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Protocols

An Improved Enzyme-Linked Immunoabsorbent Assay Protocol for the Detection of Small Lytic Peptides in Transgenic Grapevines (*Vitis Vinifera*)

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Abstract. An enzyme-linked immunoabsorbent assay (ELISA) protocol was developed for the detection of small lytic peptides in transgenic grapevines (*V. vinifera*). The protocol requires a high concentration of protease inhibitor in the extraction buffer; the use of antiserum cross-absorbed with control tissue, an increased concentration of blocking reagents in the antiserum buffer, and performing all coating and/or binding processes at 37°C while reducing the time period for each step to 1 h. The procedure greatly reduced protein degradation, increased the signal-to-noise ratio, and it allowed the effective detection of the Shiva-1 lytic peptide (5 kDa) at concentrations as low as 0.1 µM. This procedure made it possible for routine analysis of transgene expression in Shiva-1 gene-containing transgenic grape plants.

Key words: antimicrobial proteins, immunoassay, protein detection, transgene expression, transgenic grape

Introduction

Enzyme-linked immunoabsorbent assay (ELISA) is a powerful tool for the detection of specific proteins and macromolecules in biological systems. It has been the most widely used serological technique for the detection of plant pathogens, including viruses and bacteria. ELISA also has been used to reveal the production and to measure the accumulation of novel proteins resulting from transgene expression in transgenic plants. Three principal types of ELISA are generally recognized: direct ELISA, indirect ELISA, and sandwich ELISA (Crowther, 1995). Each type has advantages and disadvantages, as described by Koenig and Paul (1982). In addition, several ELISA protocols have been developed to meet the varying requirements for the detection of specific proteins or antigens (Crowther, 1995).

The use of small lytic peptides to confer resistance in transgenic plants to infection by pathogens has been reported previously (Arce et al., 1999; Reynoird et al., 1999; Cary et al., 2000; Sharma et al., 2000). These antimicrobial peptides are usually small proteins with a molecular weight of 5-10 kDa (Jaynes, 1993;

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Mourgues et al., 1998). Because of their small size and the intracellular and/or extracellular environment in which they exist, they are subject to rapid degradation. As a result, they are often difficult to detect (Florack et al., 1995; Owens and Heutte, 1997).

A number of reports have described the transfer of lytic peptide genes into transgenic plants with the subsequent enhancement of disease resistance. However, most of the previous reports failed to provide convincing evidence of the production and accumulation of lytic peptides in the transgenic plants. Rather, the observed resistance was indirectly supported by the presence of the appropriate mRNA (Arce et al., 1999; Reynoird et al., 1999; Cary et al., 2000; Sharma et al., 2000). Several studies have indicated that the production of mRNAs does not necessarily correlate with the production of the corresponding proteins (see review by Kumpatla et al., 1998). Hightower et al. (1994) demonstrated the detection of lytic peptides in transgenic tobacco plants using a competitive ELISA procedure that required the use of liquid nitrogen and a large quantity of plant tissue. We describe a protocol for the detection of Shiva-1 lytic peptide in transgenic grapevine plants. The protocol requires only small amounts of plant tissue for analysis and does not require the use of liquid nitrogen.

Materials

Plant materials, specific chemicals, solutions, and equipment required

A polypeptide corresponding to the Shiva-1 protein (Jaynes, 1993) was chemically synthesized. A stock solution of the Shiva-1 protein was prepared by dissolving in sterile distilled water at 10 μg per μL[1]. Reagents for buffer preparation were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Fisher Scientific (Suwanee, GA, USA). EDTA-free complete protease inhibitor cocktail tablets (Cat. No. 1 836 170) were purchased from Roche Molecular Biochemicals, Mannheim, Germany. Goat anti-rabbit IgG (H+L) horseradish peroxidase (HRP) conjugate (EIA-grade affinity purified) (Cat. No. 172-1019) and the peroxidase substrate 2-component kit (Cat. No. 172-1064) were purchased from BioRad Laboratories (Hercules, CA, USA).

