

# Zantedeschia mild mosaic virus, a new widespread virus in calla lily, detected by ELISA, dot-blot hybridization and IC-RT-PCR

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A new potyvirus, *Zantedeschia* mild mosaic virus (ZaMMV), was recently identified in calla lily (*Zantedeschia* spp.) in Taiwan. The sequenced 3'-terminal region indicated a unique amino acid sequence of ZaMMV, the N terminus of the capsid protein (CP), containing 39 glutamine residues before the DAG motif. In order to obtain antiserum for subsequent studies, a recombinant ZaMMV CP without polyglutamine was successfully expressed in *Escherichia coli* and used as the antigen. The specificity of ZaMMV antiserum was confirmed by immunoblot and ELISA analyses. Three detection methods, ELISA, dot-blot hybridization and immunocapture reverse transcription PCR (IC-RT-PCR), were developed and were able to detect ZaMMV successfully. During 2003–2004 field surveys, the results demonstrated that ZaMMV, as well as *Zantedeschia* mosaic virus (ZaMV), are prevalent in calla-growing areas. In contrast, infection by *Dasheen mosaic virus* (DsMV), the most important virus of aroid plants, decreased dramatically because DsMV-free calla lily seedlings were grown in the field. Accordingly, the detection methods developed in this study will be useful in studying ZaMMV and also in producing ZaMMV-free calla lilies.

**Keywords:** Araceae, *Zantedeschia* mosaic virus, *Dasheen mosaic virus*, potyvirus, virus detection, *Zantedeschia* spp.

## Introduction

Calla lily (*Zantedeschia* spp.), originating from Africa, has been imported into Taiwan from New Zealand, the Netherlands and the USA for breeding and production of cut flowers. Bacterial soft rot and viral diseases are the limiting factors for calla lily production in Taiwan, as well as in other countries. Many viruses have been reported to infect calla lily including *Dasheen mosaic virus* (DsMV), the most important virus of aroid plants (Rana *et al.*, 1983; Chen *et al.*, 2001), *Cucumber mosaic virus* (CMV) (Mokra & Gotzova, 1994), *Konjak mosaic virus* (KoMV), *Bean yellow mosaic virus* (BYMV), *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Alfalfa mosaic virus* (AMV), *Arabidopsis mosaic virus* (ArMV), *Potato virus X* (PVX), *Tobacco rattle virus* (TRV) (Pham *et al.*, 2002), *Turnip mosaic virus* (TuMV) (Chen *et al.*, 2003) and *Zantedeschia* mosaic virus (ZaMV) (Chang *et al.*, 2001; Kwon *et al.*, 2002). A new calla lily-infecting potyvirus, *Zantedeschia* mild mosaic virus (ZaMMV) was recently identified in Taiwan (Huang & Chang, 2005). After cloning and sequencing the 3'-terminal

region of the virus, a unique polyglutamine sequence was discovered spanning the N-terminal region of the capsid protein (CP) (Huang & Chang, 2005).

In order to obtain antiserum for further studies, bacteria-expressed CP was used to produce ZaMMV antiserum, due to the problem of purification of virus particles from aroid plants, described by Li *et al.* (1998). The CP gene of ZaMMV without the polyglutamine sequence was cloned into an expression vector, successfully expressed in *Escherichia coli* and used as an antigen to immunize rabbits. The specificity of the antiserum was tested by means of immunoblot and indirect ELISA (I-ELISA) assays. Moreover, three detection methods, I-ELISA, dot-blot hybridization and immunocapture reverse transcription PCR (IC-RT-PCR), were developed and used to detect ZaMMV in calla lilies from the market and field. Based on field surveys during 2003–2004, ZaMMV and ZaMV were widespread in calla lilies but DsMV became less important due to planting DsMV-free seedlings.

## Materials and methods

### Virus source and sample collection

The ZUN isolate of ZaMMV, originally from calla lily cv. Black Magic (Huang & Chang, 2005), was successfully

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Accepted 12 July 2006

transferred to *Philodendron selloum* by mechanical inoculation and then maintained on *P. selloum* and calla lily. Similarly, the ZAN isolate of DsMV (Chen *et al.*, 1998) and the ZAN isolate of ZaMV (Chang *et al.*, 2001) derived from calla lilies were also maintained on these two aroid plants. Virus-infected leaf tissues of *P. selloum* and calla lily were used as controls in immunoblot and detection experiments. Seedlings of calla lily purchased from the Taiwan Seed Improvement and Propagation Station (TSIPS) and a local flower market were used to compare the detection methods. For the disease survey, calla lily plants were collected from three different fields in central Taiwan during 2003–2004.

