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Effective application of DAS-ELISA for detection of grapevine leafroll associated closterovirus-3 using a polyclonal antiserum developed from recombinant coat protein

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Abstract

A polyclonal antiserum (As163) specific to grapevine leafroll associated closterovirus-3 (GLRaV-3) was developed using a recombinant coat protein expressed in *E. coli* from a cDNA clone identified after immunoscreening of a cDNA library. Specificity of the antiserum to GLRaV-3 was shown by Western blot and immunosorbent electron microscopy. With this antiserum, an effective double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was developed for GLRaV-3 detection. To evaluate the sensitivity of the antiserum in DAS-ELISA for virus detection, different combinations of antibodies were compared. Although best results were obtained when As163 was used for coating and a monoclonal antibody (MabNY1.1) was used as an enzyme conjugate, good results were also obtained when As163 was used both for coating and as an enzyme conjugate. Using this As163–Mab system in DAS-ELISA, we confirmed the presence of GLRaV-3 in a diverse collection of leafroll infected vines.

Abbreviations: GLRaV-3 – grapevine leafroll associated virus-3; DAS-ELISA – double antibody sandwich-enzyme-linked immunosorbent assay.

Introduction

Grapevine leafroll is one of three most important viral diseases on grapes; the other two are fanleaf and rugose wood complex. Seven types of grapevine leafroll associated closteroviruses (GLRaV-1 to -7) are associated with the disease (Boscia et al., 1995; Choueiri et al., 1996). Among these closteroviruses, however, only GLRaV-3 is transmitted by insect vectors, viz. mealybug (Rosciglione and Gugerli, 1989; Tanne et al., 1989; Engelbrecht and Kasdorf, 1990; Golino et al., 1995, 1998; Petersen and Charles, 1997) and scale insect (Belli et al., 1994).

Polyclonal (Zee et al., 1987; Zimmermann et al., 1990a) and monoclonal (Hu et al., 1990a; Zimmermann et al., 1990b) antibodies specific to GLRaV-3 were developed from purified virus preparation and effectively used in serological assays to detect GLRaV-3 in grapevine. However, developing high quality antibodies to GLRaV-3 and other GLRaVs proved rather difficult because yield of purified viruses were low (Zee et al., 1987; Hu et al., 1990b; Zimmermann et al., 1990a; Boscia et al., 1995; Choueiri et al., 1996). The 3′ two-thirds of GLRaV-3 genome (Ling et al., 1998), including the coat protein gene (Ling et al., 1997) were recently cloned and sequenced. Thus, the coat protein gene sequence provided an alternative way to produce a polyclonal antisera to recombinant coat protein expressed in *Escherichia coli*.

Relatively few reports have described the production of virus-specific antibodies to recombinant viral proteins expressed in *E. coli* (Nikolaeva et al., 1995;

Vaira et al., 1996; Jelkmann and Keim-Konrad, 1997; Rubinson et al., 1997). Although these antisera have been applied to plant virus detection in Western blot, immunosorbent electron microscopy (ISEM) and indirect enzyme-linked immunosorbent assay (ELISA), only the report by Vaira et al. (1996) showed that this type of antiserum is suitable for double antibody sandwich ELISA (DAS-ELISA) for plant virus detection. For example, an antiserum developed to the coat protein of citrus tristeza virus (CTV) reacted in an indirect ELISA and may be effective in DAS-ELISA in combination with a monoclonal antibody (Nikolaeva et al., 1995). Another antiserum produced to the coat protein of apple stem pitting virus (ASPV) (As647) was used for immunocapture PCR, Western blot, immuno electron microscopy and indirect plate trapped ELISA, but was not effective in DAS-ELISA (Jelkmann and Keim-Konrad, 1997). The other two polyclonal antisera (403 and 405) failed in all ELISA types tested (Jelkmann and Keim-Konrad, 1997). In grape, an antiserum derived from recombinant putative movement protein was useful for detecting grapevine virus A (GVA) in grapevine by Immunoblot, but not by ELISA (Rubinson et al., 1997). Due to its high specificity and convenience, DAS-ELISA is still the most desirable method for fruit tree viral disease diagnosis.