- PBS buffer (per liter) (Sambrook et al., 1989): Combine 8 g NaCl, 0.2 g KH_2PO_4, 1.1 g Na_2HPO_4, and 0.2 g KCl. Add sterile distilled H_2O to adjust to 1 L. The final pH should be 7.4.
- PBS-T buffer (per liter): This contains PBS buffer supplemented with 0.5 mL of Tween-20.
- Extraction buffer (per 8 mL): This contains 2 EDTA-free protease inhibitor tablets dissolved in 8 mL of PBS buffer[2].
- Antiserum buffer (per 20 mL)[3]: Combine 0.4 g PVP-40, 0.2 g albumin (bovine – protease-free, Sigma A-3059)[4] and 34 mg DETC (diethyldithiocarbamic acid sodium salt, Sigma D-9428). Add PBS-T buffer to a final volume of 20 mL.
- Conjugate buffer (per 10 mL): Combine 0.2 g PVP-40 and 0.1 g albumin in 10 mL of PBS-T.

Equipment

Incubator (37°C), 8-channel pipetter, reagent reservoirs for multi-channel pipetter, refrigerated microcentrifuge, refrigerated superspeed centrifuge (accepting 50 mL centrifuge tubes), and Sunrise absorbance microplate reader (Cat. No. Fo-MR20-301) by Tecan (Phenix Research Products, Hayward, CA).

Supplies[5]

Capped conical-bottom centrifuge tubes (50 mL), 15-mL capped round-bottom centrifuge tubes, 1.5-mL Eppendorf tubes, small mortars with pestles, Nunc-immuno plate with MaxiSorp surface (Nalge Nunc International, Rochester, NY, Cat. No. 439454), and sterile transfer pipettes.

Plant materials

Agrobacterium-mediated transformation (Li et al., 2001) was used to produce transgenic grapevine plants (*Vitis vinifera* cv. Thompson Seedless) containing the Shiva-1 gene and an enhanced *Aequorea victoria* green fluorescent protein (EGFP)/NPTII fusion gene. Fully expanded young leaves were collected from transgenic plants growing in the greenhouse and used for all experiments.

Notes

1. The stock solution of Shiva-1 protein was stored at -20°C. The solution and diluted solutions were thawed immediately before use. Thawed solutions were kept on ice.
2. The protease inhibitor cocktail tablets were stored at 4°C. Tablets were dissolved completely in ice-cold PBS buffer immediately before use.
3. Antiserum buffer and conjugate buffers were both prepared immediately before use and subsequently kept on ice. Although the antiserum buffer–containing DETC can be stored and reused, we noted that freshly prepared antiserum incubation mixtures containing plant extracts were unsuitable for repeated use because of strong protease activity.
4. Blocking reagents, such as albumin, were normally used at 0.1% (w/v) in other ELISA protocols (Crowther, 1995). We found that the use of 1% albumin as a blocking reagent in antiserum buffer is essential for minimizing background signal and enhancing the detection specificity for Shiva-1 protein.
5. All tubes were chilled on ice before use. Mortars and pestles were wrapped with aluminum foil, sterilized, air-dried, and chilled overnight. Mortars and pestles were kept at 4°C until use.

Methods

1. Record sample labels according to assigned positions in the microplate. Leave blank wells in positions surrounding the sample wells. Mark one set (total number of test samples) of 1.5-mL Eppendorf tubes with appropriate label.