### Construction and expression of the recombinant ZaMMV CP gene

Two pairs of primers, ZUNCPF1/ZUNCPR1 and ZUNCPF2/ZUNCPR1 (Table 1), were used to amplify the CP gene of ZaMMV (GenBank accession number AY626825) from a cDNA clone, pZUN7 (Huang & Chang, 2005). The primers are located at nt -9 to +12 of the CP gene for ZUNCPF1, nt +139 to +159 for ZUNCPF2 and nt +948 to +969 for ZUNCPR1. PCR reactions contained 5 µL of plasmid DNA, 12.5 pmol forward primer, 12.5 pmol reverse primer, 10 nmol dNTPs, 0.5 U Vent<sub>R</sub> DNA polymerase (NEB Inc.) and 1x ThermoPol reaction buffer (NEB Inc.) in a total volume of 50 µL. The PCR reaction was performed using GeneAmp® PCR system 2400 (Applied Biosystems) with an initial denaturation at 94°C for 2 min, 35 cycles of 94°C/30 s, 55°C/30 s, 72°C/1 min, and a final extension at 72°C for 5 min. The PCR products were purified by GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech). After digestion with *NotI*, the fragments were further purified for ligation with the pET-29a(+) expression vector (Novagen) pretreated with *NotI* and *EcoRV*. The clones were first screened with enzyme digestion and then confirmed by sequencing. Selected clones were used to transform into *E. coli* BL21 (DE3) and express the CP of ZaMMV with a His tag fused to the C terminus and 30 amino acids from the vector fused to the N terminus. Five hundred microlitres of bacterial suspension was collected hourly, after 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induction, and centrifuged at 1764 g for 10 min. The supernatant was discarded and the pellets were resuspended in 50 µL of SDS sampling buffer and 15 µL was analyzed by 12% SDS-PAGE (Sambrook & Russell, 2001). The recombinant CPs were purified using Ni-NTA agarose following the protocol of the QIAexpressionist™ (QIAGEN).

### Immunoblot analysis

Crude plant extracts were prepared by grinding in 10 volumes of 0.01 M sodium phosphate buffer (pH 7.2). The supernatants were collected after centrifugation at 10 160 g for 5 min and used for immunoblot assays. The bacterial lysates and total proteins of plant tissue were

Table 1 Oligonucleotide primer sequences used in this study

Primer <sup>a</sup>	Sequence <sup>b</sup>	Reference
PNibF1	5'GGBAAYAATAGTGGNCAACC3'	Hu & Chang, 2004
PCPR1	5'GGGGAGGTGCCGTTCTCDAT-RCACCA3'	Hsu <i>et al.</i> , 2005
ZUNCPF1	5'GTCTTGCAAGCAGACGATCAG3'	This study
ZUNCPF2	5'AATAAACAGTTGATGCTGGT3'	This study
ZUNCPR1	5'GCTAGCGGCCGCTGCAAG-GCTGAGATCCCAGT3'	This study
ZUNF1a	5'CTCACATATGATGAGGATGAGGG3'	This study
ZUNR1a	5'CAGATCTCTTTGGCCTTGGGTT3'	This study
ZUNF3	5'GCCTTGCAGTAAAGGAGCTG3'	This study
ZUNR0	5'GGAACGACAAACATTGCCGTA3'	This study

<sup>a</sup>F and R indicate forward and reverse primers, respectively.

<sup>b</sup>Nucleotide at degenerate positions are represented by a single letter code; R = A and G; Y = C and T; B = C, G and T; D = A, G and T; N = A, C, G and T. The created *NotI* site is underlined.

