binding transcription factor activity, DNA binding, multicellular organismal development
CW-Inh-65	StInvInh2B	*S. berthaultii*	Invertase, putative	Sucrose alpha-glucosidase activity, sucrose metabolic process
CW-Inh-42	StInvInh2B	*S. berthaultii*	Dehydrin	Response to water, response to stress
CW-Inh-52	StInvInh2B	*S. berthaultii*	GAL83-like protein	Kinase activity, protein binding, phosphorylation
CW-Inh-55	StInvInh2B	*S. berthaultii*	Histone deacetylase complex subunit sap18	Fatty acid catabolic process, protein binding, response to abscisic acid stimulus, response to salt stress
CW-Inh-6	StInvInh2B	*S. berthaultii*	Elongation factor 1-alpha	Translation elongation factor activity, sulfate adenylyltransferase (ATP) activity, translational elongation, GTP catabolic process
AC-Inv-1	StvacINV1	AC035-01	Zinc finger (C3HC4-type RING finger) family protein	Zinc ion binding, ubiquitin-protein ligase activity, protein ubiquitination evidence
AC-Inv-2	StvacINV1	AC035-01	Kunitz-type serine protease inhibitor DrTI	Serine-type endopeptidase inhibitor activity
AC-Inv-6	StvacINV1	AC035-01	Kunitz-type elastase inhibitor BrEI	Serine-type endopeptidase inhibitor activity

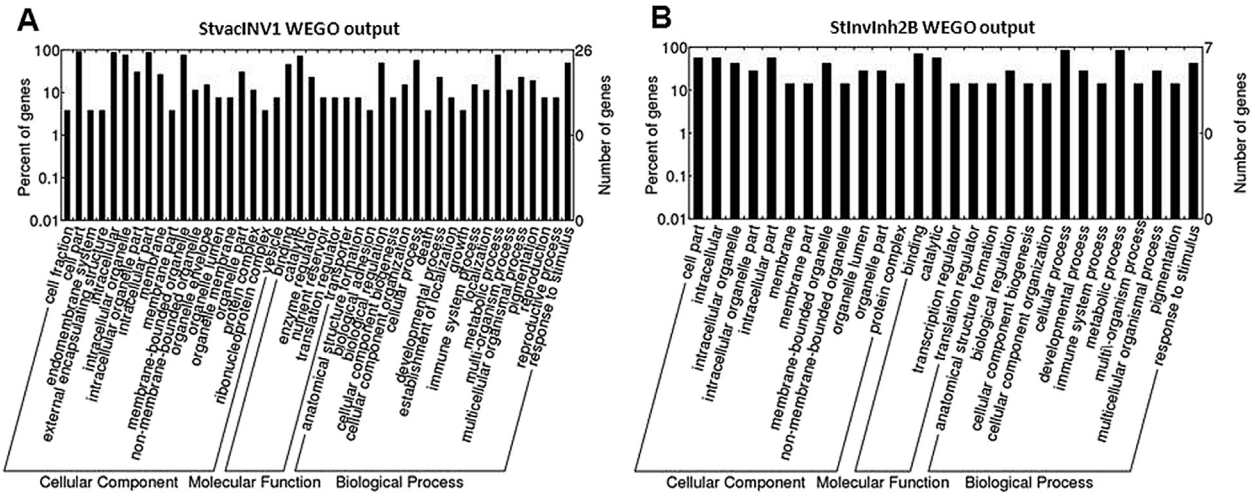

Fig. 3. Gene ontology annotations of the potential proteins captured by StvacINV1 and StInvInh2B. The GO results of all target proteins caught by the StvacINV1 (A) or StInvInh2B (B) were grouped for detailed cluster analysis using Web Gene Ontology Annotation Plotting (BGI WEGO, http://wego.genomics.org.cn/cgi-bin/wego/index.pl) and visualized on level 3.

StInvInh2B that may be due to a difference between potato genotypes in abundance of the target proteins.