This report describes the development of a polyclonal antiserum to the recombinant coat protein of GLRaV-3, characterization of its serological properties and its application in DAS-ELISA.

Materials and methods

Virus sources

The NY1 isolate of GLRaV-3 (Zee et al., 1987; Hu et al., 1990b), which is also referred to as isolate GLRaV 109 (Golino, 1992), was used throughout this work. Other isolates that were used in the validation test were grape accessions collected from various parts of the world and deposited in the USDA/ARS Plant Genetic Resources Unit at Geneva, New York.

Fusion protein production and immunization

We previously identified three cDNA clones that reacted to the antibody specific to GLRaV-3 (Ling et al., 1997). The clone pCP10 in the protein-expression vector pBluescript SK was used to induce expression of the fusion protein under the control of the lac promoter (Stratagene). Sequence analysis of clone pCP10 revealed an open reading frame (ORF) of 1167 nucleotides (nt) which consisted of 900 nt of the GLRaV-3 cp gene, an *Eco*RI linker sequence and the 3′ terminal region of the *Lac*Z gene (Figure 1). This ORF encodes a fusion protein with a calculated molecular mass of 43 kDa that consists of the N-terminal 33 kDa of the GLRaV-3 coat protein and the C-terminal 10 kDa of β-galactosidase. The coat protein coding region covered 96% of the entire coat protein gene, only lacking 13 amino acid residues of the C-terminus (Figure 1).

To obtain fusion protein for use as an immunogen, *E. coli* (strain XL1-blue) containing pCP10-1 was cultured overnight at 37 °C with vigorous shaking in 500 ml LB medium containing 50 μg/ml ampicillin. When growth of bacteria reached the log-phase ($\sim 0.5\ OD_{600\ nm}$), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM and further incubated for 3 h. After centrifugation at 4000*g* for 10 min, pelleted bacterial cells were resuspended in 20 ml of ice-cold phosphate buffer saline (PBS, 0.05 M potassium phosphate, pH 7.2, 0.15 M NaCl) and kept on ice until used. Bacterial cell walls were disrupted by sonication three times, 30 s each. Supernatant was collected after centrifugation at 14,500*g* for 15 min. Initially, different saturations of ammonium sulfate (20%, 40%, 60% and 75% saturation) (Englard and Seifter, 1990) were evaluated for their effectiveness in precipitating fusion protein. ELISA monitoring showed that the fraction with 40% saturation of ammonium sulfate (2.54 g of ammonium sulfate in 20 ml of supernatant) was most effective for an enrichment of the fusion protein. The enriched fusion protein was dialyzed and lyophilized. The lyophilized protein was resuspended in protein degradation buffer (750 mM Tris–HCl, pH 8.8, 4% (W/V) SDS, 4% (W/V) β-mercaptoethanol, 40% sucrose) and denatured by boiling for 10 min. Denatured preparations were loaded on a preparative 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Laemmli, 1970) and electrophoresed along with prestained protein molecular weight standards (Bio-Rad). Proteins were visualized by KCl staining (0.25 M KCl and 1 mM DTT) (Hager and Burgess, 1980) and the fusion protein was located after a comparison to a Western blot. The excised gel was rinsed with three changes of ice-cold water for 5 min and kept at