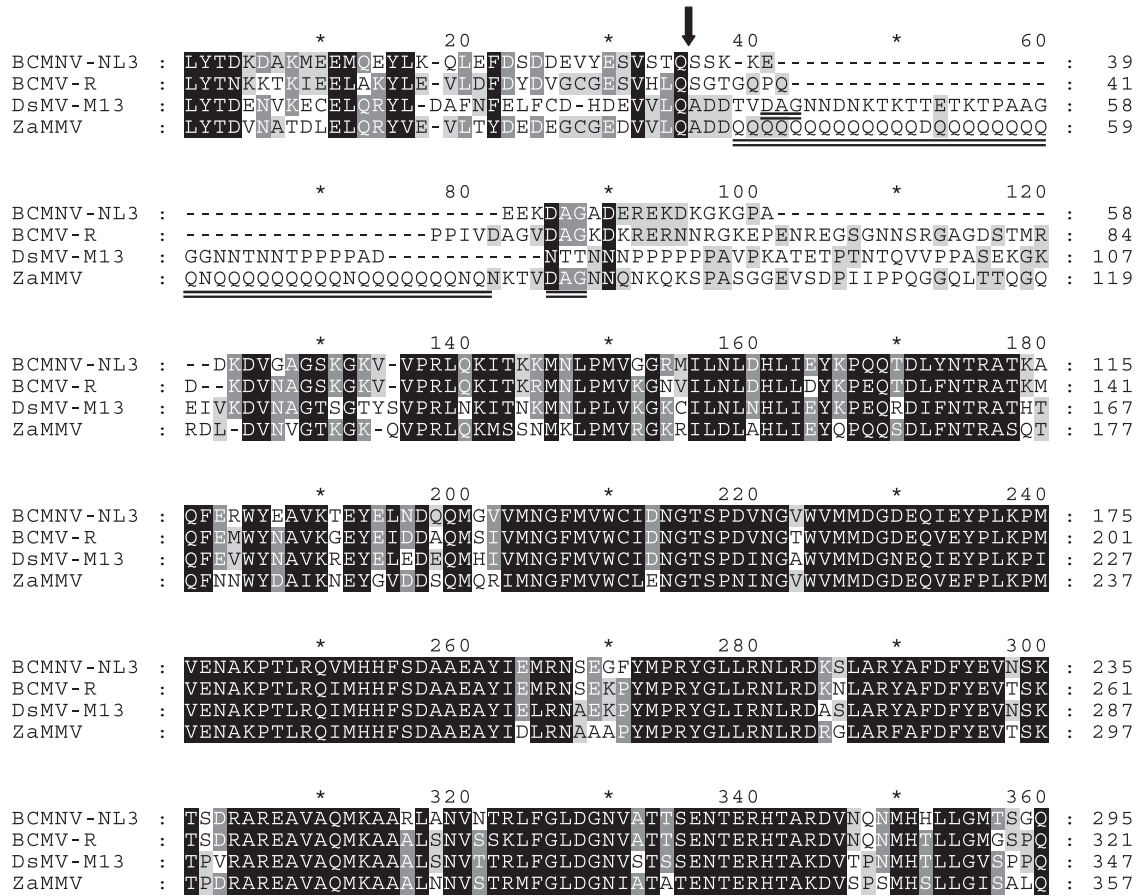
separated by 12% SDS-PAGE, in duplicate. One gel was stained with Coomassie brilliant blue and the other was transferred to PVDF membrane (Osmonics). After blocking with 0.3% non-fat milk solution, the membrane was incubated in 1x gelatin-NET (0.25% gelatin, 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.05% Tween-20, pH 8.0) containing anti-ZaMMV antiserum diluted 1 : 8000 at room temperature for one hour. Finally, the membrane was probed with a goat anti-rabbit secondary antibody conjugated with alkaline phosphatase and then reacted with the colorimetric substrate, NBT/BCIP (Roche Applied Science).

### Indirect ELISA

Antisera of DsMV and ZaMV were previously prepared in the laboratory using recombinant CPs as the antigens (Huang & Chang, 2000). Indirect ELISA (I-ELISA) was performed according to the protocol of Agdia Inc. Each sample had duplicate wells. The values of OD<sub>405</sub> of each sample were measured by the Spectra MAX 340 (Molecular Devices Co.) after 30 min incubation. A sample was considered positive if the absorbance value was greater than twice the mean of the healthy controls.

### Dot-blot hybridization

Specific DNA probes of ZaMMV were labeled with Digoxigenin (DIG) by PCR reaction. A plasmid clone, pZUN7 was used as template, and 1175-, 311- and 251-bp cDNA fragments were separately amplified by PNibF1/PCPR1, ZUNF1a/ZUNR1a and ZUNF3/ZUNR0 primer pairs (Table 1). The PCR reaction solution was set up as before except dNTPs were replaced by a 1x PCR DIG labeling mix (Roche Applied Science). The PCR program included a denaturing step at 94°C for 2 min, 35 cycles of 94°C/30 s, 53°C/30 s, 72°C/45 s, and a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel



**Figure 1** Alignment of the partial amino acid sequences of Zantedeschia mild mosaic virus (ZaMMV) and three closely related potyviruses as previously described (Huang & Chang, 2005). BCMNV-NL3: NL3 strain of *Bean common mosaic necrosis virus* (U19287); BCMV-R: R isolate of *Bean common mosaic virus* (AJ312437); DsMV-M13: M13 isolate of *Dasheen mosaic virus* (AJ298033) and ZaMMV (AY626825). The predicted cleavage site between N1b and CP is indicated by the arrow. The polyglutamine sequence and the DAG motif in the CP of ZaMMV are underlined.