The potential target proteins of each bait were subjected to the Web Gene Ontology Annotation Plotting. The highest percentage of the genes encoding potential target proteins of StvacINV1 included those having catalytic functions in intracellular organelles for metabolic process and those responsive to stimulus (Fig. 3A). Similar results were obtained for the potential target proteins of StInvInh2B although the genes performing molecular function of binding also showed a high percentage (Fig. 3B). These results reflected the main pathways of the bait proteins that are largely involved in carbohydrate metabolism.

The detail function prediction of each putative interaction protein is shown in Table 3. Seven proteins interacted with StvacINV1 in both CW2-1 and AC035-01 libraries, representing the common interacting proteins of StvacINV1 in the two potato species. This overlap consisted of two types of proteins, three members of zinc

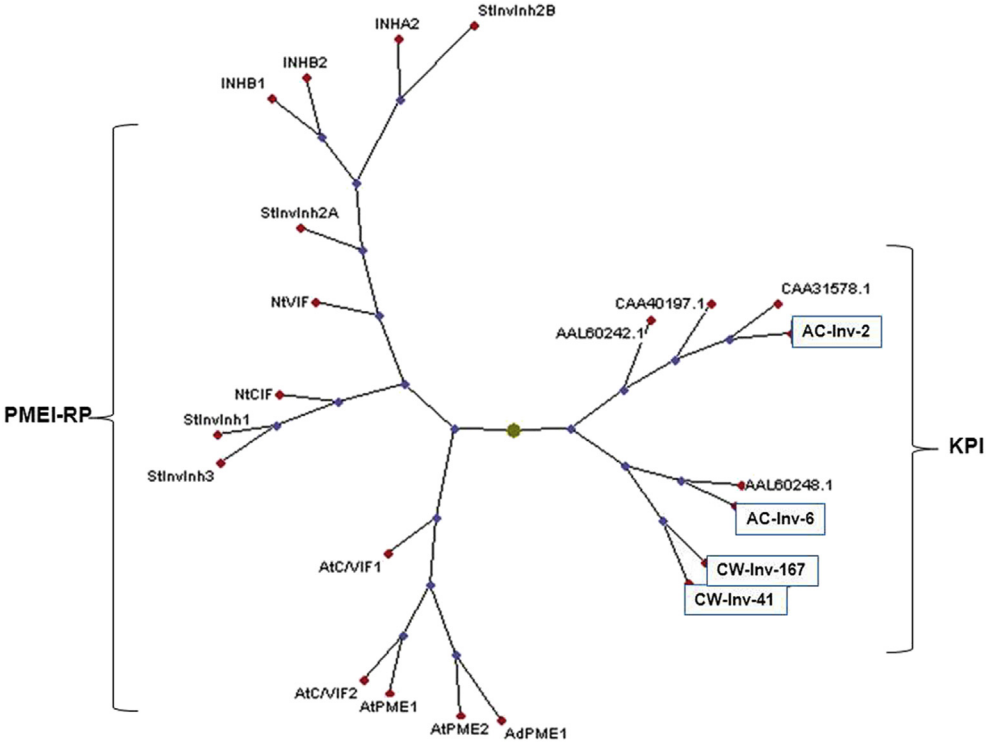

Fig. 4. Phylogenetic relationship of invertase inhibitors. The phylogenetic tree was constructed using Clustal W 2.0 program by neighbor-joining 1000 bootstrap analyses and viewed in PhyloDraw Ver. 0.8. The tree depicts the amino sequences of InvInhs from potato (CW-Inv-41, AGF39573; CW-Inv-167, AGF39572; AC-Inv-6, AGF39575; AC-Inv-2, AGF39574; StInvInh1, ADM49012; StInvInh2A, ADM49015; StInvInh2B, ADM49016; StInvInh3, ADM49013; INHB2, ADZ54777; INHA2, ADZ54775; INHB1, ADZ54776; CAA31578.1; CAA40197.1; AAL60242.1; AAL60248.1), tobacco (NtVIF, CAA73333; NtCIF, CAA73334), *Arabidopsis thaliana* (AtC/VIF1, NP_564516; AtC/VIF2, NP_201267; AtPMEI1, NP_175236; AtPMEI2, NP_188348) and Kiwi fruit (AdPMEI, P83326) searched in NCBI (access number is given after the name). Four putative protease inhibitors (CW-Inv-41, CW-Inv-167, AC-Inv-6 and AC-Inv-2) captured by StvacINV1 in Y2H libraries were marked by frames, they all assigned to the Kunitz-type protein inhibitor (KPI) family.