2. Prepare 8 mL of fresh extraction buffer. Chill on ice.
3. Collect approximately 200 mg of leaf tissue from each plant[1] to be analyzed. Weigh and record fresh weight of each sample. Immediately rinse leaves in tap water and blot dry. Wrap leaf tissue samples in individual foil sheets and store on ice.
4. Place leaf tissue in an ice-cold mortar, add 2 X volume (w/v, i.e., 400 μL for 200 mg of tissue) of extraction buffer. Grind leaf tissue into a fine slurry using an ice-cold pestle[2]. Use a sterile transfer pipette to immediately transfer the slurry into a 1.5-mL tube. Maintain tubes on ice.
5. Centrifuge microtubes at 13K rpm for 2 min at 4ºC[3]. After centrifugation, place tubes on ice.
6. Transfer 100 μL of the supernatant into the appropriate well of a microplate. Avoid contaminating the side of the wells during sample transfer. During the loading process, keep the microplate on ice (place a piece of plastic wrap between the plate and the ice).
7. Prepare negative controls by using samples from nontransformed plants or transgenic plants containing no Shiva-1 gene. Load into corresponding microplate wells.
8. Prepare protein controls by mixing 5 μL of Shiva-1 stock solutions containing a known amount of Shiva-1 protein with 95 μL of sample solution from a negative control plant. Mix briefly. Load into designated microplate wells.
9. Cover the microplate with a piece of plastic wrap. Place on top of wet paper towels in a plastic container and incubate at 37°C for 1 h.
10. Prepare cross-absorption antiserum buffer and place on ice.
11. Collect 1 g of young leaf tissue from a negative control plant (preferably from a non-Shiva-1 gene–containing transgenic plant). Rinse leaves in tap water and briefly blot dry on clean paper towels.
12. Pour about 2 mL of antiserum buffer into a cold mortar. Add negative control leaf tissue (from 11) and grind the leaf sample into a fine slurry. Add the remaining 18 mL of buffer and grind briefly. Transfer the slurry into a 50-mL conical bottom centrifuge tube. Centrifuge at 3K rpm for 5 min at room temperature.
13. Transfer 5 mL of supernatant (from step 12) into a 15-mL centrifuge tube. Add 10 μL of rabbit anti-Shiva-1 antiserum stock solution (final ratio of supernatant to antiserum stock: 1:500) and mix by vortexing for 5 s. Cap the tube and incubate at 37°C for 30 min to 1 h.
14. Remove the microplate from the incubator. Discard the sample solutions by inverting the microplate quickly several times. Tap the inverted microplate on clean paper towels to remove any bubbles from the sample wells. Briefly rinse the sample wells by adding 200 μL of PBS-T to each well with a multichannel pipetter. Remove the rinse solution by quickly inverting the plate.
15. Add 200 μL of PBS-T buffer to each well, incubate for 3 min without agitation, and then discard the solution. Repeat this washing step 2 more times. Remove the final wash solution from the wells by vigorously tapping the microplate upside down several times on clean paper towels.
16. Remove the cross-absorption antiserum mixture from the incubator. Add 100 μL of the mixture to each sample well using a multi-channel pipetter. Cover

the microplate with plastic wrap and place it inside a plastic container with moist paper towels. Incubate for 1 h at 37°C.

17. Prepare fresh conjugate buffer in a 15-mL tube. Place on ice.
18. Remove the microplate from the incubator. Discard antiserum solution from sample wells. Rinse the wells 3 times with PBS-T buffer and remove all residual liquid from the sample wells as described previously.
19. Transfer 8 mL of conjugate buffer into a 15-mL tube. Add 8 μL of goat anti-rabbit IgG HRP conjugate and vortex for 5 s to mix. Place the conjugate solution on ice.
20. Add 100 μL of conjugate solution to each sample well. Cover the microplate with plastic wrap and place it in a plastic container with moist paper towels. Incubate at 37°C for 1 h.
21. Remove the HRP substrate kit from the refrigerator and allow the solutions to warm to room temperature before use.
22. Remove the microplate from the incubator. Discard the conjugate solution from the sample wells, rinse the wells 3 times with PBS-T buffer and remove all residual liquid from the sample wells.
23. Prepare the HRP substrate working solution by mixing 9 mL of solution A with 1 mL of solution B (9:1, according to manufacturer's instruction) in a 15-mL tube.
24. Add 100 μL of HRP substrate working solution to each sample well using a multichannel pipetter. Allow the reactions to proceed at room temperature. Start timing immediately.
25. Measure the absorbance at 405 nm at set times (e.g., 10, 20, and 40 min) after initiation of the reaction. A shaking period of 5 s before measurement should be set with the instrument. Remove the microplate from the reader immediately after each reading and continue the incubation at room temperature. Repeat the absorbance readings until reaction signals from the positive controls containing the lowest concentrations of Shiva-1 protein begin to increase. This may take up to 40-45 min of total incubation time.