and quantified by Kodak Digital Science™ 1D image analysis software (Eastman Kodak Company). To compare the specificity and sensitivity of three ZaMMV probes, ZaMMV, DsMV and ZaMV plasmid clones previously obtained in the laboratory were used to prepare 10-fold serially diluted samples. These samples were immobilized onto nylon membrane and then separately hybridized with the same quantity of each probe. For dot-blot hybridization analysis, 5  $\mu$ L of crude plant sap prepared with indirect sample extraction buffer (Agdia Inc.) was transferred onto the nylon membrane (Millipore) presoaked in 20x SSC. After UV cross-linking, the membranes were prehybridized, hybridized, washed and detected for hybridization signals as previously described (Hsu *et al.*, 2005).

#### Immunocapture-RT-PCR (IC-RT-PCR) with specific primers

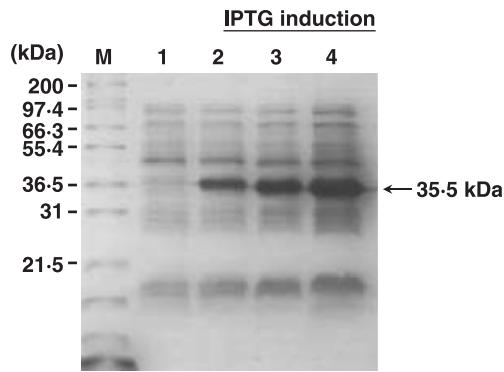
Plant extracts were prepared by grinding fresh tissue in 10 volumes of general extract buffer (Agdia Inc.). After centrifugation at 11 925  $g$  for 10 min, the supernatant was kept on ice until use. Reaction tubes were coated with

anti-ZaMMV antiserum, incubated at 37°C for 1 hour, and then washed three times with PBST buffer. The coated reaction tubes were incubated with 100  $\mu$ L of plant extracts at 37°C for 1 hour, and then washed three times with PBST buffer. After drying, the coated reaction tube was vortexed with 5  $\mu$ L of 0.5% Triton X-100 that was preheated to 65°C in order to release the viral RNA from the trapped virus particles (Wetzel *et al.*, 1992). The RT reaction was carried out with the ZUNR1a primer at 42°C for 40 min in a total volume of 12.5  $\mu$ L. The RT product was used as a template in the subsequent PCR reaction with ZUNF1a/ZUNR1a primers and the PCR condition was the same as before. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel.

## Results

### Construction and expression of the recombinant ZaMMV CP gene

Initially, a 980-bp fragment encompassing the intact CP gene with polyglutamine sequence (Fig. 1) was amplified

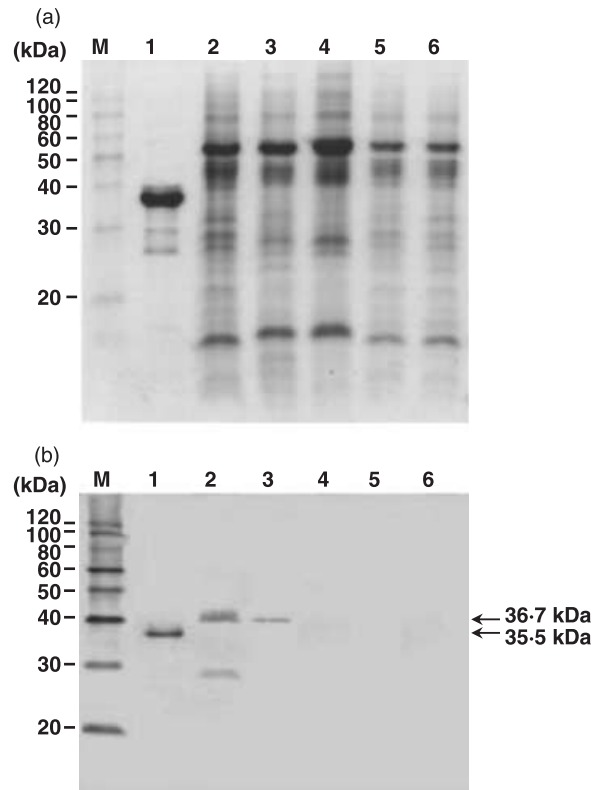


**Figure 2** Bacterial lysates of *Escherichia coli* BL21 (DE3) transformed with pZUNCPF2-7-1 were analyzed by electrophoresis in a 12% SDS-polyacrylamide gel. Lane M, Mark12™ MW Standard (Invitrogen); lane 1, no IPTG induction; lane 2, one hour after IPTG induction; lane 3, three hours after IPTG induction; lane 4, four hours after IPTG induction. Molecular weights of the markers are indicated on the left. The position of the expressed protein is indicated by an arrow and the number represents the deduced molecular weight.

