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Full Length Research Paper

Quantificational analysis of NPT-II protein from genetically modified *Vitis vinifera* L.

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Widely distributed inhibitors in grapevine extracts make it difficult to improve analytical procedures for protein detection. In this study, acidity in grapevine extracts was one of the major factors inhibiting the detection of neomycin phosphotransferase II via enzyme-linked immunosorbent assay. Leaf and berry extracts with low pH (3.0 – 4.0) strongly inhibited NPT-II detection, while root and xylem sap extracts (normally pH 5.5 to 7.0) allowed the successful detection of NPT-II. The other inhibitory effect against the detection was successfully solved by heat treatment to samples extracted. Boiling leaf extract prior to ELISA, in conjunction with pH adjustment (to 7.0) was essential to improve NPT-II detection, while with berry extracts only pH adjustment was required. In the basis of above results, NPT-II protein contents in transgenic grapevine tissues possessing a NPT-II gene were successfully measured. The results here may be useful to help in evaluation of the bio-safety whether the transgenic grapevines were released or contaminated on the grapevine cultivation area by NPT-II protein detection.

Key words: pH adjustment, polyphenol, protein detection, protein inhibitor, transgenic.

INTRODUCTION

The quantification of localized proteins in plant tissues is important to understand their biological activities by the movement of proteins passing through long distance transport system. However, the quantification is restricted in certain plant extracts caused by molecular interaction, such as protein-protein and/or protein-secondary metabolite interaction. Grapevines are rich in polyphenolic compounds (Cantos et al., 2002) and polysaccharides (Moser et al., 2004). Secondary compounds in grapevine extracts make it difficult to purify DNA (Demeke and Adams, 1992; Angeles et al., 2005), RNA (Moser et al., 2004; Tattersall et al., 2005) and enzymes (Loomis and Battaile, 1966). Certain grape polyphenols are also used either as inhibitors of enzyme activity in pharmacological products (Larrosa et al., 2004; Jo et al., 2005), or as protein precipitators in the food industry (Mouécoucou et al., 2003; Weinbreck et al., 2004; Cabello-Pasini et al., 2005). The presence of assorted secondary compounds interferes with the investigation of protein metabolism in grapevine tissues.

Difficulties in protein detection in grape extracts are likely caused by several factors, including pH, interaction between secondary compounds and proteins (Eom and Reisch, 2008), as well as the action of proteases. Adding protease inhibitors gave partial improvement in ELISA detection of a small lytic protein (< 5 kDa) in grapevine leaf extracts (Li et al., 2001). Although some macro-molecules in grapevines, such as tannins and poly-saccharides, are known to be obstacles to purifying proteins by binding to each other (Demeke and Adams, 1992), other inhibitors of protein detection in grapevine remain to be elucidated. Recently, it was found that polyphenols affected NPT-II detection using ELISA (Eom and Reisch, 2008). NPT-II is often used as a selectable marker in transgenic plants. However, the protein is difficult to detect in certain tissues of grapevines, even though NPT-II gene expression can be verified (Eom and Reisch, 2008). Certain acidic molecules, such as tartaric acid and ellagic acid, acted as inhibitors of NPT-II detection via ELISA (Eom and Reisch, 2008). As reported above,

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although we found certain inhibitory factors interfering protein detection on ELISA, the improved methods are still not investigated. In addition, the efficiency of the protein detection in each tissue of grapevine is also not known.

To improve detection of transgenic proteins by manipulating characteristics of grapevine extracts, non-transgenic Chardonnay (*Vitis vinifera*) clone 95 (CdCl.95) and transgenic (CdEN15 and CdEN18) Chardonnay producing NPT-II (29 kDa) and endochitinase (43 kDa) were investigated and we reported here the improvements in NPT-II detection in grapevine tissues by pH adjustment and heat treatment prior to ELISA. It was also investigated whether the improved procedures are applicable to the detection of other transgenic proteins, such as chitinase.