finger protein family (CW-Inv-102, CW-Inv-124 and AC-Inv-1) and four Kunitz-type protease inhibitors (KPIs) (CW-Inv-41, CW-Inv-167, AC-Inv-2 and AC-Inv-6). There were three proteins interacted with both StInvInh2B and StvacINV1, including two subunits of sucrose non-fermenting-1-related protein kinase 1 (CW-Inv-91 encoding the α-subunit and CW-Inh-52 encoding the β-subunit) and a DNA-directed RNA polymerase (CW-Inh-22 and CW-Inv-40). These overlapped proteins captured by both StvacINV1 and StInvInh2B implied a potential protein complex for regulation of StvacINV1.

2.3. Interaction confirmation

The four identified KPIs potentially interacted with StvacINV1 share 81–99% identity with the reported KPIs and possess four conserved Cys to form disulfide bridges which were believed to be important in forming correct three-dimensional structure of invertase inhibitor protein [25]. Although they all belong to KPI family, phylogenetic analysis could classify them into two subgroups as AC-Inv-2 was separated from the other three (Fig. 4).

To reconfirm the potential interactions identified, two KPIs (CW-Inv-167 and AC-Inv-2) and one invertase (CW-Inh-65) were selected for further bimolecular fluorescence complementation (BiFC) test. Pairwise expressions of StvacINV1/CW-Inv-167 (Fig. 5A), StvacINV1/AC-Inv-2 (Fig. 5B) and StInvInh2B/CW-Inh-65 (Fig. 5C) resulted in YFP fluorescence signals in the bombardment transformed tobacco cells while the negative controls of these proteins showed no detectable fluorescence (Fig. 5D–G), verifying a high reliability of the Y2H results.

3. Discussion

Previous research reported that the acid invertase StvacINV1 plays important roles in potato CIS by hydrolyzing sucrose into glucose and fructose, and the invertase inhibitor StInvInh2B could be an interacting protein of StvacINV1 to modulate the invertase

