Proceedings of ANAS (Biological Sciences), 65(5-6): 152-157 (2010)

Single-Stranded DNA Plant Viruses in Azerbaijan, the State of the Art

Ioana Grigoras[1], Heinrich-Josef Vetten[2], Nargiz F. Sultanova[3], Stephan Winter[4], Irada M. Huseynova[3], Alamdar Ch. Mamedov[3], Ahmed Kheyr-Pour[5], Bruno Gronenborn[1]*

[1]*Institut des Sciences du Végetal, CNRS, 91198 Gif sur Yvette, France*

[2]*Julius Kühn Institute, Bundesforschungsinstitut für Kulturpflanzen, Institut für Epidemiologie und Pathogendiagnostik, D-38104 Braunschweig, Germany*

[3]*Institute of Botany, Azerbaijan National Academy of Sciences, 40 Badamdar Shosse, Baku AZ 1073, Azerbaijan*

[4]*DSMZ Plant Virus Department, Inhoffenstrasse 7B, 38124, Braunschweig, Germany*

[5]*31 Allée des Joncherettes, 91190 Gif sur Yvette, France*

We describe an initial assessment of plant viruses with a single-stranded DNA genome in Azerbaijan. In the years 2009 and 2010, a limited survey of cultivated and wild legumes as well as of tomato has uncovered the presence of three different nanoviruses, two distinct faba bean necrotic yellows viruses and one faba bean necrotic stunt virus. In addition, tomato plants showing symptoms typical of tomato yellow leaf curl virus, a geminivirus, were encountered. The results presented provide proof for the efficiency of combining serological diagnosis with the power of DNA rolling circle amplification (RCA) to rapidly obtain genetic information on viral plant pathogens with a circular DNA genome.

Keywords: *single-stranded DNA virus, geminivirus, nanovirus, rolling circle amplification, legumes*

INTRODUCTION

The remarkable floral biodiversity of Azerbaijan has triggered some recent interest in the biodiversity of endemic plant viruses as well, in particular plant viruses with a single-stranded (ss) DNA genome. Plant viruses with an ssDNA genome have been assigned to two families, *Geminiviridae* and *Nanoviridae* (Stanley et al., 2005; Vetten et al., 2005).

Geminiviruses are distributed worldwide and some of them cause very severe and economically important diseases. Examples include viruses responsible for maize streak disease (the maize streak virus group), cassava mosaic virus disease (the African and Indian cassava mosaic viruses), and viruses causing tomato (yellow) leaf curl disease, the tomato-infecting geminiviruses. Whereas the former two groups are restricted to Africa and the Indian Subcontinent, members of the latter are found worldwide in tropical, subtropical, semi-arid and Mediterranean climate zones. For a comprehensive recent assessment of the disease caused by tomato yellow leaf curl virus, the molecular biology of the virus and its worldwide agronomical impact on tomato cultivation see contributions in (Czosnek, 2007). Geminiviruses are transmitted by different leafhopper species or the whitefly *Bemisia tabaci* and are assigned, based upon their transmission vector in combination with their respective genome organization, to four genera in the family *Geminiviridae*. All tomato-infecting geminiviruses belong to the genus *Begomovirus* with an ever-increasing number of tomato (yellow) leaf curl viruses transmitted by *B.tabaci*.

Compared to the geminiviruses, members of the family *Nanoviridae* are by far less numerous and also much less studied and understood. Nanoviruses are transmitted by various aphid species in a persistent and non-propagative manner. An economically very important nanovirus causes bunchy top disease in banana and is indigenous in Southeast Asia and the Pacific region including Australia (Nelson, 2006). Banana bunchy top virus, the causal agent of the disease, currently invades Africa and the Indian subcontinent (Khalid and Smooro, 1993; Karan et al., 1994). The other very important nanovirus is faba bean necrotic yellows virus (FBNYV), indigenous in regions of West Asia and North Africa. In some years yield losses of faba bean production due to FBNYV were considerable and have had serious impacts on food supply in Egypt during the 1990ies (Makkouk and Kumari, 2009). In recent years, diseases caused by nanoviruses emerged in numerous countries of the

