Studies on Viruses Infecting the Family of *Amaryllidaceae* and Development of Their Detection Techniques

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ABSTRACT

The family of *Amaryllidaceae* contains abundant plant species adaptive to diversified environment and numerous of them are artificially grown as ornamental plants. Taiwan is the native country of many amaryllis plant species such as the well known golden spider lily (*Lycoris aurea* Herb.). Many species in amaryllis family are considered to have great potential transforming into novel ornamental crops in the future. To pursue this goal, we believe detailed understanding of virus diseases that may threaten the growth and production of amaryllis plants is crucial. Most amaryllis plants and many other ornamentals are propagated solely by vegetative means so that their seedlings will be jeopardized once mother stock planting materials are infected by viruses. During last four years, virologists in Taiwan Agricultural Research Institute have been in close cooperation with scientists from USDA/ARS, Beltsville Agricultual Research Center, USA to survey the viruses infecting various species of the family *Amaryllidaceae*. At present, we have succeeded in identification and characterization of six viruses infecting amaryllis plant species including *Lycoris*, *Narcissus*, *Hippeastrum* and *Agapanthus*. At least four of these six viruses are new records in the literature and named by this research team. Specific antibodies and nucleic acid primers and probes against these viruses were successfully produced and their standard detection procedures were established. These detection kits and protocols are essential and vital to the development of virus-free amaryllis plant propagation system. It is hoped that with this accomplishment the future development of amaryllis ornamental industry in Taiwan and USA will both be benefited.

Key words: *Amaryllidaceae,* Viruses, Detection, Characterization, *Lycoris, Narcissus, Hippeastrum, Agapanthus.*

INTRODUCTION

There are 90 genus and probably over 1200 plant species recorded in the family of Amaryllidaceae [(8)]. Among them several Amaryllis genus such as *Narcissus*, *Hippeastrum*, and *Nerine* are well known world widely for their ornamental importance [(8)]. In recent years, another genus of this family, *Lycoris*, has absorbed attention from botanists, breeders and horticulturists around the world for its

diversities in the color and morphology of flower parts and especially its broad adaptation abilities to different environmental conditions [8]. It is proposed that *Lycoris* shall be one of the most promising new ornamental crops in the coming twenty-first century. Since Taiwan is the origins of several *Lycoris* ornamental plant species, we certainly are advantaged in the development of this ornamental crop. However, in the history of ornamental plant industry development, the grasp of disease control techniques especially those for viral agents are known to be one of the keys to success. The experience of Dutch ornamental plant industry and their leading technology in virus indexing program certainly have explained very well this situation. Therefore, to ensure the strong competition in the future's *lycoris* industry, we proposed that the research program on the virus diseases of *lycoris* or even in the whole family of Amaryllidaceae should be established as soon as possible. Since 2001, we started a three-year collaborative project on "Studies on viruses infecting the family of Amaryllidaceae and development of their detection techniques" with the Floral and Nursery Plants Research Unit (FNPRU, formerly Floral and Nursery Crops Laboratory) of the U.S. National Arboretum of the United States Department of Agriculture (USDA). Presently, we have succeeded in identification and characterization of six viruses infecting amaryllis plant species including *Lycoris*, *Narcissus*, *Hippeastrum* and *Agapanthus* (Table 1)[1, 2, 3, 4, 5]. Four of these six viruses are new records in the literature and named by this research team. Specific antibodies and nucleic acid primers and probes against these viruses were successfully produced and their standard detection procedures were established. We are confident that with this accomplishment the future development of amaryllis ornamental industry in Taiwan and USA will both be benefited.

ACCOMPLISHMENT

1. Lycoris viruses

Golden spider lily (*Lycoris aurea* Herb.) is an indigenous species and currently a popular cut flower ornamental bulb crop in Taiwan [8]. There have been twenty-four different viruses documented in the literature infecting plants of *Amaryllidaceae* [2]. Most of these 24 viruses are from the reports of three currently most well-known genera in the *Amaryllidaceae*, i.e. *Narcissus*, *Hippeastrum*, and *Nerine*. For *Lycoris*,

Table 1. Viruses found in Taiwan that infect species of *Amaryllidaceae*.

Virus	Genus	Host
Lycoris mild mottle virus (LyMMV)	*Potyvirus*	Golden spider lily (*Lycoris*)
Lycoris potyvirus (LPV)	*Potyvirus*	Lycoris, Narcissus
Lycoris virus T (LPV)	*Carlavirus*	Lycoris, Narcissus, Hippeastrum
Hippeastrum mosaic virus, isolate HIP4	*Potyvirus*	Hippeastrum
Narcissus virus isolate WN9 could be a strain of *Narcissus yellow stripe virus*	*Potyvirus*	Narcissus
African lily mosaic virus	*Potexvirus*	African lily (*Agapanthus*)

