Session 2D

Viruses, Phytoplasmas and their Transmission

Chairman & Session Organiser:	Dr Matt Dickinson *University of Nottingham, UK*
Platform Papers:	2D-1 to 2D-4
Poster Presentations:	P2D-5 to P2D-10

Control of phytoplasma vectors

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Phytoplasmas were first observed in plant phloem four decades ago. Novel molecular methods now allow not only the detection of new phytoplasma infections but also the systematic differentiation of these pathogenic agents far beyond the initial serological distinctions. In addition to these impressive taxonomical developments, other areas, such as elucidating many insect vectors and characterizing the specifics of phytoplasma-vector relationships, have considerably advanced. Yet despite all these improvements, from the grower's perspective, it is principally a matter of luck whether a crop will suffer losses from phytoplasma infections, or not. For most crops in most countries or geographical regions, the damage inflicted by phytoplasma infection, originating from vectors that acquired these pathogens from native wild plants, could range anywhere from negligible to total economic loss from one year to another.

Traditional vector-control methods are insufficient to control the disease (Weintraub & Beanland, 2006). The most reliable means of vector control is by covering the crop with insect-proof screening. Papaya is subject to three different phytoplasma diseases: dieback (causing 10–100% tree death per season in Australia), yellow crinkle and mosaic (chronic diseases). Management practices consist of rogueing yellow crinkle- and mosaic-infected trees and ratooning (removing symptomatic shoots and allowing lateral shoot development) dieback-infected trees to reduce the inoculum load. More recently, it has been demonstrated that the pathogen vectors could be 100% controlled by covering the trees with insect exclusion netting. Screening was compared to systemic insecticide (imidacloprid) treatments and non-treated control – there was no difference in disease incidence between the insecticide and control trees. However, these authors concluded that due to the cost of erecting a support structure and the reduced pollination within the screening, only high-cash crops could justify the expense.

While, indeed, screening is the only method to attain good vector control, its applicability is so severely limited in major crops – sugar cane, corn, rice, fruit trees, grapes – that its use can not even be contemplated. On the other hand insecticides, even when used on a weekly basis, will not control the appearance of disease because pathogen transmission occurs faster than insecticides can act. At best, use of insecticides can help control vector populations and thus reduce intra-crop transmission. While chemical control of vectors likely will continue for the foreseeable future, vector management will slowly shift to genetic manipulation of crops.

The future, however, is not bleak. A new and potentially very powerful tool for controlling pathogen transmission involves the manipulation of symbiotic bacteria, termed symbiont-based protection (SyBaP). Many arthropods carry a diverse assembly of symbiotic microorganisms, which are maternally inherited and which profoundly affect their hosts. These bacteria can be genetically modified to prevent transmission of pathogens; arthropods carrying these transformed bacteria are called paratransgenic. To develop such symbiotic-control methods it is necessary to first identify microorganisms in the target vector whose characteristics appear promising: 1) the symbiont is present in the same organs as the

pathogen, 2) the symbiont exhibits a potential for spreading rapidly into the pathogen, and 3) the symbiont exhibits a potential for spreading rapidly into the host populations. Once identified, cultured and modified, these bacteria could compromise transmission by reducing vector competence, by expressing a gene product that would kill the pathogen, by causing high offspring-mortality rates through cytoplasmic incompatibility, or by physically competing for space that the pathogenic bacteria would otherwise normally occupy.

This symbiont-based strategy is already being applied against several insect-borne pathogens of human diseases, including the Chagas' disease agent. Research with leafhoppers transmitting the xylem-limited bacterium *Xylella fastidiosa* (causative agent of Pierce's Disease) which replicates in the foregut of the glassy-winged sharpshooter (*Homalodisca coagularta*) is also progressing apace. A symbiont, *Alcaligenes xylosoxidans* subsp. *denitrificans*, has been identified, cultured, modified and successfully reintroduced into leafhoppers via several plants (citrus, chrysanthemum, grape, periwinkle and Crepe Myrtle) and is expected to be competing with the pathogen for space and resources, thus reducing the vectoring capacities of the host. Similarly, another bacterium, *Cardinium hertigii*, has been identified and localized in the fat bodies and salivary glands of the leafhopper *Scaphoideus titanus*. Because this insect is the vector of the phytoplasma-causing Flavescence dorèe (FD), its presence in the same locations as the FD phytoplasma may eventually be instrumental in symbiotic control efforts. One of the major challenges in this field is the delivery of the transgenic bacteria to the target vectors without adversely affecting the environment or other insect populations.