*E-mail: gronenborn@isv.cnrs-gif.fr

Mediterranean Basin and the Near East. First reports of nanoviruses from European countries date from 2005 for Spain (Ortiz et al., 2006), 2008 for Azerbaijan (Kumari et al., 2009) and 2009 for Germany (Grigoras et al., 2010). Contrary to the well studied geminiviruses, knowledge on the biology and molecular genetics (Grigoras et al., 2010). Contrary to the well studied geminiviruses, knowledge on the biology and molecular genetics of nanoviruses is still less advanced (Gronenborn, 2004), yet recent progress in diagnosis and reverse genetics of faba bean necrotic stunt virus (FBNSV) is expected to allow for rapid compensation for the deficit (Grigoras et al., 2009).

The advent of the multiple displacement amplification or rolling circle amplification (RCA) technique has considerably facilitated diagnosis and molecular identification of viruses with ssDNA genomes (Haible et al., 2006). RCA and subsequent cloning and sequencing have allowed us to identify three distinct nanoviruses from Azerbaijan. In the following a short summary of the progress in the molecular characterization of nanoviruses from Azerbaijan is provided.

MATERIAL AND METHODS

Field prospecting

In June 2009 and in June 2010 several tomato (*Solanum lycopersicum*) fields as well as fields and gardens with chickpea (*Cicer arietinum*), lentil (*Lens culinaris*) and faba bean (*Vicia faba*) were surveyed for plants showing symptoms of a potential infection by a geminivirus and/or a nanovirus. Surveys of tomato plantings were near Khachmaz, Goychay, Masalli (Onjagala) and Lenkoran; legumes were surveyed in Lahij-Arakit (faba bean, wild legumes), Farzili near Jalilabad (chickpea), Peshtatuk (chickpea, lentil), and Jangamiran near Lerik (chickpea). The locations in Lahij-Arakit and Onjagala were visited in both 2009 and 2010 as samples of the 2009 survey had yielded interesting results. Samples were collected as fresh leaf material until they were frozen at -80°C. In 2009, sample tissue was also blotted onto Whatman FTA® classic cards for later DNA analyses.

Serological analyses were done as described in Grigoras et al. (2009).

Molecular analyses and sequencing

Frozen plant samples were ground in liquid nitrogen, and DNA was prepared using the method of Edwards et al. (1991), modified as described by Grigoras et al. (2009). We used 5-µL aliquots of the amplified DNA for diagnostic digests by restriction endonucleases, see examples shown in Figure 4. Preparative restriction endonuclease digests for cloning of the respective nanovirus genome components were carried out using between 10 and 50 µL of RCA product and subsequent resolution by preparative 0.7% agarose gel electrophoresis. One kb fragments were cut from the gel, purified, cloned and sequenced as described by Grigoras et al. (2009).

RESULTS

Tomato

The tomato fields visited appeared generally healthy and productive. In particular, no wide-spread symptoms of tomato yellow leaf curl virus (TYLCV), a very devastating virus in neighbouring Iran, were noticed. However, a particular case of damage of tomato crops was encountered in the Goychay region in 2009, where tomato was grown in plastic greenhouses. Here, the plants suffered from severe infestation with probably several pathogens (Figure 1A), and the fruits were of non-marketable quality (Figure 1B). Analyses carried out at the DSMZ, Braunschweig, Germany, identified tomato bushy stunt virus, a soil- and waterborne pathogen, as the major cause of the detrimental disease.