by primers ZUNCPF1/ZUNCP1 and then cloned into a pET-29a(+) expression vector. Six independent clones were identified by enzyme digestion, and one clone designated pZUNCP7-4 was sequenced from both directions. After transformation and IPTG induction, the predicted 41.2-kDa recombinant protein was not expressed in *E. coli* BL21 (DE3) by pZUNCP7-4 nor in the remaining five clones (data not shown). Another forward primer, ZUNCPF2, was therefore used together with ZUNCP1 to produce a PCR fragment of 830 bp that excluded the polyglutamine sequence from the N terminus of the CP gene of ZaMMV. Two correct clones were obtained and confirmed by enzyme digestion. One sequenced clone, pZUNCPF2-7-1, was used to transform *E. coli* BL21 (DE3). The predicted 35.5-kDa recombinant protein was successfully expressed in *E. coli* after induction for 1 h by IPTG and gradually increased for another 3 h (Fig. 2). The large quantities of expressed protein were purified as antigens and the antiserum of ZaMMV was prepared by a local private company.

#### Specificity of ZaMMV antiserum analyzed by immunoblot and ELISA assays

To test the specificity of the ZaMMV antiserum, the expressed recombinant CP of ZaMMV and total proteins of healthy and diseased plants were assayed by immunoblot analysis. The DsMV- and ZaMV-infected plants were chosen for analysis because these potyviruses are frequently found to infect calla lilies in Taiwan (Chen *et al.*, 1998; Chang *et al.*, 2001). The results showed that ZaMMV antiserum only reacted with the CP of ZaMMV in the crude extracts from ZaMMV-infected calla lily and *P. selloum* plants and did not react with any proteins from other diseased or healthy plants (Fig. 3). The predicted molecular weight of recombinant ZaMMV CP (35.5 kDa), although containing 30 amino acids of expression



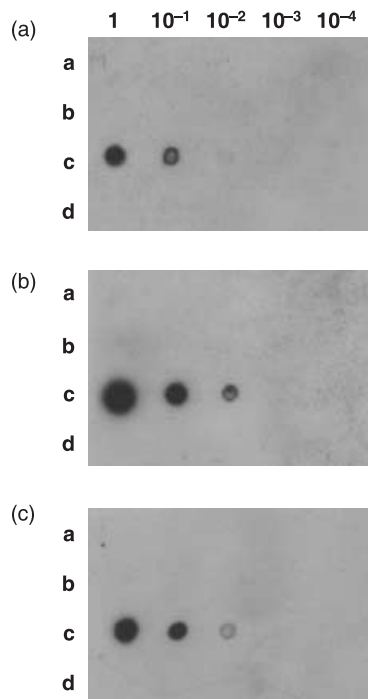
**Figure 3** Specificity of Zantedeschia mild mosaic virus (ZaMMV) antiserum by immunoblot analysis. Expressed protein and crude extracts of diseased and healthy plants were separated by 12% SDS-PAGE and either stained with Coomassie brilliant blue (a) or transferred to a PVDF membrane and reacted with anti-ZaMMV antiserum (b). Lane M, MagicMark™ Western Standard (Invitrogen); lane 1, purified recombinant protein of ZaMMV CP; lanes 2–3, crude extracts of ZaMMV-infected calla lily and *Philodendron selloum*, respectively; lane 4, crude extract of DsMV-infected calla lily; lane 5, crude extract of ZaMV-infected calla lily; lane 6, healthy calla lily. Molecular weights of the markers are indicated on the left. The positions of native and recombinant proteins of ZaMMV CP are indicated by arrows, and the numbers represent the deduced molecular weights.

vector and a His tag, was still smaller than that of native CP (36.7 kDa) due to its lack of the polyglutamine region (equivalent to 46 amino acids) (Fig. 3). A smaller protein ~27 kDa in size, possibly a degraded product of the CP, also reacted to ZaMMV antiserum. A recent immunoblot assay indicated that CPs of TuMV and BYMV were not recognized by ZaMMV antiserum (data not shown). According to the results of I-ELISA, ZaMMV antiserum did not react to DsMV, ZaMV, TuMV or BYMV but did react to ZaMMV-infected samples (data not shown). The ZaMMV antiserum is apparently highly specific and can be used for detection and diagnosis.