Notes

1. Fully expanded young leaves should be collected. For best results, all plants to be analyzed should be moved into the laboratory before sampling to reduce the delay between sample collection and analysis. This will reduce target protein degradation.
2. Liquid nitrogen is not required in this procedure. However, maintaining all utensils at 4°C is essential to minimize degradation of the target protein. The time required for grinding samples should be uniform (within 1-2 min). The fineness of the sample slurry should be uniform across samples. We are currently evaluating the effectiveness of other grinding devices, such as the Berea hand-held homogenizer (STA Laboratories, Inc., Longmont, CO).
3. It is important to obtain a clear supernatant after centrifugation. Samples with excessive cell debris and insoluble cellular components may interfere with antigen binding on the surface of the microplate wells and, thus, limit the detection sensitivity.

Results and Discussion

ELISA has been widely used to detect antigenic proteins in biological samples. However, the ELISA protocols currently available are limited in their ability to detect small antigens such as the Shiva-1 protein. The most commonly reported difficulties have been the instability of these proteins in plant extracts and the low detection sensitivity of available procedures (Florack et al., 1995). However, strategies have been developed to increase the stability of these proteins (Owens and Heutte, 1997; De Lucca et al., 1998). We initially evaluated several ELISA protocols that were developed for the detection of viral proteins. However, none of the tested protocols yielded satisfactory results or permitted the unambiguous identification of Shiva-1 proteins in transgenic grapevines. To improve the efficacy of the ELISA procedure for detecting Shiva-1 protein, we incorporated several modifications to an indirect enzyme immunoassay procedure described by Tijssen (1985). These modifications included adding more than double the manufacturer's recommended dosage of protease inhibitor reagents to the extraction buffer, using antiserum cross-absorbed with control tissue extracts, incorporating up to 1% blocking reagents in the antiserum buffer, and carrying out all coating and/or binding processes at 37°C while limiting the time to 1 h for each step.

To determine the detection sensitivity of the modified procedure, we quantified chemically synthesized Shiva-1 protein over a range of concentrations (Figure 1). The lowest concentration of Shiva-1 protein used was 0.1 μM (equivalent to 0.5 ng/mL). Three minutes after the addition of the substrate reagent to the sample wells, Shiva-1 protein at this concentration resulted in an OD_{405} that was 30% higher than that of the background (0.058 vs. 0.044). When the reactions were continued for extended periods, no increase in the absorbance of the background sample was observed. However, the absorbance value for the 0.1 μM of Shiva-1 protein sample increased by 64.4%, 91.1%, and 131.1% above the background values after 13, 20, and 45 min of incubation time, respectively.

With the use of higher concentrations of Shiva-1 protein, significantly higher values of absorbance were observed (Figure 1). In all time-series absorbance studies, Shiva-1 protein at concentrations ranging from 1-100 μM produced OD_{405} values as much as 90-fold higher than the background values. In addition, the increase in absorbance over time remained linear relative to the concentration increases from 0-10 μM (Figure 1). Subsequent experiments also indicated that the linear relation between the absorbance change and target protein concentration remained unchanged, even with the use of up to 50 μM of Shiva-1 protein (data not shown). Thus, it is possible to accurately measure the amount of Shiva-1 protein in unknown samples using the procedure, as long as the protein concentration in the sample falls within a range of 0.1-50 μM.

Previous modifications to ELISA procedures have included the attachment of small antigens to particles larger than the antigen before reaction with antiserum. This modification effectively enhanced detection sensitivity for small antigens and antigens at low concentrations. For example, Demski et al. (1986) demonstrated the successful detection of 0.1-0.5 μg/mL of 6 rod-shaped viruses by using a procedure that involved the use of latex agglutination. Our procedure