Other tomato samples collected in 2009 and 2010 were analyzed in part at the Institute of Botany, Azerbaijan National Academy of Sciences, Baku, and at the ISV, Gif sur Yvette, France. Contrary to earlier surveys of tomato and cucurbits in 2003 and 2005, when no TYLCV or watermelon chlorotic stunt virus were detected (A. Kheyr-Pour), a few tomato plants showing symptoms typical of TYLCV infections were found in a small tomato plot at Onjagala, south of Masalli, both in 2009 and 2010 (Figure 2). In 2009, one single plant with an apparently late infection was encountered. In 2010, several plants with serious infections were scored, which means that the virus and its vector are established in the area.

First molecular analyses of the extracted DNA after RCA at Baku hinted at a geminivirus as the causative agent. However, the final confirmation of the specific TYLCV species or strain by polymerase chain reaction (PCR), RCA-based RFLP analysis or molecular cloning is still pending.

An epidemiological follow-up of these findings is of major importance, as in southern Azerbaijan the climatic conditions for the viral vector *B.tabaci* are ideal and the as yet only sporadic occurrence of the virus may turn into a serious threat for tomato cultivation in that region.

Legumes

Since in late June there were not too many


A


B

Figure 1. Damaged and non-marketable tomatoes from plastic greenhouses in the region of Goychay (2009).

Figure 2. Typical symptoms of tomato yellow leaf curl virus. Left: Example from 2009; right: example from 2010, same field.

legume crops in the field anymore, only two larger chickpea fields were inspected, one near Jalilabad (Farzili) in 2009, and one near Lerik (Jangamiran) in 2010.

No obvious symptoms of a viral infection were observed in both cases. This assessment was later confirmed by the serological analyses carried out at the Julius Kğhn Institute (JKI), Braunschweig, Germany, where a panel of antisera specific for various legume-infecting viruses was used for diagnosis. Only one chickpea sample from Farzili near Jalilabad was diagnosed as infected by a (non-specified) luteovirus.

Therefore, we specifically looked in more remote areas and in private gardens to find and identify potential reservoir plants containing legume-infecting gemini- or nanoviruses. All samples collected originated from small gardens with only a few faba bean plants intercropped with potato (Lahij-Arakit). The lentil and chickpea samples from Peshtatuk were also from garden-sized plots with about 50 to 100 plants each. In addition, a number of wild legume species were sampled both around Lahij as well as in the Lerik area, but none of them proved to be infected with a nanovirus or a geminivirus.

A selected number of samples were analyzed at the Institute des Sciences du Vygytal, CNRS, Gif sur Yvette, France, by molecular DNA analyses, and in parallel at the JKI, Braunschweig, Germany, by ELISA-based serology. For instance, out of 32 wild- and cultivated legume samples collected in 2010 two were found infected by alfalfa mosaic virus (AMV), two by bean yellow mosaic virus (BYMV), one by bean leaf roll virus (BLRV), one with both AMV and BLRV, and two with a nanovirus.

Whereas one garden in Lahij-Arakit (family Muslim) in 2009 was infested by a nanovirus and luteoviruses (not specified) at about 50% incidence, no nanovirus infection was detected in the same two gardens in Arakit (family Muslim and Gilexanim) when visited in 2010. This contrasts the increased incidence of TYLCV in Onjagala (Masalli) in 2010 compared to that of 2009.

Figure 3 shows two examples of nanovirus-infected legumes, faba bean infected by (as we now know from the molecular analyses) faba bean necrotic yellows virus (FBNYV) and lentil infected with (as we now know from the molecular analyses) faba bean necrotic stunt virus (FBNSV). The faba bean sample was collected at Arakit in

Figure 3. Faba bean necrotic yellows virus-infected faba bean from Lahij-Arakit (left) and faba bean necrotic stunt virus-infected lentil from Peshtatuk (right).

2009, and the lentil sample is from Peshtatuk collected in 2010. The identity of the two aforementioned nanoviruses has been confirmed by both serology and molecular analyses (see below).