A possible new avenue would be genetic manipulation of plants (such as *Arabidopsis* or *Medicago*, for which the genomes are almost completely known) harboring traits that will not interact with the invading pathogens, thus preventing the commonly observed phytoplasma reactions involving cell proliferation and phloem blockage. Preliminary work has shown that systemic acquired resistance (SAR) on benzothiadiazole-treated *A. thaliana* reduced leafhopper survival significantly (Bressan & Purcell, 2005).

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A secreted effector protein of AY-WB phytoplasma accumulates in nuclei and alters gene expression of host plant cells

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The genome of Aster Yellows phytoplasma strain Witches' Broom (AY-WB) was sequenced to completion (Bai *et al.*, 2006; http://www.jic.ac.uk/staff/saskia-hogenhout/genome.htm). AY-WB belongs to subgroup 16SrIA within *Candidatus* Phytoplasma asteris. AY-WB and other phytoplasmas are insect-transmitted plant pathogens. They are mainly limited to the phloem of their plant hosts, but invade and replicate in most organs of their insect vectors, which is the leafhopper *Macrosteles quadrilineatus* for AY-WB. Phytoplasmas cannot yet be cultured in cell-free culture media.

To identify potential virulence (effector) proteins, the AY-WB genome was mined for the presence of membrane-targeted proteins by determining whether deduced protein sequences carry N-terminal signal peptide (SP) sequences. This resulted in the identification of 76 AY-WB proteins with SPs. Of these 76, 20 proteins contained a SP and additional transmembrane domains. Thus, these 20 proteins are apparently initially secreted but remain associated with the membranes. AY-WB proteins 016, 395, 432, 599 and pIV04 have one additional transmembrane domain at their C-termini and therefore are predicted to locate at the exterior of the phytoplasma cell. The accuracy of the SP and transmembrane domains predictions were validated by the presence of AY-WB_599 among the list of 20 proteins. AY-WB_599 has similarity to the Antigenic Membrane Protein (AMP) of Onion yellows phytoplasma strain M (OY-M). AMP possesses a cleavable SP, is secreted by the Sec-dependent translocation system, and is an abundant cell surface protein (Kakizawa *et al.*, 2004). AMP determines insect vector specificity (Susuki *et al.*, 2006), and is therefore involved in phytoplasma virulence.

The remaining 56 proteins contain only SPs and no additional transmembrane domains, and hence are probably secreted into the extracellular environment of AY-WB. Most of the proteins have unknown functions. However, the list of 56 proteins contains the five solute-binding proteins (SBPs) ArtI (AYWB_263), DdpA (AYWB_529), NlpA (AYWB_588), ZnuA (AYWB_624) and MalE (AYWB_667). SBPs are secreted through the Sec-dependent pathway in other bacteria (Higgins, 2001), thereby validating our SP predictions. Additional independent evidence that the secreted AY-WB proteins (SAPs) are effector proteins was obtained. Two phytoplasma effectors, SAP11 and SAP30, contain eukaryotic nuclear localization signals (NLSs) that are functional in plant cells. Indeed, SAP11 localizes in host nuclei of AY-WB-infected China aster plants, and needs the NLS domain for this nuclear localization. In *Nicotiana benthamiana*, nuclear localization of SAP11 requires a host factor, importin α. Also, SAP11 alters gene transcription, notably of several transcription factors, in tomato.

Immunofluorescence microscopy experiments detected SAP11 beyond the phloem tissue in mesophyll and other cell types, suggesting that SAP11 moves out of the phloem into adjacent tissues. In agreement with this finding, Imlau *et al.*, (1999) have shown that GFP specifically produced in phloem cells under control of the *AtSUC2* promoter in *Arabidopsis* can unload from the phloem into mesophyll cells and other cell types of developing (sink) tissues, including young rosette leaves, petals, anthers and roots, whereas this post-phloem transport of GFP was not detected in fully developed (source) tissues. Indeed, the size exclusion limits (SELs) of plasmodesmata change during organ development. The SELs of sink tissue plasmodesmata range between 10 and 40 kDa, whereas those of source tissues are much smaller (Imlau *et al.*, 1999). The majority of secreted AY-WB proteins (51 of 56), including SAP11 and SAP30, are smaller than 40 kDa, indicating that most secreted protein of AY-WB may move out of the phloem and target developing tissues of plants. This is consistent with the appearance of symptoms predominantly in developing tissues of phytoplasma-infected plants. Typical symptoms include the retrograde metamorphosis of the floral organs to the condition of leaves (phyllody), and clustering of branches of developing tissues (Witches' broom).