MATERIALS AND METHODS

Plant materials

CdCl.95 and transgenic lines, CdEN15 and CdEN18, in which the NPT-II and En42 (endochitinase) genes are constitutively expressed (Vidal et al., 2003), were used for this experiment. Grapevines were grown in a greenhouse, with 250 μmol m^{-2} s^{-1} photosynthetic photon flux light supplement during the daytime (14 h) at 22 - 28°C. Plants were watered with distilled water and 7 to 10 g of fertilizer (Osmocote® Plus, Scotts Co., OH, USA) was added to each pot (15 cm in diameter) every four months.

Sample extraction

An NPT-II ELISA kit containing protein extraction buffer (PEB1, pH 7), standard NPT-II protein, 1X phosphate buffered saline Tween-20 (PBS-T), DeMan-Rogosa-Sharpe solution (MRS-2), enzyme conjugates (NPT-II monoclonal antibody and polyclonal antibody), 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution and 3M sulfuric acid was purchased from Agdia (Cat. No. PSP 73000, Agdia Inc., Elkhart, Indiana, USA). Leaf and root tissue extraction of CdCl.95 was conducted by the following procedures: 9.9 mL of 1X PEB1 (Protein Extraction Buffer) solution was prepared and placed in a 50 mL tube on ice. 2 g of fresh young leaves and secondary hairy roots samples were placed into a pre-cooled 50 mL Falcon tubes. 100 μL of phenylmethylsulphonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, Missouri, USA) was added to the samples, to give a final concentration of 20 μg/mL PMSF. Samples were ground using a mortar and pestle in the PEB1/PMSF solution. Some samples were processed without PMSF to test the effect of protease inhibitor. Samples were centrifuged at 13,000 g, 4°C, for 3 min. Supernatant was collected and filtered using a sterile syringe filter (0.2 μm, Corning®, Corning, NY, USA). 0, 0.30, 0.75 and 1.50 ng NPT-II standard was added per 1 mL of sample. The pH was adjusted to 7.0 with sodium hydroxide and the samples were incubated at 100°C in a water bath for 10 min.

Xylem sap and berry extraction from CdCl.95 was collected by the following procedure. Xylem sap dormant grapevines held at 4°C were placed in a greenhouse (22 - 28°C). After 8 weeks, stems were cut 50 cm above the soil surface and the outer bark peeled (2 cm from the cut stem) to remove the phloem and epidermal cells. The cut surface was washed using distilled water and a 15 mL plastic-tube collector was positioned below the stem. Airflow was blocked between the cut stem and the collector by wrapping the cover of the 15 mL tube with Parafilm. Xylem sap was collected for 24 h and was stored at –80°C. Both immature and mature berries were collected from greenhouse grown vines. Juice was extracted by hand pressing and collected in pre-cooled 50 mL Falcon tubes. Juice was filtered using a sterile syringe filter (0.2 μm, Corning®, Corning, NY, USA). NPT-II (0, 0.30, 0.75 and 1.50 ng per 1 mL juice filtrate) was added to juice and/or xylem sap samples.

Procedures for sample collection and extraction from line CdEN18 followed the same process described above, except for the addition of NPT-II.

NPT-II ELISA

Both 100 μL of sample extracts and NPT-II standard series that were dissolved in PEB1 were loaded into wells on a pre-coated 96-well plate. The plate was incubated at 28°C for 2 h. All subsequent ELISA steps followed the procedures outlined by Agdia, Inc., except for the incubation temperature, which was increased from 22 to 28°C (Agdia Inc., Elkhart, Indiana, USA). Optical density was read at 450 nm in a spectrophotometer. NPT-II in each sample based on calculations using positive controls was quantified.