#### Comparison of three specific DNA probes of ZaMMV

The probe 1175 was prepared by PCR with PNIbF1/PCPR1 primers, and spans the entire 3' region of the NIb gene and the 5' region of the CP gene. The probe 311





**Figure 4** Dot-blot hybridization assay for comparison of the specificity and sensitivity of three *Zantedeschia* mild mosaic virus (ZaMMV) probes. The membranes were hybridized with three ZaMMV DNA probes, probe 1175 (a), probe 311 (b), and probe 251 (c), respectively. Plasmid DNAs of DsMV (a), ZaMV (b) and ZaMMV (c) clones were 10-fold serially diluted and dotted on the membranes together with H<sub>2</sub>O (d) as negative control. The original concentration of plasmid DNAs was 45 ng.

covering the junction region of the Nib and CP genes, the sequence of which is variable among potyviruses, was amplified with ZUNF1a/ZUNR1a primers. The probe 251 corresponding to the 3'UTR of ZaMMV was PCR-amplified with ZUNF3/ZUNR0 primers. The cDNA clones of ZaMMV, DsMV and ZaMV were used to compare the specificity and sensitivity of the probes. The results indicated that all probes reacted with the ZaMMV cDNA clone only (Fig. 4). Probe 311 and probe 251 could detect at least 0.45 ng of ZaMMV plasmid DNA, whereas probe 1175 had a detection limit ten times lower (Fig. 4). Because probe 311 showed better sensitivity than probe 251 due to its stronger signals (Fig. 4), it was used to perform dot-blot hybridization to detect ZaMMV in field samples. A recent result confirmed the high specificity of probe 311, which did not hybridize with TuMV or BYMV cDNA clones (data not shown).

#### Detection of ZaMMV in calla lilies by I-ELISA, dot-blot hybridization and IC-RT-PCR

To see whether ZaMMV already exists in some areas of Taiwan, calla lilies were collected from the field in central Taiwan, purchased from the flower market and the TSIPS, and were tested with ZaMMV antiserum by I-ELISA. The numbers of infected calla lilies were 10/13 from the field, 4/24

from the market, and 2/19 from TSIPS. This result indicates that 10.5–77% of samples tested were infected by ZaMMV.

Calla lilies from the same three sources were randomly selected to test ZaMMV by I-ELISA, dot-blot hybridization and IC-RT-PCR at the same time. All three samples from the field (Fig. 5a, a1-a3), two samples from the market (Fig. 5a, b1 and b3) and one sample from TSIPS (Fig. 5a, c2) reacted with antiserum to ZaMMV. Crude saps of the same samples were checked by dot-blot hybridization with probe 311 and also by IC-RT-PCR with ZaMMV-specific primers, ZUNF1a and ZUNR1a. The result of IC-RT-PCR was similar to that of dot-blot hybridization (Fig. 5b and c), and also agreed with the result of I-ELISA. Some samples (Fig. 5, a2 and b3) having weak signals in dot-blot hybridization and low ELISA values produced the same amount of ZaMMV cDNA fragment in the IC-RT-PCR assay.