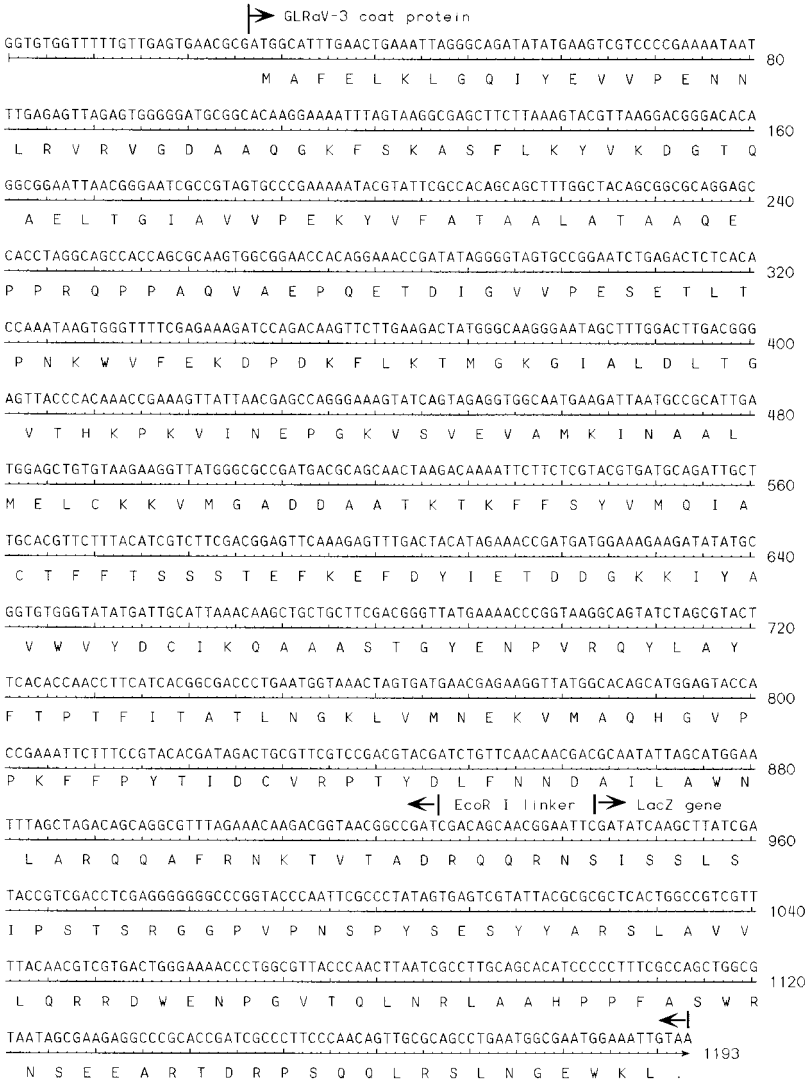

Figure 1. Nucleotide and deduced amino acid sequences of fusion coat protein of GLRaV-3. A complete ORF including 900 bp of the coat protein gene followed by 17 bp *Eco*R I linker and 250 bp 3′ *Lac*Z gene is shown. The calculated size of the fusion protein is 43,313 D.

−20 °C or ground immediately in a mortar and pestle. The fusion protein was eluted with 10 volumes of elution buffer (0.05 M Tris–HCl, pH 7.9, 0.1% SDS, 0.1 mM EDTA and 0.15 M NaCl) by gently shaking for 6 h to overnight at 4 °C. The gel slurry was filtered through a Whatman 3 mm filter paper and washed with 2 ml of elution buffer and re-extracted again. Eluates were combined and the fusion protein was precipitated with 20% (final volume) trichloroacetic acid at 4 °C overnight. The purified fusion protein was collected after centrifugation at 14,500*g* for 10 min, and resuspended in 1 ml of 0.01 M Tris–HCl, pH 8.2 and dialyzed.

One mg of purified fusion protein (1 mg/ml) was emulsified with 1 ml of Freund's complete adjuvant and injected subcutaneously at multiple sites along the back of a New Zealand white rabbit, followed by two more weekly injections with protein emulsified with Freund's incomplete adjuvant. One week after the last injection, the rabbit was bled weekly and the titer was evaluated by SDS-immunodiffusion (Purcifull and Batchelor, 1977), ELISA (Clark and Adams, 1977)

or Western blot (Hu et al., 1990b) against bacterium expressed fusion protein and plant extracts. An additional booster shot with 1 ml of 1 mg/ml purified fusion protein emulsified with 1 ml of incomplete adjuvant was given after the tenth weekly bleeding. Thereafter, the blood was collected weekly for 3 more months. The antiserum was designated as As163.