however, there hasn't any viruses been reported until recently our laboratory documented that at least two new potyviruses and a unreported carlavirus were found associating with severe mosaic disease of golden spider lilies (*Lycoris aurea*) [1, 2, 5]. The 3'-terminal region of the genome of these two potyviruses were separately amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using a potyvirus-specific degenerate primer set. The amplicons were cloned and subsequently sequenced. They were found corresponding to the genome organization of potyviruses with a coat protein (CP) gene followed by a 3'-noncoding region. Comparing these sequences with each other and with those reported in the GenBank, these two lycoris potyviruses were revealed to be different from each other and also unique to other known *Potyvirus* species [2]. One of the lycoris potyvirus was named as *Lycoris mild mottle virus* (LyMMV) because it was frequently found singly infected some golden spider lily plants developing mild mottling symptom on their leaves [2]. The second potyvirus was provisionally designated as *Lycoris potyvirus* (LPV) for its specific symptom on golden spider lily was not yet conclusive [2]. PCR primers for specific amplification of LyMMV and LPV were designed based on their 3-terminal genome sequences and the primers were successfully used for the detection and identification of these two potyviruses in golden spider lilies. Specific antibodies against LyMMV and LPV were separately prepared in rabbits by immunizing viral coat protein expressed in *E. coli* using expression vector plasmid pET28b(+) (Novogen, Inc., Madison, WI, USA). The antibodies were shown feasible to differentiate these two lycoris potyviruses from each other and also from other known potyviruses. They could also be applied in ELISA, SDS-immunodiffusion and Western blotting tests for the virus indexing in plants.

In subsequent studies, another virus isolate was obtained from lycoris plants exhibiting strong mosaic symptoms. Different from LyMMV and LPV, the isolate was able to induce local lesions on *Chenopodium quinoa*, which was therefore routinely used to maintain pure culture of this new virus isolate. After characterization studies, this new isolate was found to be a carlavirus [5]. Its virus particles are flexuous rod shaped, about 617 nm in length, typical of carlaviruses. A sequence of 8047 nucleotide representing almost entire genome, except probably less than a hundred nucleotides from the 5'-terminal non-coding region, of this isolate was sequenced. The genome organization was found corresponding to carlaviruses with a typical triple gene block in the 3' part. A coat protein gene containing 894 nucleotides which encodes 298 amino acid residues was revealed. Comparing with other known carlaviruses in the GenBank, the percent identities of the nucleotide and amino acid sequence of CP gene were less than 60%, showing that the isolate obtaining from lycoris should be recognized as a new carlavirus species [5]. We proposed that the virus is named as *Lycoris virus* T (LVT). A polyclonal antiserum against LVT was successfully prepared in rabbits by immunizing virus particles purified from infected tissues of *C. quinoa*. The antiserum was found to be useful to detect LVT infection in ELISA and many other serological tests. Specific PCR primers for the detection and identification of LVT was

also designed and routinely applied in regular virus surveys task.

2. *Narcissus viruses*

Narcissus is an important and popular ornamental bulbous crop worldwide. Two species of narcissus, namely the Chinese narcissus (*Narcissus tazetta* L. var. orinentalis Hort.) and the western narcissus (*N. poeticus*) are the major commercial ones in Taiwan. Virus symptoms of mosaic, mottling and yellow striping on the leaves are commonly seen on narcissus plants for sale in the markets. Using potyvirus-specific degenerate primers, two molecularly different potyvirus isolates, namely CN4-2 and WN9, were frequently found associating with Chinese narcissus plants bearing viral symptoms [9]. However, in western narcissus only WN9-related virus isolates were detected [9]. Based on our observation, WN9-infected narcissus plants always exhibited yellow striping and mild mosaic symptoms on their leaves, which was similar to those described for *Narcissus yellow stripe virus* (NYSV) in the literature. However, due to the lack of documentation of NYSV's genome sequence in GenBank, we are not sure presently whether our WN9 isolates are strains of NYSV or a unique potyvirus species since its CP gene sequence shares less than 80% identities with other known potyviruses.

CN4-2 from Chinese narcissus was later determined to be molecularly similar to LPV found in golden spider lily [9]. Comparative studies showed that they shared more than 95% of identities in nucleotide and amino acid sequences of CP gene, indicating they are strains of the same virus species. An antiserum prepared against *E. coli* expressed coat protein of CN4-2 was found reacting strongly in ELISA with isolates of LPV from lycoris [3]. Using this antiserum, LPV isolates from lycoris and CN4-2 from narcissus were found serologically indistinguishable in SDS- immunodiffusion test [3]. We have also prepared antiserum against bacteria expressed CP of WN9 and shown that it could be applied in ELISA and many other serological tests to detect and identify virus infection in narcissus and other ornamental plants [3].