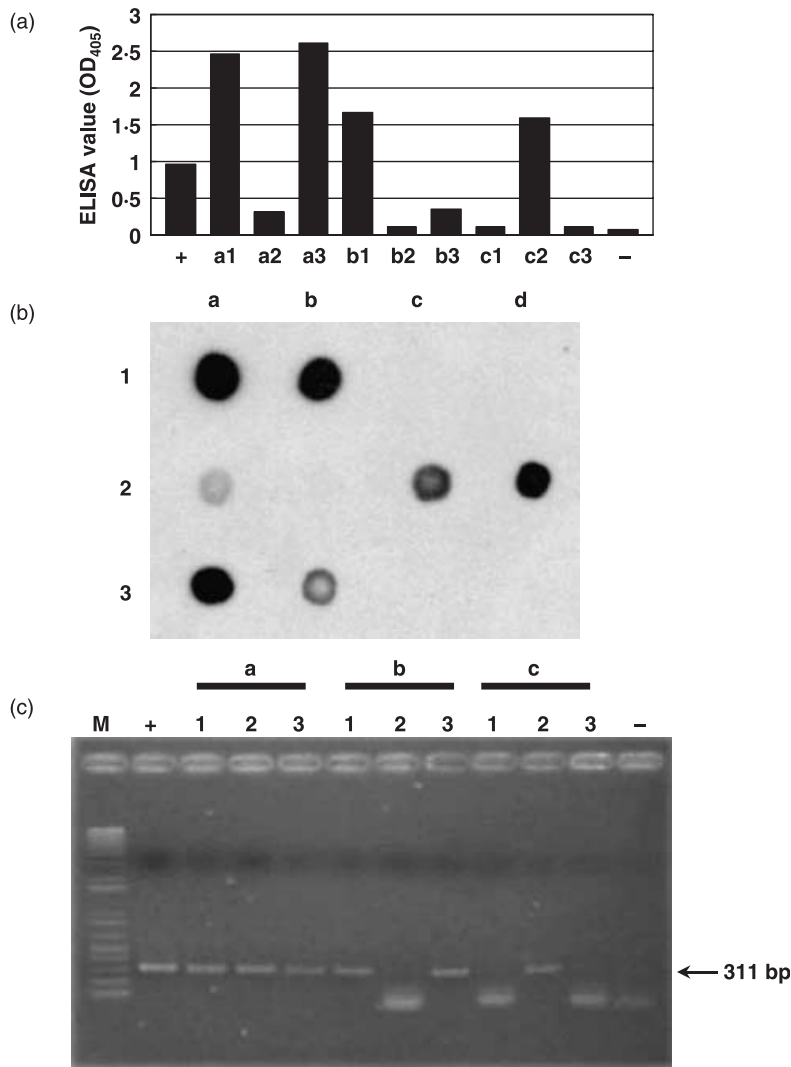
#### Occurrence of ZaMMV, DsMV and ZaMV in calla lilies

To evaluate the disease incidence caused by ZaMMV, DsMV and ZaMV in calla lilies in Taiwan, two field surveys were done during 2003 and one during 2004. A total of 101 samples collected from three fields on different dates were tested by I-ELISA using ZaMMV, DsMV and ZaMV antisera simultaneously. The results indicated that 55% and 38.5% of calla lilies in 2003 and 42.9% in 2004 detected positive for ZaMMV (Table 2). Among samples tested, 72.5% and 65.4% in 2003 and 60% in 2004 were infected by ZaMV (Table 2). Although 27.5% and 30.8% of calla lilies were infected by DsMV in the 2003 survey, none of the samples tested positive in 2004 (Table 2). According to the ELISA data, only 20–34.6% of sampled calla lily plants did not react to any of the antisera. In addition, 38–63% of infected plants contained two or even three kinds of viruses (Table 2).

#### Discussion

The purification of virus particles from aroid plants is difficult and resulted in the failure to produce good quality antiserum (Li *et al.*, 1998). A similar situation arose when purifying virus particles from calla lilies and *P. selloum*, probably due to the mucilage in the plant tissues. Expression of the recombinant CP in *E. coli* solved this problem and the purified protein was a good antigen to produce antiserum (Li *et al.*, 1998). A similar approach was therefore employed to produce ZaMMV antiserum, which had very good specificity. Bacteria-expressed viral CP can therefore be used as an alternative method to produce antiserum for viruses that are difficult to purify.

The dominant epitopes of potyvirus virions are the variable terminal parts of the CP, especially the N-terminal part. It has been reported that about 30 amino acids of the N terminus of the CP were exposed on the surface of the virus particle (Shukla & Ward, 1989). Therefore, the unique repetitive sequence of ZaMMV CP is composed of 39 glutamine residues which are likely to be exposed on the surface of the virion, if not degraded or processed



**Figure 5** Detection of *Zantedeschia* mild mosaic virus (ZaMMV) in calla lilies collected from three sources. (a) Indirect-ELISA assay. Samples a1-a3, calla lilies from the field; samples b1-b3 calla lilies from the market; samples c1-c3, calla lilies from the Taiwan Seed Improvement and Propagation Station (TSIPS). Sample +, ZaMMV-infected calla lily; sample -, healthy calla lily. The absorbance at 405 nm was measured 30 min after addition of substrate. (b) Dot-blot hybridization assay of the same set of samples as (a). Crude extracts of calla lilies were dotted onto the membrane and hybridized with probe 311. Dots d1 and d2 represent total RNA of healthy and ZaMMV-infected calla lilies, respectively. (c) IC-RT-PCR assay of the same set of samples as (a). The DNA products amplified by ZaMMV specific primers, ZUNF1a/ZUNR1a, were analyzed in a 1.5% agarose gel. Lane M, 1 kb Plus DNA Ladder (Invitrogen); lane +, positive control; lane -, negative control. The position of expected amplified product is indicated by an arrow.