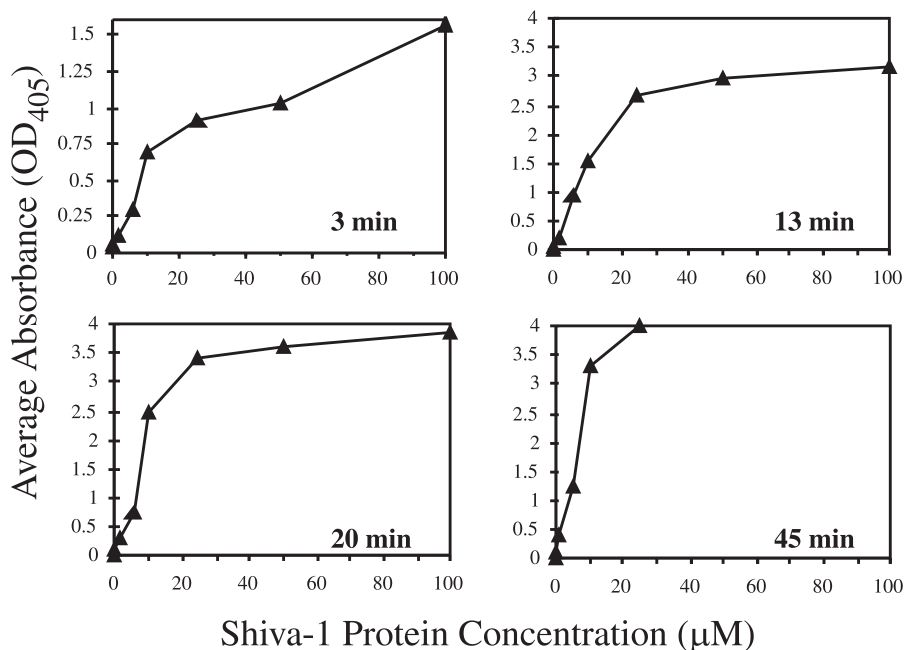

Figure 1. Quantitative measurement of Shiva-1 protein using an improved ELISA procedure. Shiva-1 protein was added to the extraction buffer and then subjected to ELISA analysis using the described procedure. Absorbance at 405 nm was measured at designated time intervals after the initiation of enzymatic reaction. Average values from 3 independent experiments were plotted. An OD_{405} value of 4 was the upper limit set by the ELISA reader.

allowed the detection of a small antigen (Shiva-1 protein) at an even lower concentration (5 ng/mL) without the use of any high molecular weight carriers.

Plant extracts generally contain sufficient proteolytic activity to destroy small proteins (Florack et al., 1995). Our preliminary experiments indicated that the addition of Shiva-1 protein at a concentration as high as 100 μM to a grapevine leaf extract resulted in complete degradation of the protein within minutes and the subsequent failure to detect any Shiva-1–specific signals using a previously reported ELISA assay (Koenig and Paul, 1982). The addition of EDTA-free protease inhibitor to the extract buffer reduced proteolytic activity and minimized the degradation of Shiva-1 protein. We found that an increased concentration of the protease inhibitor reagent (up to 1 tablet per 4 mL of extraction buffer, i.e., twice the manufacturer's recommended concentration) provided more effective inhibition to the proteolytic activity present in grapevine leaf extracts (data not shown).

Various concentrations of Shiva-1 protein were mixed with aliquots of leaf extract prepared from young leaves of a nontransformed cv. Thompson Seedless plant and loaded into the wells of a microplate. Higher absorbance values were observed in leaf extract samples without the Shiva-1 protein than for a control buffer solution (Figure 2 vs. Figure 1). The absorbance value of the negative control leaf sample increased as the enzymatic reaction proceeded (Figure 2). These