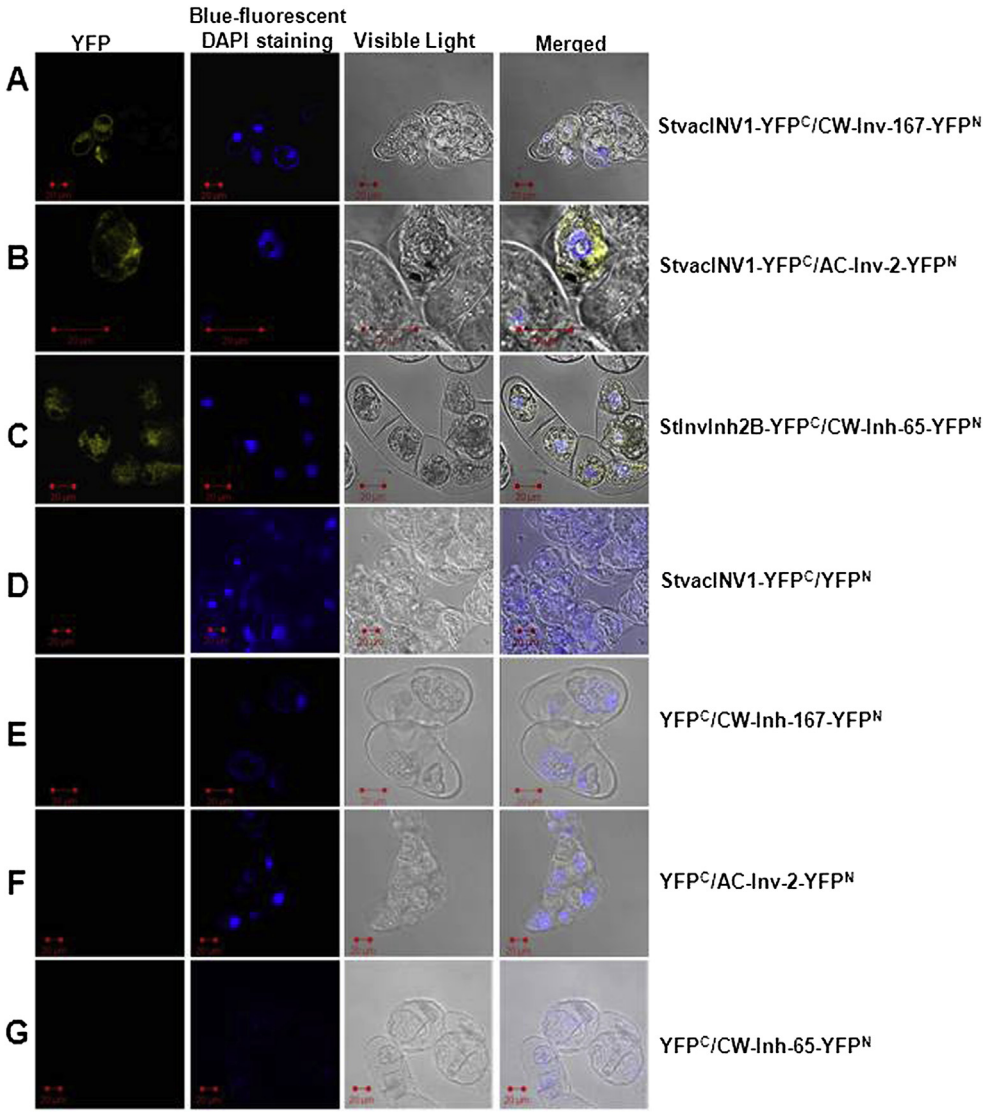

Fig. 5. BiFC visualization of interactions between each bait–target pairwise in tobacco BY-2 cells. The full length of the two bait proteins (StvacINV1 and StInvInh2B) and all the target proteins were used. CW-Inv-167 and AC-Inv-2 are two KPIs targeted by StvacINV1 in the Y2H while the putative invertase peptide CW-Inh-65 is a target of StInvInh2B. YFP signals represent the interaction between bait and target. DAPI is a stain that binds to DNA to show alive cells that have blue fluorescence. Bars indicate the image magnification. Four pairwise expressions, four target genes along with empty vectors, were served as negative controls (D–G). The experiment was repeated by three times (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

activity at post-translational level [7,22,26]. Based on these findings, two Gal4-based Y2H libraries were separately constructed with a CIS-resistant wild species *S. berthaultii* (CW2-1) and a CIS-sensitive cultivated dihaploid clone (AC035-01). The StvacINV1 and StInvInh2B proteins served as baits to globally identify possible partners that may be involved in the post-translational regulation of StvacINV1. From millions of library clones, 27 and eight potential interaction proteins were captured by StvacINV1 and StInvInh2B, respectively (Table 3). Of them, putative invertase and invertase inhibitors were respectively captured by StInvInh2B and StvacINV1 (Fig. 2), and then reinforced by BiFC assay (Fig. 5). Based on the advantages of the Y2H and BiFC assays [27], these results showed a high creditability of the constructed libraries and considerable quality of library screen.