Western blot

Western blotting was done according to the procedure described by Hu et al. (1990b) with some minor modifications. Denatured virus-infected crude extract was applied directly onto a 12% SDS-PAGE gel and electrophoresis was carried out at 180 V until the green colored band reached the bottom of the gel. After transfer, the blotted Immobilon membrane (Millipore) was blocked in 2% milk–PBS solution for 1 h at room temperature and further incubated with 1 : 1000 dilution of alkaline phosphatase conjugated GLRaV-3 specific monoclonal antibody (MabNY1.1) (Hu et al., 1990a), polyclonal antisera As131 (Zee et al., 1987) and As163 (this study) for 4 h at 30 °C. After thorough washing with PBS–Tween-20, membranes were treated with freshly prepared nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and coat protein bands were revealed.

Immunosorbent electron microscopy (ISEM)

For negative staining, Formvar-covered grids were placed on a drop of purified virus preparation for 30 s and stained with a drop of 2% uranyl acetate. For immunodecoration of virus particles, we followed the ISEM procedure described by Milne and Luisoni (1977) as modified by Namba et al. (1991). Decorated virus particles were observed after staining with 2% uranyl acetate on a JEM-100SX transmission electron microscope (Joel Ltd., Tokyo). Decoration of virus particles by As163 were compared to decoration by polyclonal As131 (Zee et al., 1987) and by MabNY1.1 (Hu et al., 1990a).

ELISA

All ELISA tests were performed using the DAS method (Clark and Adams, 1977) essentially as described by Ling et al. (1995). Polyclonal and monoclonal antibodies to GLRaV-3 were produced earlier (Zee et al., 1987; Hu et al., 1990a). In some cases, crude antiserum diluted (1 : 1000, 1 : 2000 and 1 : 4000) in coating buffer (0.2 M sodium carbonate buffer, pH 9.6) were used for coating and the grape extraction buffer (200 mM Tris–HCl, pH 8.2, 140 mM NaCl, 2% PVP40) (Walter and Etienne, 1987) was used for sample preparation (1 : 10). The plant tissue extract was prepared by powdering about 200 mg of grape bark tissue in liquid nitrogen with a mortar and pestle and ground in 2 ml of grape extraction buffer. After a brief centrifugation, 100 µl of upper clean supernatant was loaded and incubated overnight at 4 °C. Plates were then blocked with 1% BSA for 1 h at room temperature. Alkaline phosphatase conjugation was performed as described (Clark and Adams, 1977). Preparations of enzyme conjugates in ELISA plates were incubated at 30 °C for 4 h. Substrate at 1 mg/ml was added and incubated at room temperature. Absorbances at $OD_{405\,nm}$ were recorded on a computer programmed ELISA reader (STL Spectra, Tecan U.S. Inc., Research Triangle Park, NC).

Results

Preparation of the fusion protein

The sequence of GLRaV-3 cp gene and its surrounding nucleotides were determined in our earlier work (Ling et al., 1997). Clone pCP10 was selected for production of the recombinant protein because it produced a protein that reacted specifically to polyclonal and monoclonal antibodies to GLRaV-3 (Ling et al., 1997) and sequence analysis revealed that pCP10 contained the entire cp gene except 10 amino residues on C-terminus (Figure 1). About 5 mg of fusion protein was produced from 500 ml of starting bacterial culture. Western blot analysis showed that the isolated fusion protein reacted strongly to antibodies specific to GLRaV-3 (Figure 2).