Chitinase assay

CdEN18 was analyzed for endochitinase activities of root, leaf, xylem sap and berry by using the umbelliferyl fluorescence assay (Carsolio et al., 1994). Root and leaf tissues (200 mg fresh weight) were ground in liquid nitrogen and then placed in 1 ml of PEB1 protein extraction buffer (Agdia. Elkhart, Indiana). After a 5 min extraction process in room temperature, debris was removed by centrifugation at 13,000 x g for 15 min at 4°C. Berry and xylem sap were used directly without the extraction buffer. Extracted samples for experiments involving pH adjustment and heat treatment used the same procedures. An aliquot (50 μL) of the supernatant was mixed with an equal volume of methyl umbelliferyl-N,N',N"-triacetyl chitobioside (MuchB) 240 μg/ml, in sodium acetate buffer. The substrate MuchB produced a fluorescent product, methylumbelliferone (MU), after hydrolysis by chitinase. The reaction mixture was tested in a Cytofluor II microplate reader (CytoFluor®II, Fluorescence Multi-well Plate Reader, PerSeptive Biosystems, Framingham, MA, USA) set to 360 nm excitation and 460 nm emission levels. Absorbance readings were recorded every 5 min for a total of 30 min. The amount of protein in the root sample was determined using the Bio-Rad Bradford dye-binding protein assay according to the manufacturer's recommendation (Bio-Rad Co, Hercules, California, USA). Chitinase activity was expressed as nM MU/min/μg protein.

Other analyses

Total protein content in grapevine extracts was measured by the Bradford method (Bradford, 1976). Different amounts of sample extracts, including 100 μL from 200 mg F.W./mL PEB1 of leaf and root extracts, were assayed by the Bradford method. For xylem sap, 1 mL without PEB1 was utilized. To analyze fruit, 100 μL of juice without PEB1 was used directly. Total polyphenol content was measured using gallic acid as a standard (Singleton and Rossi, 1965).

Statistical analysis

Data are presented as means with standard errors for each treatment. Means of all data were subjected to standard ANOVA procedures using the SAS software (SAS version 8.02, SAS Institute Inc., Cary,

NC). Significant differences among treatment means were determined at the 5% level using Fisher's protected least significant difference (LSD) tests.

RESULTS AND DISCUSSION

Inhibitory effects of grapevine extracts

The ability to detect NPT-II additions to CdCl.95 tissues, including leaf, berry, root and xylem sap, was evaluated. NPT-II detection was strongly inhibited in leaf and berry extracts but not in xylem sap (Figure 1). Although an inhibitory effect was seen with root extracts, the NPT-II standard curve was linear ($y = 0.0051x + 0.0654$, $r^2 = 0.99$). Such a linear value of regression and a positive slope allow the use of a standard curve to calculate NPT-II levels in transformed plants (Figure 1). Therefore, the ELISA technique required modification before application to grapevine berry and leaf extracts.

It has been demonstrated that grape leaf and berry extracts are acidic, ranging from pH 3 to 4 (Manteau et al., 2003). In this experiment, grapevine leaf and berry extracts were also acidic compared to other extracts (Table 1). Although, leaf tissues were extracted using pH 7 PEB1 buffer, the extracts were still in the range of pH 3.0 to 3.5. Younger leaf extracts were less acidic than older leaf extracts. However, the rate of NPT-II detection did not significantly differ between extracts made using different leaf stages (Table 1). NPT-II detection was poor in juice extracts from both immature as well as mature berries (Table 1). Extracts from leaves and berries were both highly acidic, even though leaves were extracted in a neutral pH buffer and berries were not.

Change of grapevine components affected by pH and heat treatment

The addition of protease inhibitors to plant extracts prevents protein degradation and the concentration of protease inhibitors is a critical factor (Li et al., 2001). NPT-II protein was likely not sensitive to protease activity in grape extracts because PMSF (a protease inhibitor) addition to extracts did not improve NPT-II detection via ELISA (Figure 2). Addition of PMSF improved detection of endochitinase activity in mature leaves, while it had a negative effect on detection in immature leaves (Figure 2). It is suggested that the addition of a protease inhibitor to aid in the detection of certain proteins should be done with careful consideration of the characteristics of target protein and plant tissues, like mature leaves of grapevine that possess abundant proteases. pH adjustment and boiling treatments in transgenic grapevine tissues result either in degradation or inactivation of chitinase activity in a fluorometric assay (data not shown). Thus, the assay for endochitinase activity should be conducted without heat treatment and pH adjustment.