Molecular characterization

Only a fraction of all samples has been analyzed to date. Legume samples, in which a nanovirus was serologically detected, were given priority and analyzed at greater details. DNA of selected samples was prepared, subjected to RCA and analyzed by restriction endonuclease treatment. Resulting DNA fragments were resolved by agarose gel electrophoresis, and eventually cloned and sequenced (ISV, Gif).

An example of the molecular analyses of four legume samples from Peshtatuk collected in 2010 is shown in Figure 4.

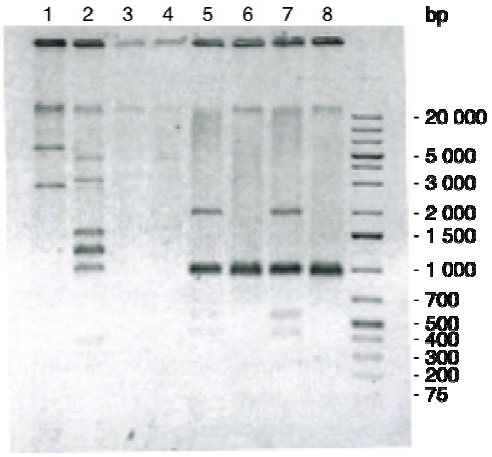

Figure 4. Restriction analysis of RCA-amplified DNAs of chickpea and lentil from Peshtatuk.

DNA of two chickpea (Figure 4, lanes 1-4) and two lentil (lanes 5-8) samples was amplified by RCA, restricted by endonuclease *Aat*II (lanes 1, 3, 5, 7) or *Hind*III (lanes 2, 4, 6, 8), and resolved by agarose gel electrophoresis. The DNA in lanes 5-8 was restricted into a major fragment of 1 kb; in case of the *Aat*II digests (lanes 5 and 7) also a minor product of 2 kb was produced. Our experience with nanovirus DNA segments shows that without exception so far, at least some genomic DNAs of a nanovirus have an *Aat*II site. Hence, the predominant 1-kb fragment produced by *Aat*II was a strong indication of the presence of a nanovirus in the two samples from lentil. No nanovirus-specific DNA pattern after restriction with *Aat*II or *Hind*III was obtained for the two chickpea samples shown. The same two lentil samples also reacted not only with broad-spectrum nanovirus antibodies but also with FBNSV-specific antibodies.

Consequently, DNA of the sample shown in lanes 7 and 8 (Peshtatuk #12b) was amplified at a preparative scale, restricted by *Aat*II or various other restriction endonucleases, and the purified 1kb fragments were cloned and sequenced. In a similar way, DNA of faba bean samples collected in Lahij-Arakit in 2009 was analyzed, and from two distinct locations about 0.5 km apart, garden of family Muslim (example shown in Figure 3, left), and garden of Gilexanim, several nanovirus positive samples were identified by serology and DNA analyses.

As of October 2010, all eight DNAs of a typical faba bean necrotic yellows virus were identified in a faba bean sample from Lahij-Arakit (collected in 2009 from family Muslim's garden), and were cloned and sequenced. From a sample collected in the garden of Gilexanim, Lahij-Arakit, in 2009 six DNAs of a typical, yet different faba bean necrotic