Production and characterization of antibody

Since preliminary ELISA and Western blot analysis suggested that the first several batches of antiserum (As163) had relatively low antibody titers to GLRaV-3 infected tissues, the rabbit was given a booster shot after the 10th weekly bleeding. Initial analysis on Western blot of antiserum collected after the boost showed that it gave a stronger reaction than those antisera collected before the boost (data not shown). The reactivity of As163 was further compared to the polyclonal (As131) and Mab to GLRaV-3 (Zee et al., 1987; Hu et al., 1990a)

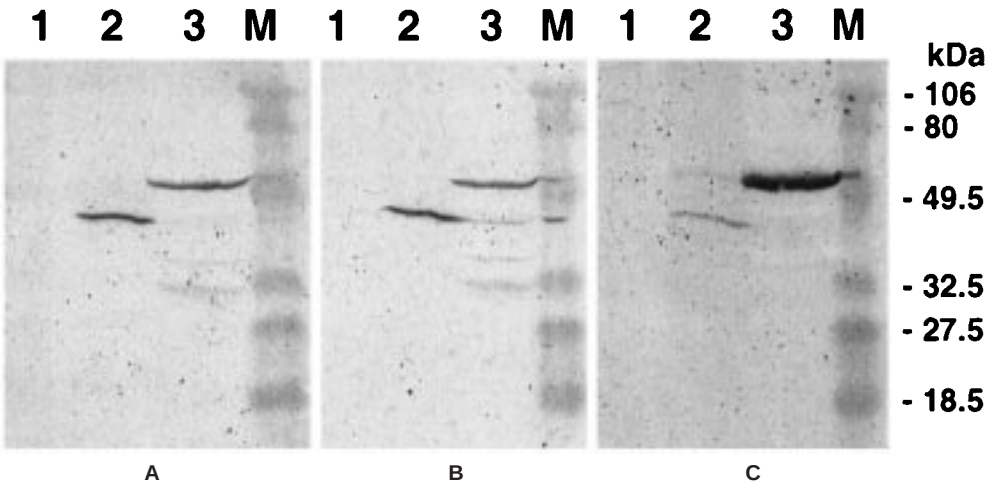

Figure 2. Comparative effectiveness of three GLRaV-3 specific antibodies on Western blots. Similar banding patterns were observed when monoclonal antibody (MabNY1.1) (panel A), polyclonal As131 (panel B) and As163 (panel C) were used to decorate the membrane. Three replica of samples including healthy control (lane 1), infected grapevine tissue (lane 2) and bacterium expressed fusion protein (lane 3). Lane 4 is a prestained protein molecular weight standards (Bio-Rad, low range). MabNY1.1: monoclonal antibody NY1.1 (Hu et al., 1990a); As131: polyclonal antiserum (Zee et al., 1987); As163: polyclonal antiserum (this study).

in Western blot. As shown in Figure 2, all three antibodies provided similar reactions to GLRaV-3 infected tissue extract and purified fusion protein, confirming that As163 was specific to the coat protein of GLRaV-3. Immuno-electron microscopy also showed that As163, like As131 and MabNY1.1, decorated GLRaV-3 particles. Comparatively, As131 gave the strongest reaction, followed by As163 and MabNY1.1 (Figure 3).

Comparative effectiveness of As163 in ELISA tests

We were particularly interested in developing a DAS-ELISA system with As163 for detection of GLRaV-3 in grapevine because this assay method is being widely used. To evaluate the effectiveness of As163 in DAS-ELISA, comparative studies were carried out with combinations of three antibodies (As131, As163, and MabNY1.1) for coating and as an enzyme conjugate. Absorbance readings were recorded at 30 min, 1 and 2.5 h intervals after start of substrate hydrolysis. Best readings were obtained from a combination of As163 for coating and MabNY1.1 as a conjugate (As163–MabNY1.1), with average OD readings of 1.43 after 1 h. As expected, high absorbances were also observed from coating–conjugate combinations of As131–MabNY1.1 (0.85 at 1 h) and As131–As131 (0.77 at 1 h). Clear results were also achieved when As131 or As163 was used for coating in combination with As163 as a conjugate (0.27–0.34 at 1 h). However, only marginal readings were obtained from MabNY1.1–MabNY1.1 or As163–As131 (data not shown). Mab was not suitable as a coating antibody in combination with either As163 or As131 as a conjugate (data not shown). At 2.5 h, all readings were enhanced while background levels remained below 0.10. The experiments were repeated at least three times with similar results.