In previous research, the acidic components of leaf extracts were hard to remove from solution containing proteins for ELISA (Eom and Reisch, 2008). Both the methanol soluble fraction of the leaf extract as well as the residue containing protein fractions retained their inhibitory effect on ELISA detection of NPT-II (Eom and Reisch, 2008). It can be assumed that pH also affected protein detection as a result of the highly acidic condition of the extracts. We therefore evaluated whether the adjustment of leaf and berry extracts to a neutral pH could improve protein detection using ELISA.

It has been shown that heat treatment of ground tissues may induce an increase in the quantity of polyphenols and acidic polysaccharides following extraction, resulting in short chained forms of these components (Kim et al., 2007). High pH improved the detection of activity of certain proteins (Diakou et al., 2000). Also, oxidized polyphenols could covalently bind with proteins during extraction resulting in inactivation of the proteins and causing extracts to turn brown (Angeles et al., 2005). However, in the present work, pH change did not affect the total phenolic content of grape leaf extracts, but resulted in a slight increase in the content in berry extracts (Table 2).

Grape leaf and berry extracts had a low pH (Table 1) and turned brown when boiled. In contrast, extracts adjusted to a neutral pH kept their original color after boiling and less precipitation was observed. It was found that total protein content was greater in samples adjusted to pH 7.0 (Table 2). Thus, it is suggested that at higher pH values, there may be less binding of NPT-II to other molecules. However, heat treatment tended to reduce the yield of total protein in leaf extracts compared with other treatments. The total protein content in berry extracts varied much less in comparison to the changes observed in leaf extracts. Heat treatment after pH adjustment may affect the degradation of heat sensitive leaf proteins. Thus, it is suggested that boiling at a neutral pH might improve NPT-II detection via ELISA by denaturing proteins negatively interacting with NPT-II in leaf extracts.

NPT-II detection in leaf extracts was improved by the combination of pH adjustment (pH 7) and boiling (Table 2). However, either pH adjustment or boiling alone did not improve protein detection in leaf extracts. In berry extracts, NPT-II detection was also improved when samples were neutralized and boiled. Unlike leaf extracts, NPT-II detection in berry extracts was also improved when samples were neutralized without boiling.

Protein contents in transgenic grapevines

The above experiments suggest that adjusting extracts to a neutral pH combined with boiling may be helpful to improve the detection of NPT-II via ELISA. However, these treatments negatively affect the detection of endochitinase activity. For protein analysis, greenhouse grown CdEN15 and CdEN18 tissues were used, which were previously selected as either low or high NPT-II