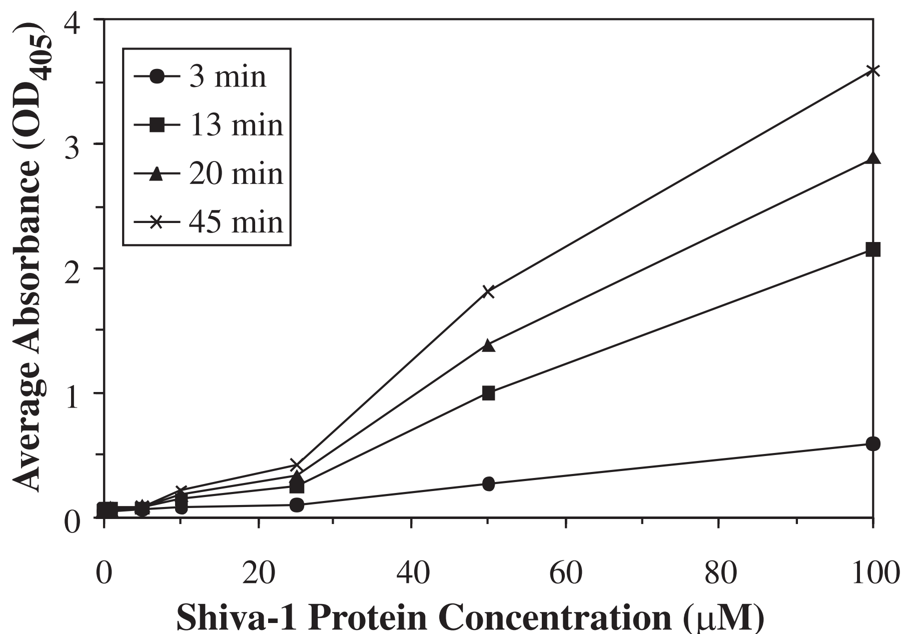

Figure 2. Quantitative measurement of Shiva-1 protein in leaf extract of grape using an improved ELISA procedure. Shiva-1 protein was mixed in leaf extract and subjected to ELISA analysis using the described procedure. Leaf extract was prepared using young leaves of a nontransformed cv. Thompson Seedless plant. CK samples contained leaf extract without the addition of Shiva-1 protein. Absorbance at 405 nm was measured at designated time intervals after the initiation of enzymatic reaction. Data lines represent the average values from 3 independent experiments. An OD_{405} value of 4 was the upper limit set by the ELISA reader.

elevated signal values from the control wells probably resulted from nonspecific antibody binding activity in the leaf extract. When Shiva-1 protein was added to the grape leaf extract at a concentration of 1 μM, no significant increase in absorbance was observed. The addition of Shiva-1 protein at concentrations of ≥5 μM into the leaf extract was required to produce absorbance values that allowed the positive identification of the presence of Shiva-1 protein when compared to the negative control sample. Also, at least 40 min of enzymatic reaction period was required to obtain discernible signals (Figure 2). Thus, the detection sensitivity, even using the modified procedure, was reduced by up to 50-fold when the assay system included leaf extract (0.1 μM vs. 5 μM).

Twelve transgenic grape plants (cv. Thompson Seedless) containing the Shiva-1 and/or EGFP genes were analyzed using the improved protocol to determine protein expression. Since all the Shiva-1 gene–containing transgenic plants also contained an expressible EGFP gene, 2 plants transgenic for the EGFP gene but not the Shiva-1 gene were used as negative controls. Positive signals for Shiva-1 protein were successfully detected in all Shiva-1 gene–containing transgenic plants, but not in negative control plants (Figure 3). Table 1 summarizes the absorbance values and protein concentrations of Shiva-1 protein in transgenic