In a further study on the effectiveness of using As163 for virus detection, fourteen samples were tested with various combinations of antibodies in DAS-ELISA (Table 1). Eight GLRaV-3 infected and six healthy samples had been previously characterized biologically and serologically (Forsline et al., 1996). All three antibodies (As163, As131 and MabNY1.1) were used for coating in combination with two enzyme conjugates (As163 and MabNY1.1). Eight GLRaV-3 infected and six healthy samples were readily identified when either As163 or As131 was used as coating antibody in DAS-ELISA (Table 1). The best results were obtained when As163 or As131 was used for coating and MabNY1.1 as an enzyme conjugate. Good results were obtained when As163 alone was used for both coating and conjugate. Only marginal positive readings were observed when combinations of As131–As163 was used. MabNY1.1 used as coating and as conjugate

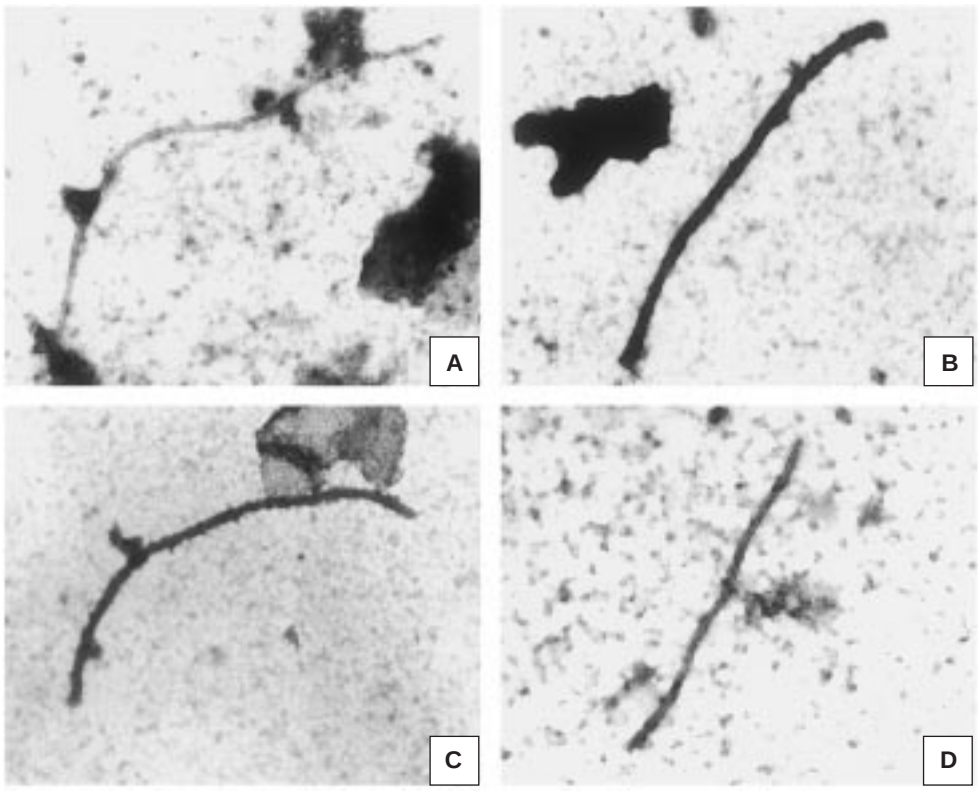

Figure 3. Decoration of GLRaV-3 particles by three GLRaV-3 specific antibodies observed under the electron microscope. GLRaV-3 virus particles were decorated with three GLRaV-3 specific antibodies (see Materials and methods for details), As131 (panel B, Zee et al., 1987), AS163 (panel C, this study) and MabNY1.1 (panel D, Hu et al., 1990a) respectively. Panel A was a negative staining showing long flexuous virus particles.

produced positive results, however, combinations of MabNY1.1 for coating and As163 as a conjugate did not work (Table 1).