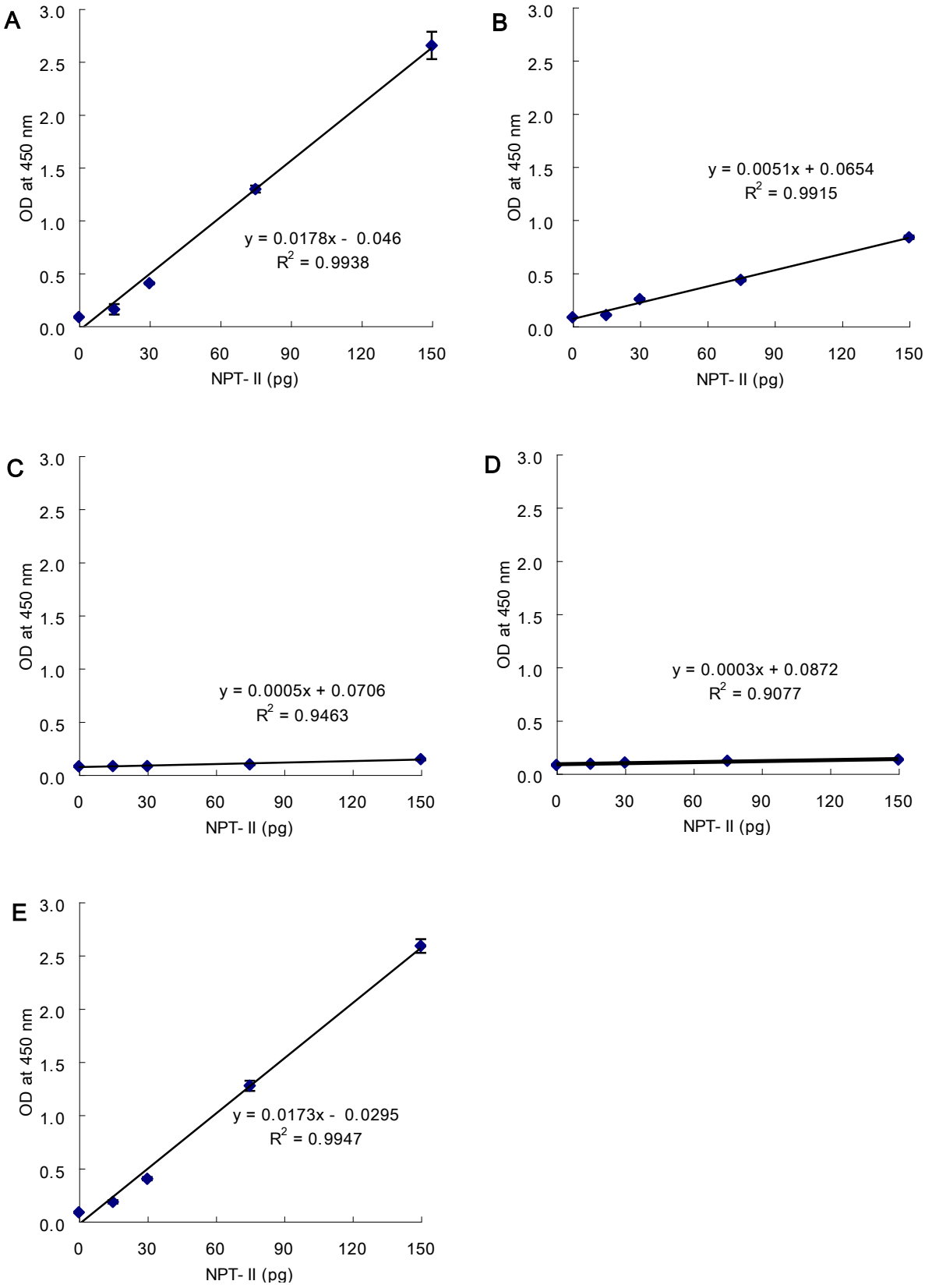

Figure 1. NPT-II detection in grapevine extracts. Grapevine tissues (leaf, berry and root) were extracted in PEB1 (200 mg F.W./mL). Xylem sap was directly tested without a PEB1 extraction. NPT-II standard amounts were dissolved into grapevine extracts. (A) PEB1 only, (B) root extract, (C) leaf extract, (D) berry extract and (E) xylem sap. Error bars represent standard deviations.

Table 1. pH and NPT-II detection in CdCl.95 grapevine tissues with additions of NPT-II.

Grapevine tissues	pH (± SE)	NPT-II detection rate (%)[+]
Immature leaves at nodes 1 to 3	4.09 ± 0.22	2.64
Fully expanded-young leaves at nodes 3 to 5	3.55 ± 0.14	1.57
Mature leaves at nodes 5 to 8	3.10 ± 0.23	1.07
Immature berries within 3 weeks	3.01 ± 0.12	3.14
Ripened berries	3.39 ± 0.12	2.15
Xylem sap during breaking of dormancy	5.83 ± 0.29	98.35
Roots	6.09 ± 0.17	29.09

Leaf and root tissues were extracted as 2 g fresh weight into 10 mL of PEB1. Then 50 μL of each extract with the addition of 50 μL of NPT-II (75 pg) solution in PEB1 were loaded into wells of an ELISA plate. Berry juice and xylem sap were mixed with 50 μL of NPT-II (75 pg) solution in PEB1 and loaded into wells of an ELISA plate. Calculations of NPT-II detection rates were based on NPT-II detection in a PEB1 solution as a control.