It is accepted that proteins can interact with more than one target to implement diverse functions in different biological processes [28]. Through function alignment of all the interaction proteins, two overlaps (the same proteins captured by StvacINV1 in the two prey libraries, and the same proteins captured by both StvacINV1 and StInvInh2) of the target proteins were clarified. The first overlap includes four members of the KPI proteins and three zinc finger proteins captured by StvacINV1 in the two libraries (i.e. overlapped in both CIS-resistant and CIS-sensitive genotypes). These results suggested that invertase activity could be modulated by different inhibitors, and this modulation might be complemented with upstream transcription factors harboring zinc finger structure. Up to now, there is little information available for the functions of zinc finger proteins in potato CIS but worth further investigation. More evidently, a potato KPI gene, *St-inh*, was reported to have inhibitory function of invertase and, hence, to impact potato CIS [20]. A recent report also documented that a potato PMEI-RP type invertase inhibitor, StInvInh2, could specifically inhibit the activity of StvacINV1 in potato tubers [23]. The question may be raised from the absence of PMEI-RPs in the captured proteins of StvacINV1. Also, StvacINV1 was not caught by StInvInh2B. A possible explanation could be that the current Y2H system is favorable for the expression of nuclear localized proteins [29]. Frequently, this system is likely to produce false negatives for membrane localized proteins, like StvacINV1 and StInvInh2B, because of its different natural environment from the membrane system, especially the lack of some specific post-translational protein modifications [30]. Nevertheless, our results speculated that StvacINV1 may have a multi-inhibitor interaction mechanism varied in organelles or tissues with different regulation patterns in response to diverse environmental or biological conditions *in vivo*. This speculation is in accordance with Link et al. who showed that while both the two invertase inhibitors AtC/VIF1 and AtC/VIF2 of *Arabidopsis thaliana* could impact on the vacuolar invertase, the later specifically targeted the cell wall invertase, these two invertase inhibitors also had distinct expression profiles in different plant tissues [31].

A common target of different proteins is most likely a part of the complex composed of each partner. To approach the potential mode by which the VI activity could be subtly regulated, the previously identified counterparts, StvacINV1 and StInvInh2B, were used as baits in present research and common target proteins, including two subunits of sucrose non-fermenting-1-related protein kinase 1 (SnRK1) and a DNA-directed RNA polymerase, were captured by both baits. While the function of DNA-directed RNA polymerase in potato CIS remains unknown, SnRK1 was reported as a key regulator that is most likely involved in sucrose regulation [32,33]. More relevantly, overexpression of *SnRK1* was able to decrease glucose content in potato tubers [34], suggesting that *SnRK1* could be associated with potato CIS. These findings lead us to hypothesize that SnRK1 could be involved in VI activity regulation by enhancing or inhibiting its interacting proteins in a potential protein complex which is at least composed of VI, VIH and SnRK1.

4. Conclusion

The high throughput Y2H screen revealed potential target proteins interacted with StvacINV1 and StInvInh2B. The activity of StvacINV1 could be regulated post-translationally by multi-inhibitors responsive to diverse factors. A more complex model for modulating invertase activity was suggested which may be composed of at least StvacINV1, StInvInh2B and SnRK1.

5. Materials and methods

5.1. Plant materials

A CIS-sensitive potato (*Solanum tuberosum*) dihaploid clone AC035-01 [22] and a CIS-resistant potato wild diploid species *S. berthaultii* (accession CW2-1) were grown in greenhouse under normal conditions at National Centre for Vegetable Improvement (Central China), Huazhong Agricultural University in Wuhan, China. The mature tubers were kept at 20 °C for one week in dark for skin set and then stored under 4 °C for 5 days, during this period the distinct invertase activity was detected between CW2-1 and AC035-01 while similar level of the invertase gene expression was observed [6], The same treatment used for isolation of the genes involved in potato CIS [35]. Sampled tubers were immediately frozen by liquid nitrogen and stored at −80 °C.

5.2. Modification of StvacINV1 and bait-BD vectors

Two genes, *StvacINV1* (GenBank ID: AY341425, the potato vacuolar invertase gene) and *StInvInh2B* (GenBank ID: GU321342, the potato invertase inhibitor gene) which were previously cloned by Liu et al. [7,22], were employed as bait genes for the Y2H system. The BD Matchmaker™ Screening Kit (Clontech, Germany) was used for the bait vectors construction and testing. The *StvacINV1*-BD and *StInvInh2B*-BD bait vectors were constructed. However, StvacINV1 did not have any hybrid colony in a primary testing (data not shown), suggesting that the full length of StvacINV1 may not fit this Gal4-based Y2H system (the interactions mainly occur in yeast nucleus) as described previously [29]. To circumvent this limitation, a truncated version of *StvacINV1* gene by excising the predicted anchor region (1–168 bp) was employed, similar strategy was reported for other genes [36,37]. For clarifying the potential changes caused by this modification, both intact and truncated StvacINV1 proteins were expressed in the eukaryotic system followed by an enzyme-inhibitor assay as described by Liu et al. [22]. Constructed *StvacINV1* and *StInvInh2B* vectors were also subjected to transcriptional activation and toxicity test to ensure these two baits were suitable for the Y2H system (data not shown) as addressed in the protocols of the BD Matchmaker™ Screening Kit.