Based on results from the comparative experiments, the combination of As163 for coating and MabNY1.1 as an enzyme conjugate (As163–Mab) was selected to test its effectiveness to detect GLRaV-3 in the grapevine collection at the USDA/ARS Plant Genetic Resources Unit, Geneva, New York, which contains grapevine germplasm from around the world. Extensive efforts have been carried out since 1988 in identifying GLRaV-3 infected vines from this collection both biologically and by ELISA (Forsline et al., 1996) to select nominees for virus elimination. On the basis of screenings carried out both in 1988 and 1989 growing seasons by DAS-ELISA with As131, 39 accessions representing 39 different cultivars or hybrids collected from USA (30), France (6), Canada (2) and Germany (1) were shown to be infected by GLRaV-3 (Forsline et al., 1996, unpublished data). In the 1997 and 1998 growing seasons, we tested these 39 accessions using As163–MabNY1.1 system in DAS-ELISA. The results not only confirmed the earlier testings in 1988 and 1989, but also clarified three ambiguous identifications. The ELISA readings (specific data not shown) were clean and clear with a minimum absorbance at 405 nm of over 0.550 from infected vines and an average of 0.022 from healthy controls.

Discussion

An antiserum produced to a recombinant viral protein and used in combination with a monoclonal antibody was shown to be effective in the detection of a woody plant virus by DAS-ELISA. Although, a number of polyclonal antisera have been raised against recombinant viral proteins (Nikolaeva et al.,

Table 1. Validation test using field collected grapevine samples with different antibody combinations in DAS-ELISA

Vine	As163[a]		As131		Mab		Status[b]
	As163	Mab	As163	Mab	As163	Mab	
18-1-2	0.189	0.871	0.133	0.446	0.090	0.144	(+)
18-4-4	0.268	1.359	0.184	0.681	0.115	0.316	(+)
18-5-3	0.257	1.230	0.190	0.629	0.082	0.294	(+)
18-5-4	0.289	1.302	0.207	0.714	0.094	0.318	(+)
19-4-1	0.261	1.280	0.188	0.660	0.156	0.368	(+)
19-4-3	0.340	1.426	0.265	0.849	0.128	0.389	(+)
19-4-4	0.245	1.271	0.177	0.706	0.110	0.241	(+)
19-5-3	0.103	0.368	0.116	0.202	0.094	0.097	(+)
17-6-1	0.033	0.022	0.061	0.021	0.111	0.025	(−)
18-3-5	0.033	0.022	0.060	0.022	0.092	0.028	(−)
18-4-3	0.035	0.023	0.056	0.022	0.101	0.031	(−)
18-4-5	0.033	0.026	0.056	0.023	0.106	0.032	(−)
19-6-4	0.030	0.021	0.051	0.022	0.058	0.029	(−)
Healthy	0.036	0.022	0.046	0.023	0.051	0.034	(−)

[a] Coating antibody is shown over the line and enzyme conjugate is shown under the line for each DAS-ELISA. Coating with 1 : 2000 dilution of antisera (As131 and As163) or 2 μg/ml MabNY1.1. Enzyme conjugate used were diluted to As163 (1 : 500) or MabNY1.1 (1 : 1000). The absorbance at $OD_{405\,nm}$ was taken 1 h after substrate hydrolysis.

[b] Status for GLRaV-3 infection was previously determined by graft inoculation to the biological indicator Pinot noir and by ELISA.