African lily

African lily (*Agapanthus africanus* L.), a species of *Amaryllidaceae* and native from South Africa, has become a popular ornamental crop in Taiwan and many other countries. It can be used as bedding plants or intensively grown for cut flower production. In 2002, we observed a diseased African lily plant with mosaic symptom on its leaves in Nantou County [4]. The plant was brought back to the lab and inoculated on various indicator plants. Local lesions were found on *Chenopodium quinoa* 7-10 days after mechanical inoculation. Single lesions were taken and re-inoculated on the same plant and this procedure was repeated for three times to obtain pure culture of this virus isolate which was named as AL-1 [4]. Serologically, none of the antisera against more than 30 different known viruses in our collection reacted with AL-1 [4]. In electron microscopy, flexuous rod shaped virus particles with 508 nm in length were consistently observed in negatively stained leaf dips and samples purified from *C. quinoa* [4]. Analyses of purified virus sample in SDS-PAGE showed that the virus contained a single species of protein subunit with a molecular mass about 23 kDa [4]. An antiserum prepared against purified virion of AL-1 reacted strongly with its homologous antigen from *C. quinoa* and also

with diseased African lily plants, but not with non-symptomatic African lilies and non-inoculated *C. quinoa* in SDS-immunodiffusion and ELISA tests. Interestingly, a potexvirus-specific degenerate primer set was shown to react with AL-1 and to amplify a 280 bp product equivalent to the size of amplicons amplifying from potexviruses [4]. Subsequent molecular studies have cloned and sequenced a 2.6 kb fragment corresponding to the 3'-terminal region of AL-1's genome, which was also found coinciding with that of potexviruses [4]. Triple gene blocks containing a CP gene were detected in this 2.6 kb sequence. CP gene of AL-1 was found to contain 660 nucleotides with 220 deduced amino acid residues. Comparing with 18 CP gene sequences of known potexviruses in GenBank, AL-1 shared only 54% and 38% identities in nucleotide and amino acid sequences, respectively [4]. This result indicates AL-1 isolated from African lilies is a newly recognized species in the Genus of *Potexvirus* and it was named as *African lily mosaic virus* [4].

Amaryllis

Amaryllis is commonly grown in Taiwan as potting or bedding ornamentals. We obtained a virus isolate (HIP) from a diseased amaryllis plant showing systemic mosaic symptoms, which is commonly seen in amaryllis nurseries [6,7]. A 2-kb DNA product was amplified by RT-PCR from HIP by the use of potyvirus-specific degenerate primers [6,7]. The PCR product was subsequently cloned and its sequence analyzed. It was found comprising of 1851 nucleotides (nts) corresponding to the 3'-terminal region of potyviral genome. The deduced amino acid sequence contained 576 residues encoding part of the 3'-terminal region of NIb gene (264 residues) and the complete sequence of coat protein (CP) gene (312 residues) [6, 7]. A 122 nts of non-coding region (NCR) was found locating at the 3'-terminal region of the DNA. Comparing HIP CP gene with those of known potyviruses in GenBank, the percent identities of nucleotide and amino acid sequence were less than 53% and 45%, respectively [6, 7]. Since genome sequence of *Hippeastrum mosaic virus* (HiMV), the only potyvirus reported to infect amaryllis, has not been reported yet, the identities of HIP and its relationship with HiMV remain to be further explored. Based on HIP sequence, we developed a specific primer set for RT-PCR detection of HIP and confirmed that HIP is consistently associated with amaryllis plants showing mosaic symptoms [6,7]. Using directional cloning techniques, a 37-kDa fusion protein containing complete CP sequence of HIP and partial sequence encoded by the expression vector plasmid (pET-28b, Novagene), was highly expressed and purified from *E. coli* cell cultures [6, 7]. Antiserum prepared against this fusion protein was applicable in ELISA and SDS-immunodiffussion test for the detection of HIP in amaryllis plants. It did not react with antigens of healthy amaryllis plant and at least 20 different potyviruses in our collections [6, 7].

In a recent survey, some amaryllis plants were found positively reacted in ELISA with antiserum against LVT, a carlavirus found in Taiwan infecting golden spider lilies. Diseased plants were tested in RT-PCR by the use of LVT-specific primers and a DNA product equivalent to the expected size was consistently amplified. The DNA product was cloned and

sequenced. The sequence was found almost identical to that of LVT, confirming that a strain or even the same virus was capable of infecting amaryllis plants.

CONCLUSION

It is generally accepted that successful control of plant virus diseases relies on early and accurate identification of the causal viruses. Knowing the identity of causal virus leads to correct understanding of the mode of transmission and type of vector possibly involved in the disease epidemic. Appropriate control strategy can be thus proposed and implemented in time to suppress disease epidemic. For vegetatively propagated crops such as most ornamentals, accurate identifying mother stock growing materials to be free from virus infection is a crucial step before mass production. Therefore, development of accurate and sensitive virus detection protocol is inevitable for almost any ornament crops attempting to set up industrialized production system. Since 2000, we have been in close cooperation with Dr. Hei-Ti Hsu and his associates from USDA/ARS, Beltsville Agricultual Research Center, USA to survey the viruses infecting golden spider lily, narcissus, African lily and amaryllis in the family *Amaryllidaceae*. At present, we have successfully identified and characterized six viruses including four potyviruses, one carlavirus and one potexvirus. At least four of the viruses are identified to be new species and named by this research team. Specific antibodies and nucleic acid primers and probes against these viruses were successfully produced and their standard detection procedures were established. These detection kits and protocols are essential and vital to the establishment of virus-free planting material propagation system. This fruitful results accomplished by the joint efforts from virologists of TARI and USDA-ARS will certainly benefit the future development of amaryllis ornamental plant industry not only in Taiwan and USA but also other countries around the world.

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