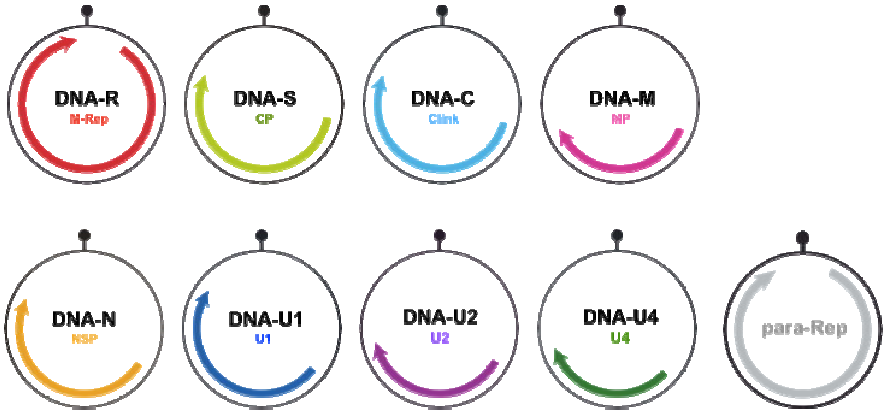

Figure 5. Genome organization of a typical nanovirus. The eight single-stranded virus DNAs are shown as circles with the common short inverted repeat sequences displayed as a stem loop. All are about 1 kb in size, coding regions for the eight different proteins are symbolized by proportionally sized arrows. Genome component names are shown along with the names of the encoded proteins. M-Rep, master replication initiator protein (Timchenko et al., 2000); CP, capsid protein; Clink, cell cycle link protein (Aronson et al., 2000); MP, movement protein; NSP, nuclear shuttle protein (Wanitchakorn et al., 2000); U1, U2, U4, proteins of as yet unknown function. A non-essential alphasatellite DNA encoding a para-Rep is also shown.

yellows virus, were also cloned and sequenced. DNA-U2 and -U4 have not yet been identified from the latter isolate, however, an additional para-Rep encoding DNA (alphasatellite) of the para-Rep1-type (Timchenko et al., 1999) was found here.

From the lentil sample #12b (Figure 3, right) collected in Peshtatuk in 2010 (garden of Nabiyev family), seven DNAs of a typical FBNSV isolate were cloned and sequenced, with identification of a DNA-U2 still pending. Interestingly, the partial FBNYV capsid protein gene sequences of a lentil and a vetch isolate of FBNYV determined by Kumari and colleagues (Kumari et al., 2009) are similar to DNA-S of FBNYV isolate #13-5 (faba bean) from family Muslim's garden, Lahij-Arakit, and to that of a faba bean isolate from Kermanshah, Iran (GenBank acc. AM493900), whereas the DNA-S of the FBNYV isolate AZ.12 from faba bean collected in Gilexanim's garden, only about 0.5 km away from the previous one in Lahij-Arakit, is more closely related to a FBNYV isolate from Alashtar, Iran, originating from chickpea (GenBank acc. AM493899). The sequences of the Iran isolates had been determined some years ago in our laboratory (Bananej K., Timchenko T., Gronenborn B.). The relationships between the Azerbaijani nanoviruses with the rest of the virus family were established by phylogenetic analysis. We aligned all available genome sequences using ClustalW and calculated the respective genetic distances based upon their pairwise sequence similarity. The phylogeny revealed that the FBNSV isolate from Peshtatuk is more closely related to a FBNSV isolate from Morocco (Abraham et al., 2010) than to FBNSV from Ethiopia, where the virus was found first. This finding raises some intriguing questions about the natural evolution of that virus or its eventual human distribution. Further surveys may shed more light on the phylogeography of these emerging pathogens.

CONCLUSION

In summary, the still limited analyses of tomato and legume samples collected in our surveys of 2009 and 2010 have already led to the identification of three distinct nanoviruses from Azerbaijan. The symptoms of a TYLCV-like infection in tomato make it almost certain that this geminivirus is present also in southern Azerbaijan, notwithstanding the fact that final molecular proof is pending. A thorough epidemiological follow-up of these findings will be important, as the climatic conditions in the Jalilabad, Masalli, Lenkoran region are ideal for *B.tabaci*, the viral vector of TYLCV and other begomoviruses. Therefore, the as yet only sporadic occurrence of TYLCV may turn into a serious threat for tomato cultivation in that region.

The floral biodiversity of Azerbaijan may well harbour also quite diverse ssDNA plant viruses, and that our initial analyses may have only touched the tip of the iceberg. We are convinced that further extended surveys and in-depth characterization of the material collected might reveal more ssDNA viruses and maybe even ones that were never before described.