1995; Vaira et al., 1996; Jelkmann and Keim-Konrad, 1997; Rubinson et al., 1997), only the antibody produced to tomato spotted wilt virus nucleoprotein (Vaira et al., 1996) was effective in detecting the virus by ELISA. For woody plant viruses, this type of antibodies was effective only for immunocapture PCR, Western blot, immuno-electron microscopy or indirect ELISA (Nikolaeva et al., 1995; Jelkmann and Keim-Konrad, 1997; Rubinson et al., 1997). Nikolaeva et al. (1995) mentioned that their antibody produced to the recombinant coat protein of CTV was effective in DAS-ELISA in combination with a monoclonal antibody as an enzyme conjugate, however, no data were presented. Reasons for the ineffectiveness of these polyclonal antisera in DAS-ELISA are generally attributed to their relatively low performance for trapping antigens or the high background reaction generated from healthy controls (Nikolaeva et al., 1995; Jelkmann and Keim-Konrad, 1997; Rubinson et al., 1997). In the present study, high background reactions were also experienced in healthy controls with antiserum collected before the last booster shot was used (unpublished results). However, satisfactory results were obtained with antisera collected after the booster shot that was given 10 weeks after the initial immunization. Western blot analysis of antisera collected after the booster shot provided clear evidence that reactivity of the antisera to GLRaV-3 had been enhanced (unpublished results).

Several other reasons may account for the adequate reaction of our antiserum in DAS-ELISA. It is possible that the immunogen was renatured during the purification process before immunization. Although the proteins had been denatured prior to electrophoresis, subsequent elution and precipitation steps might have caused the protein to renature. A similar technique was employed to isolate the recombinant viral proteins to GVA (Rubinson et al., 1997). However, the denatured GVA recombinant viral protein in the SDS-PAGE gel was used directly to immunize rabbits without elution and concentration. The antiserum was useful for immunoblotting, but not for ELISA (Rubinson et al., 1997). Another possible reason for the effectiveness of GLRaV-3 As163 in DAS-ELISA is that the ratio of coat protein to vector sequence in the fusion protein of pCP10 is relatively high (77%). In the case of CTV, the ratio is about 30% (Nikolaeva et al., 1995). This high proportion of viral protein in a fusion protein should increase the chances that the

animal would have a good immune response to the viral protein.

Direct use of cDNA clones for protein expression is time-saving and trouble-free as compared to the subcloning into a protein expression vector (Nikolaeva et al., 1995; Rubinson et al., 1997). Since pBluescript SK was able to express a fusion protein, clone pCP10 was used directly without further subcloning into a protein-expression vector. This method of cDNA preparation (Ling et al., 1997) should be useful to identify coat protein genes of other GLRaV types since antibodies to these types are available (Boscia et al., 1995; Choueiri et al., 1996). Once a cDNA clone expressing the fusion coat protein is identified, a high quality polyclonal antiserum can be prepared according to the procedure outlined in the present study.

The approach of using recombinant viral protein for the antibody production should be a good alternative for producing high quality antibodies to other types of closteroviruses associated with grapevine leafroll. There are seven serologically distinct types of closteroviruses that are associated with leafroll (Boscia et al., 1995; Choueiri et al., 1996). In general, it is rather difficult to obtain a sufficient amount of purified virus for production of high quality antibodies. Furthermore, it is more complicated to produce a true-to-type polyclonal antiserum by the fact that mixed infections of different types of GLRaV in a field collected sample is common (Agran et al., 1990; Credi and Giunchedi, 1996; Monis and Bestwick, 1997). With the exception of GLRaV-2 (Boscia et al., 1995; Goszczynski et al., 1996), these viruses are not able to be separated biologically. Therefore molecular cloning provides an alternative means for virus separation. We recently cloned and sequenced most of the GLRaV-2 genome (Zhu et al., 1998). A polyclonal antiserum to GLRaV-2 was also developed to the recombinant coat protein (unpublished results). As sequence information from other types of closteroviruses becomes available, high quality and type-specific polyclonal antibodies could be developed using recombinant viral protein technology.

In summary, the polyclonal antiserum (As163) to recombinant coat protein was effectively used in DAS-ELISA to detect GLRaV-3. Comparative studies with different combinations of antisera for coating and enzyme conjugate suggested that As163 can effectively replace the original polyclonal antiserum (As131) produced from purified viruses (Zee et al., 1987). Although the best result was obtained when As163 was used for coating in combination with monoclonal antibody as an enzyme conjugate, good results were observed in DAS-ELISA tests with As163 coating and enzyme conjugate. Furthermore, this antiserum detected GLRaV-3 in grapevines collected around the world.

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