Table 2. NPT-II detection rate and concentration of total polyphenol as influenced by pH and heat treatment in fully expanded young leaf (FEYL) and ripened berry (RB) extracts of CdCl95.

Parameter		pH 3 – 4 (Raw)		pH 7 (adjusted)	
		Not boiled	Boiled	Not boiled	Boiled
NPT-II detection rate (%)	FEYL	1.95 c	4.79 b	1.25 c	78.24 a
	RB	4.54 c	4.95 c	71.74 b	94.50 a
Total protein (mg/mL)	FEYL	0.30b	0.29b	0.36a	0.21c
	RB	0.15b	0.11c	0.17a	0.15b
Total phenolics (mg/mL)	FEYL	30.69 a	27.93 c	30.30 a	29.25 b
	RB	10.51 b	10.61 b	11.62 a	10.96 ab

Standard NPT-II (75 pg) was dissolved into sample extracts (100 μL) after the treatment of pH and heat processes. Values in the same row followed by different letters are significantly different at the 5% level based on Turkey's Studentized Range Test (n = 4). Abbreviations in the second column represent: FEYL - fully expanded-young leaf at nodes 3 to 5 from the shoot tip, RB - ripened berry.

Table 3. Transgenic proteins in transgenic grapevine tissues by the application of amended protocol.

Tissue	Total protein (μg/g F.W. tissue)		NPT-II (μg/g protein)		Chitinase activity (mM/min/g protein)	
	CdEN15[*]	CdEN18	CdEN15	CdEN18	CdEN15	CdEN18
Leaf (Young)	6811	6035	0.22 ± 0.04[z]	0.35 ± 0.01	19.80 ± 0.78	15.53 ± 1.78
Root	5380	5455	0.91 ± 0.08	2.37 ± 0.16	21.54 ± 0.97	26.95 ± 5.59
Berry	496	771	1.92 ± 0.13	1.48 ± 0.04	92.63 ± 2.45	90.35 ± 4.11
Xylem sap	75	61	0.19 ± 0.15	3.52 ± 0.24	7.15 ± 0.22	9.42 ± 0.39

*CdEN15 and CdEN18 mean transgenic chardonnay clone No. 15 and 18 line grapevines that are expressing endochitinase.
[z] indicates detection values expressing average ± standard deviation of triplicate samples.

content in their *in vitro* roots (data not shown). Total protein content between the transgenic lines was similar in each tissue comparison, except for berries. Total protein content was the highest amount (about 6500 μg/g F.W.) in young leaf tissue among tissues evaluated, exhibiting about 5400 μg/g F.W. in root, 500 μg/g F.W. in berry and 70 μg/g F.W. in xylem sap, respectively (Table 3). In the basis of the above improved NPT-II detection method, NPT-II in different tissues was successfully measured. Table 3 also shows that distribution of NPT-II content among transgenic grapevine tissues is likely to differ. Similar results of NPT-II contents were observed in the roots, leaves and xylem saps of greenhouse grown grapevines as compared with the result of the *in vitro* root analysis, presenting the higher content in CdEN18 than that in CdEN15. Otherwise, NPT-II content in berry was higher in CdEN15 than CdEN18. In the comparison of overall tissues, the amount of NPT-II protein was relatively low in immature leaf tissues (Table 3).