The AY-WB and OY-M genomes contain several potential mobile units (PMUs) that exhibit features of bacterial pathogenicity islands (Bai *et al.*, 2006). The gene for SAP11, *AYWB_370*, is located in a PMU-like region and is the first of a series of five genes encoding predicted secreted proteins SAP68, SAP67, SAP58 and SAP66. Hypothetical proteins with sequence similarities to these secreted and membrane proteins were found in OY-M and other phytoplasmas, however they have no sequence similarities to domains and proteins with known functions in the GenBank non-redundant database.

In summary, we propose that SAP11 is an effector protein that is secreted directly inside phloem cells and then is transported to mesophyll and other plant cells where it targets nuclei to affect the expression of various genes. This is the first report of an effector protein of a plant-pathogenic bacterium with Gram-positive ancestry. Extending the concept of effectors to the phytoplasmas points to novel research strategies for unraveling pathogenicity mechanisms of these fascinating pathogens.

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Are phytoplasmas transmitted through seed?

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Introduction

Generally phytoplasmas are assumed to be phloem-limited organisms in plants that are transmitted in a circulative propagative manner by phloem-feeding insects. However, there have been recent reports in which DNA of the lethal yellowing (LY) disease of coconuts has been detected in coconut embryos, and in particular in the plumule and the cells ensheathing it (Cordova *et al.*, 2003). There are also conference abstracts that report the seed transmission of phytoplasmas in alfalfa, tomato, oilseed rape and lime (see Nipah *et al.*, 2007 for references). Generally, seed transmission of phytoplasmas has been considered unlikely because there is no direct connection between the phloem sieve elements of plants and the developing embryo or seed, but there have been reports of phytoplasmas in companion cells and parenchyma cells, and these may provide a route for the phytoplasmas to pass from the phloem into the developing embryos.

The prospect of seed transmission of phytoplasmas has major implications for quarantine services and regulatory authorities, since seed is not routinely tested for the presence of phytoplasmas. In the case of coconut production, fears that the progeny from breeding programmes undertaken in diseased areas may contain lethal-yellowing like phytoplasmas have led to research programmes involving movement of seed for conservation in disease-free regions in Ghana being turned down by funding authorities, and the local coconut research programme is prevented from exporting germplasm to neighbouring countries (Nipah *et al.*, 2007). It is recommended that all exchanges of coconut germplasm be carried out in the form of zygotic embryo *in vitro* cultures, but there is no scientific basis to conclude that this will completely remove the possibility of seed transmission. Furthermore, the recommendation is partly based on the belief that infected embryos are unlikely to germinate.

In this work, we report the use of PCR-based diagnostic techniques in two separate studies to detect the presence of phytoplasma DNA in coconut embryos in Ghana, and also in maize kernels in Peru. We also present evidence that suggests that the presence of phytoplasma DNA in coconut embryos has no detrimental affect on the ability of these embryos to develop into progeny plants.

Methods and results

Genomic DNA was extracted from Cape St Paul Wilt (CSPWD)-infected West African Tall coconut palms in Ghana and from maize plants exhibiting phytoplasma disease symptoms in Peru and screened by PCR using primers for the 16S rDNA gene. PCR confirmed the presence of phytoplasma DNA in peduncles, spikelets, male and female flowers from CSPWD-infected palms and in nine of 52 embryos, and these were confirmed as CSPWD by restriction digestion and DNA sequencing. In the Peru study, two cobs were selected from each of three maize plants (two symptomatic and one asymptomatic for phytoplasma disease).

Leaf samples from all three plants tested positive for *Candidatus* P. asteris phytoplasmas. Eight kernels were pooled from each cob and DNA extracted and tested by PCR. The 4 samples for the cobs derived from the two symptomatic plants were positive for *Ca.* P. asteris DNA whilst the two samples from the asymptomatic plant were negative.

To test for the viability of embryos that contained CSPWD DNA, 33 seeds from healthy palms and 61 from diseased palms, were nursed in mesh cages at two disease-free locations. Whilst 19 out of 33 healthy seeds (57.6%) germinated, a significantly higher number, 44 out of 61 seeds (72.1%) germinated from diseased palms. Both harvested and dropped seeds from diseased palms showed approximately equal chances of germination. Nested PCR performed on all germinated seedlings from the nurseries as well as plantlets from embryo cultures after three months and six months of nursing and culturing respectively, failed to detect the presence of phytoplasma DNA in any of the samples.