**Table 2** Number of samples (% in parenthesis) of calla lilies collected from different fields testing positive by I-ELISA using DsMV, ZaMV and ZaMMV antisera simultaneously

ELISA result <sup>a</sup>	Field A <sup>b</sup>	Field B <sup>b</sup>	Field C <sup>b</sup>
	18 April 2003	30 May 2003	26 December 2004
ZaMMV	3 (7.5)	0	5 (14.3)
DsMV	0	0	0
ZaMV	9 (22.5)	7 (26.9)	11 (31.4)
ZaMMV + DsMV	0	0	0
ZaMMV + ZaMV	9 (22.5)	2 (7.7)	10 (28.6)
DsMV + ZaMV	1 (2.5)	0	0
ZaMMV + DsMV + ZaMV	10 (25)	8 (30.8)	0
ND	8 (20)	9 (34.6)	9 (25.7)
Total	40 (100)	26 (100)	35 (100)

<sup>a</sup>OD<sub>405</sub> value greater than twice the mean of the healthy controls was considered a positive sample. ND: sample did not react to three kinds of antisera.

<sup>b</sup>Field A located in Hsinshu, Taichung County; field B located in Peitou, Changhua County; field C located in Tanzih, Taichung County.

(Huang & Chang, 2005). It has been shown that incorporation of polyglutamine results in protein oligomerization, probably because the glutamine repeats form polar zippers for protein-protein interaction (Stott *et al.*, 1995). In the current study, pZUNCP7-4 and five similar recombinant clones that failed to express recombinant protein in *E. coli* have polyglutamine at the N-terminus of ZaMMV CP. If the recombinant protein expressed from them aggregated and then interfered with the transcription of *E. coli*, it may explain why this recombinant protein cannot be overexpressed in *E. coli*.

In addition to DsMV (Chen *et al.*, 1998), ZaMV (Chang *et al.*, 2001) and TuMV (Chen *et al.*, 2003), ZaMMV is another potyvirus found to infect calla lily in Taiwan (Huang & Chang, 2005). The original source of ZaMMV might be the imported tubers of calla lily from other countries. Some imported tubers collected directly from the international airport by HsinChu Branch Office of The Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ) were shown to be infected with ZaMMV by this laboratory (data not shown). According to TSIPS,

the imported calla lilies were only screened with anti-DsMV and CMV antibodies, and then used for micropropagation. This may explain why the tissue-cultured seedlings purchased from TSIPS were still infected by ZaMMV.

In order to survey the prevalence of ZaMMV in Taiwan, three detection methods were developed and were able to detect the virus successfully. I-ELISA is the most convenient method. Dot-blot hybridization is also suitable for handling large amounts of samples. Although IC-RT-PCR is not appropriate for large scale screening, it showed better sensitivity than the other two methods as previously described (Wetzel *et al.*, 1992), and may be valuable for mother stock indexing of calla lily. So far, ZaMMV seems to infect only members of the Araceae family (Huang & Chang, 2005) but other non-aroid hosts cannot be excluded. Although ZaMMV alone caused mild mosaic on calla lilies, it was frequently found together with ZaMV and/or DsMV in the field based on this study (Table 2). It was not determined whether the infection of these three viruses was synergistic.

Although importation of ZaMMV into Taiwan may have been accidental, ZaMMV appears to be prevalent in the field. Zettler & Hartman (1986; 1987) have successfully removed DsMV by tissue culture, but tissue culture alone cannot guarantee the seedlings will be free of virus unless sensitive detection methods are available. This explains why the tissue-cultured calla lilies from TSIPS grown in the field had a lower percentage (27.5% and 30.8%) of DsMV infection in 2003 and no infection in 2004, since the DsMV antibody has been routinely used in micropropagation of calla lily by TSIPS. Because ZaMV and ZaMMV are two new viruses found in calla lily, the detection methods for these two viruses are not yet included in the TSIPS system. Therefore, the frequencies of ZaMV and ZaMMV detected by ELISA were higher than DsMV (Table 2). DsMV and ZaMV were shown to have a significant influence on the production of calla lilies. Although the significance of the newly found ZaMMV has not been determined, it was commonly observed to be the causal agent of complex infection in calla lilies. The importance of ZaMMV in the calla lily industry should not be underestimated and because calla lily has become a popular cut flower in Taiwan, a virus-free certification scheme, using the described detection methods, needs to be established.

## Acknowledgements

We thank Dr Ching-Chung Chen for helping to collect calla lily samples from Peitou, Changhua County and Dr Yung-An Lee for critically reviewing the manuscript. This study was partially supported by a grant from the Council of Agriculture (92AS-1-1-5-SS-X3(2-1), Executive Yuan, Taiwan, R. O. C.

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