In conclusion, grapevine tissue extracts included strong

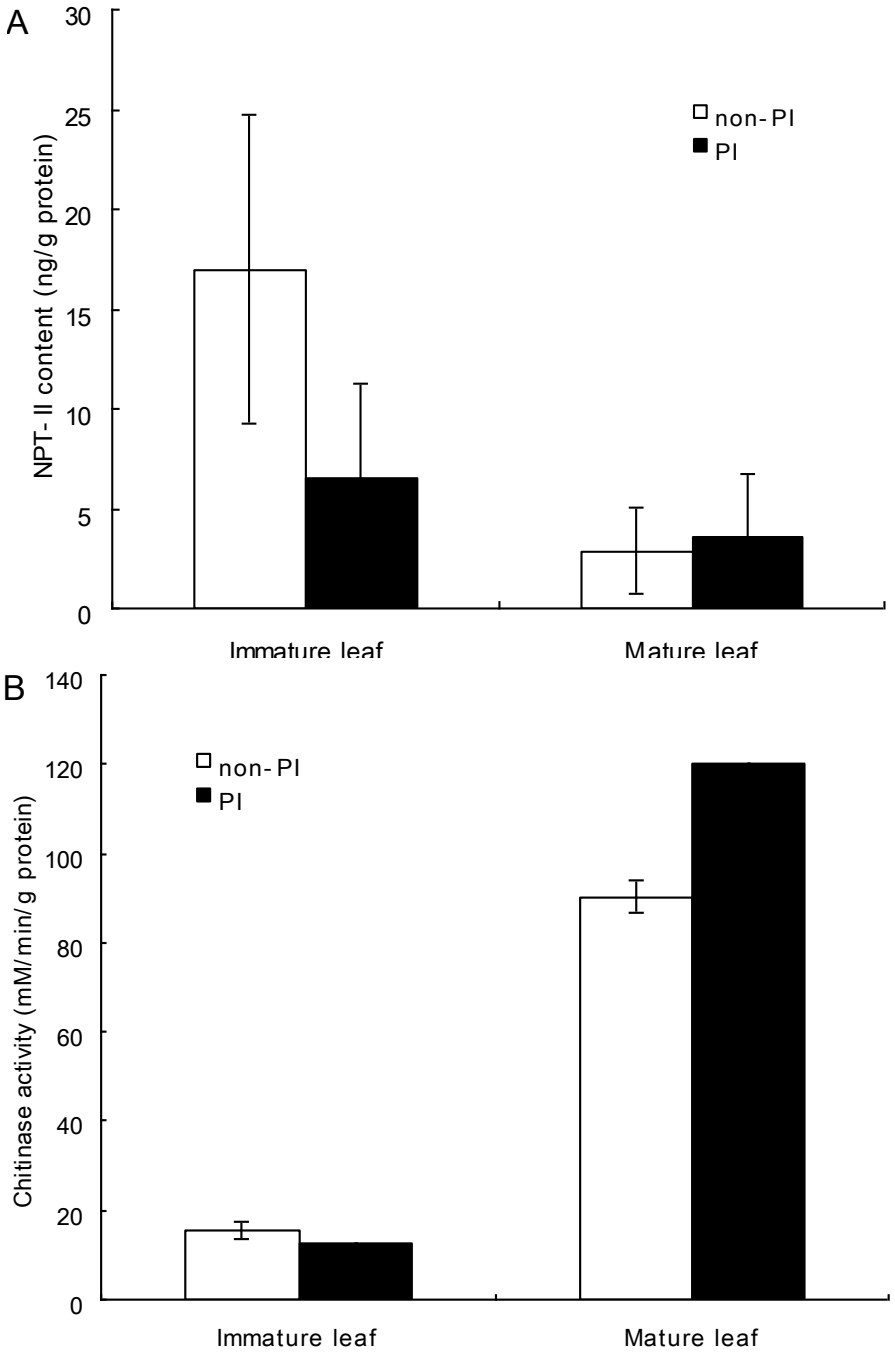

Figure 2. Effect of a protease inhibitor on NPT-II detection and endochitinase activity in CdEN18 leaves. (A) NPT-II protein content and (B) endochitinase activity. Error bars represent standard deviations.

inhibitors of NPT-II protein detection, characterizing low pH and interaction between metabolites. It was found that these inhibitory factors could be amended by neutralization of pH and heat treatment. NPT-II protein contents in transgenic grapevine tissues were successfully measured by using the improved method. These findings would be helpful to evaluate the bio-safety; whether the transgenic grapevines were released or contaminated on the grapevine cultivation area by NPT-II protein detection.

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