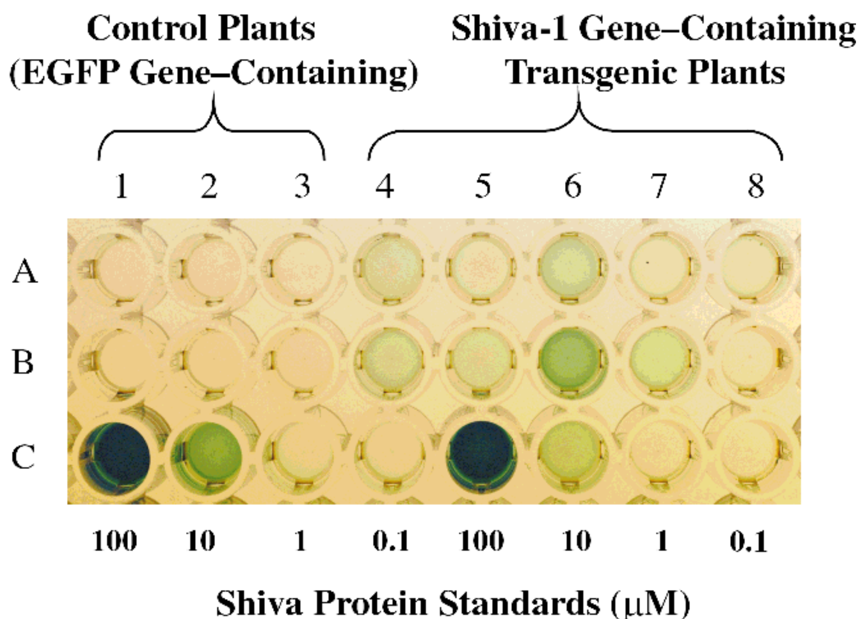

Figure 3. Detection of Shiva-1 protein in transgenic grapevine plants using a modified ELISA procedure. Leaf samples were collected from 2 enhanced green fluorescent protein (EGFP) gene–containing transgenic plants (T101-68, wells A1-A3 and T101-71, wells B1-B3, with 3 repeated wells per sample) and 10 transgenic plants containing both EGFP and Shiva-1 genes (see Table 1, wells A4-A8 and B4-B8 with 1 sample per well), and subject to ELISA analysis, along with positive controls that were prepared by adding specified amounts of Shiva-1 protein into leaf extract of T101-68 (wells C1-C8). Image was taken 40 min after the initiation of enzymatic reaction.

Table 1. Detection of Shiva-1 protein in transgenic grapevine plants (*V. vinifera* cv. Thompson Seedless) using an improved ELISA procedure.

Plant name	Transgenes	Absorbance[1] (OD_{405})	Shiva-1 Concentration (μM)	Well-No. Shown in Fig.3
T101-68	EGFP	0.098	0	A1 to A3
T101-71	EGFP	0.101	0	B1 to B3
T101-71+Shiva-1	EGFP	0.354	10.0	C2 & C6
T408R-2-1	EGFP+Shiva	0.222	6.07	A4
T408R-2-5	EGFP+Shiva	0.207	5.67	A5
T408R-2-7	EGFP+Shiva	0.342	9.92	A6
T408R-2-8a	EGFP+Shiva	0.221	6.07	A7
T408R-2-8b	EGFP+Shiva	0.245	7.89	A8
T408R-2-9	EGFP+Shiva	0.289	8.70	B4
T408R-2-12	EGFP+Shiva	0.236	6.68	B5
T408R-2-19	EGFP+Shiva	0.628	15.18	B6
T408R-2-21	EGFP+Shiva	0.284	8.50	B7
T408R-2C-2	EGFP+Shiva	0.166	3.44	B8

[1]Absorbance values were measured 40 min after reaction initiation.

grape plants. The absorbance values from EGFP gene–containing transgenic plants were similar to those generated from nontransgenic plants (Figure 2, Table 1). This suggested that the EGFP protein did not affect the detection specificity for Shiva-1 protein. Transgenic grape plants analyzed here produced Shiva-1 protein at concentrations ranging from 3.44 - 15.18 μM in the leaf extracts (Table 1). The Shiva-1 protein concentration in those plants was estimated based on a standard curve reconstructed by using absorbance values from known amounts of Shiva-1 protein in leaf extract of a transgenic grapevine plant lacking the Shiva-1 gene (T101-68) (data not shown). However, it is important to note that actual *in vivo* protein concentrations in Shiva-1 gene–containing transgenic plants may be higher because up to a 50-fold difference in protein (synthetic Shiva-1) concentration was observed with similar absorbance values in previous analysis using extraction buffer vs. leaf extract (Figures 1 and 2).

The described procedure provided a simple, reliable, and effective method for the analysis of small proteins in transgenic plants. Thus far, we have used this procedure for routine quantitative analysis of Shiva-1 protein production in a large number of transgenic grapevine plants. Information obtained through these analyses should ultimately benefit our effort to monitor and optimize transgene expression in target tissues and organs to produce more effective disease resistance.

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