ACKNOWLEDGEMENTS

We are indebted to Prof. Jalal Aliyev for his interest in and support of this research.

REFERENCES

Abraham A.D., Bencharki B., Torok V., Katul L., Varrelmann M., Vetten H.J. (2010) Two distinct nanovirus species infecting faba bean in Morocco. Arch. Virol. **155:** 37-46.

Aronson M.N., Meyer A.D., Györgyey J., Katul L., Vetten H.J., Gronenborn B., Timchenko T. (2000) Clink, a nanovirus-encoded protein, binds both pRB and SKP1. J. Virol. **74:** 2967-2972.

Czosnek H. (2007) Tomato Yellow Leaf Curl Virus Disease: Management, Molecular Biology, Breeding for Resistance. Springer, Dordrecht, The Netherlands: 350 p.

Edwards K., Johnstone C., Thompson C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19:** 1349.

Grigoras I., Gronenborn B., Vetten H.J. (2010) First Report of a Nanovirus Disease of Pea in Germany. Plant Dis. **94:** 642.

Grigoras I., Timchenko T., Katul L., Grande-Pyrez A., Vetten H.J., Gronenborn B. (2009) Reconstitution of authentic nanovirus from multiple cloned DNAs. J. Virol. **83:** 10778-10787.

Gronenborn B. (2004) Nanoviruses: genome organisation and protein function. Vet. Microbiol. **98:** 103-109.

Haible D., Kober S., Jeske H. (2006) Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. J. Virol. Methods **135:** 9-16.

Karan M., Harding R.M., Dale J.L. (1994) Evidence for two groups of banana bunchy top virus isolates. J. Gen. Virol. **75:** 3541-3546.

Khalid S., Smooro M.H. (1993) Banana bunchy top disease in Pakistan. Plant Pathol. **42:** 923-926.

Kumari S.G., Attar N., Mustafayev E., Akparov Z. (2009) First Report of Faba bean necrotic yellows virus Affecting Legume Crops in Azerbaijan. Plant Dis. **93:** 1220.

Makkouk K.M., Kumari S.G. (2009) Epidemiology and integrated management of persistently transmitted aphid-borne viruses of legume and cereal crops in West Asia and North Africa. Virus Res. **141:** 209-218.

Nelson S. (2006) Banana Bunchy Top Virus. *http://wwwctahrhawaiiedu/banana/*.

Ortiz V., Navarro E., Castro S., Carazo G., Romero J. (2006) Incidence and transmission of *Faba bean necrotic yellows virus* (FBNYV) in Spain. Span. J. Agric. Res. **4:** 255-260.

Stanley J., Bisaro D.M., Briddon R.W., Brown J.K., Fauquet C.M., Harrison B.D., Rybicki E.P., Stenger D.C. (2005) *Geminviridae*. In: Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses (Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U. and Ball L.A., eds.), Elsevier, Academic Press, London: 301-326.

Timchenko T., de Kouchkovsky F., Katul L., David C., Vetten H.J., Gronenborn B. (1999) A single rep protein initiates replication of multiple genome components of faba bean necrotic yellows virus, a single-stranded DNA virus of plants. J. Virol. **73:** 10173-10182.

Timchenko T., Katul L., Sano Y., de Kouchkovsky F., Vetten H.J., Gronenborn B. (2000) The master rep concept in nanovirus replication: identification of missing genome components and potential for natural genetic reassortment. Virology **274:** 189-195.

Vetten H.J., Chu P.W.G., Dale J.L., Harding R., Hu J., Katul L., Kojima M., Randles J.W., Sano Y., Thomas J.E. (2005) *Nanoviridae*. In: Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. (Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U. and Ball L.A., eds.), Elsevier, Academic Press, London: 343-352.

Wanitchakorn R., Hafner G.J., Harding R.M., Dale J.L. (2000) Functional analysis of proteins encoded by banana bunchy top virus DNA-4 to -6. J. Gen. Virol. **81:** 299-306.