5.3. Construction and screening of the prey libraries

For selection of the target proteins that potentially interact with invertase StvacINV1 or invertase inhibitor StInvInh2B, the prey cDNA libraries of potato AC035-01 and CW2-1 for Y2H were generated and transferred into yeast stain AH109 using BD Matchmaker™ Library Construction Kit and the Y2H protocols provided therein (Clontech, Germany). After libraries construction, the quality of the libraries such as transformation efficiency, insert size and cell density were tested as described in the Y2H protocols.

The two bait genes were individually inserted into the *Eco*RI/*Bam*HI restriction sites of pGBKT7 vector before transforming into

yeast stain Y187 as described in the protocols of the BD Matchmaker™ Screening Kit, along with the bait transcriptional activation and toxicity tests. Based on the character of the Y2H system, only the no-transcriptional activation and no-toxicity bait could be used for the subsequent library screen. Subsequently, the targeted clones were subjected to X-α-Gal (5-Bromo-4-chloro-3-indoxyl-α-D-galactopyranoside) assay by following the instruction of the Yeast Protocols Handbook (http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17602). The positive clones were sequenced by Beijing Genomics Institute (BGI, China).

After screening and selection of the libraries, positive clones were analyzed by GO pathway analysis (gene ontology, http://www.blast2go.com/b2ghome). Usually, one target protein could be localized into more than one GO pathway and have many GO numbers. All proteins caught by the same bait were grouped as a dataset, and then the GO results were employed for detailed cluster analysis using Web Gene Ontology Annotation Plotting (BGI WEGO, http://wego.genomics.org.cn/cgi-bin/wego/index.pl) and visualized on level 3.

5.4. Eukaryotic expression of StvacINV1

The eukaryotic system including pPIC9K vector and the GS115 strain (methylotrophic yeast, *Pichia pastoris*) was used for the expression of intact StvacINV1 and truncated StvacINV1 proteins. Construction of the expression vectors (pPIC9K-*StvacINV1* and pPIC9K-truncated *StvacINV1*) and proteins purification were performed according to the manual (Invitrogen, USA).

5.5. Bimolecular fluorescence complementation (BiFC)

To confirm the potential interaction, BiFC assay was used in tobacco BY-2 cell lines (*N. tabacum* cv. Bright Yellow 2). For construction of the BiFC vectors, the full-length cDNA fragments of all target genes were cloned and inserted into the *Bam*HI/*Kpn*I restriction site of BiFC vectors pUC-SPYCE/pSPYCE-35S and pUC-SPYNE/pSPYNE-35S [38]. The vectors were then transformed into the BY-2 cells by particle bombardment as previously described [39]. Each fusion construct was sequenced to ensure no mutations occurred. Detection of the yellow fluorescence was performed using a confocal laser scanning microscope (LSM510 Meta, Zeiss, Germany). Fluorescence signals for yellow fluorescence protein (excitation 514 nm) of the cells were detected and recorded by LSM Image Examiner software (Zeiss, Germany) 20 h after infiltration [22]. Four plasmids pairs *StvacINV1*-YFP^C along with empty vector YFP^N, empty vector YFP^C with *CW-Inh-167*-YFP^N, YFP^C with *AC-Inv-2*-YFP^N and YFP^C with *CW-Inh-65*-YFP^N were served as negative controls. Every transformation has three biological replicates.

5.6. Statistics analyses

Biological tri-replicates were used for each measurement, and data were presented as mean ± SD. Regression analysis of the data was conducted using the Microsoft Excel program (Microsoft Office, 2003).

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