Discussion

This study has indicated that phytoplasma DNA can be detected in embryos derived from infected plants on a regular basis, with CSPWD DNA being detected in Ghanaian coconut palms, and *Ca.* P. asteris DNA detected in Peruvian maize kernels. It therefore appears clear that phytoplasma DNA can find its way into embryos, but until microscopic evidence confirms that this is within viable phytoplasmas there remains a possibility that it is the phytoplasma DNA alone that enters the embryos. The questions that also need to be addressed are whether such embryos can germinate, and whether the phytoplasma DNA found in the embryo is associated with viable organisms that can survive the process of seedling development to result in disease. It was not possible in this study to divide an embryo to test for the presence of phytoplasma in one part and germinate the remaining part.

However, should the assumption that infected embryos can not germinate be correct, one would expect seeds from infected palms to generally record lower germination percentages in comparison to those from a healthy source. The opposite was the case in this study. Furthermore the ability to germinate was found to be independent of whether the matured seed had dropped or was freshly harvested from the diseased palm. This suggests that matured embryos containing phytoplasma DNA retain the ability to germinate regardless of whether the seed has dropped or not. Since our current study failed to detect any phytoplasmas in the seedlings and plantlets derived from infected palms, we conclude that although phytoplasma DNA can be detected in embryos, there is as yet no evidence that CSPWD can be seed transmitted to cause disease in the resultant palm.

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Emerging virus and viroid diseases - new threats and novel methods

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Despite the huge improvements made in diagnostic technologies over the last 30 years, the detection and identification of plant viruses remains a major challenge, for many different reasons. Firstly the number of pathogens faced by virologists has grown hugely, with around 1200 different plant viruses now listed as full or tentative species. Keeping pace with this ever expanding viral biodiversity is an immense challenge. Certain individual hosts, for example many of the major solanaceous crops including potato, tomato and petunia, can be infected by numerous different viruses and viroids. To provide even a basic screening package, based on pathogens known to occur frequently in these hosts, requires multiple individual tests to be performed on each sample, and the use of combinations of different technologies; predominantly immunological (e.g. ELISA) and molecular (e.g. PCR) methods, with biological testing (e.g. sap inoculation) and electron microscopy also required to detect certain pathogens. This type of parallel testing is time consuming and expensive, and requires diagnostic services to invest heavily in different technologies.

Another major challenge facing today's virology diagnostician is globalisation and the development of an international trade in agricultural and horticultural produce, which is breaking down the traditional geographical barriers to the movement of pathogens. As this trade has become more international, the potential for the importation of non-indigenous plant viruses has grown significantly. For example, twenty one new viruses have been identified in the UK since 1980; a rate of just under one a year. Of these new pathogens, two thirds of the new viruses have been in horticultural crops, in particular protected crops, while the remaining third were soil-borne diseases of arable crops. Despite some successes in eradication, in many cases these new pathogens are now established and industry is having to deal with the consequences of these introductions, and the cost implications can be considerable.

While all horticultural crops are at risk, ornamental species pose a particularly high threat, due to the huge volume of live material (young plants or cuttings) that is moving across international borders. The massive numbers involved (often millions of units per year), combined with problems in detecting viruses on young plants due to latent infections, can make visual inspection extremely difficult. The trade in ornamental plants also provides additional risks due to: the sheer diversity of species and families involved; the continual introduction of new varieties; and the speed at which these new lines can become popular (thus leading to massive demand). Often a new variety will begin as a very small number of mother plants, which will be propagated repeatedly, eventually giving rise to millions of cuttings. As a result, if the original mother plants are infected, then the infecting-virus will spread rapidly and widely. Given these factors, new diseases appear very frequently in ornamentals, sometimes simply as extensions to already broad host ranges, but more significantly through major jumps into entirely new plant families or through the appearance of completely new viruses. While these new diseases can cause losses in ornamental crop themselves, their potential to act as reservoirs of infection for other hosts of potentially greater economic importance (e.g. vegetable crops), is also a major concern.

For example, ornamentals have been suggested as possible sources of infection for viroid outbreaks in European tomato crops. The feasibility of this pathway has been recently strengthened by widespread findings of *Potato spindle tuber viroid* and other pospiviroids in ornamental hosts across Europe.

Further problems occur through the movement of material (either via trade or germplasm exchange) from regions where there is known to be a high level of virus diversity e.g. solanaceous viruses from South America. In these situations, new viruses can be easily introduced, without extremely carefully screening of material. This situation poses a particularly problem as it is often unclear which viruses pose the greatest risk and which are likely to emerge as significant problems; especially as many are relatively obscure in their place of origin. An excellent example of this is *Pepino mosaic virus* (PepMV). The sudden appearance of this South American pathogen, in European tomato crops in 1999 and the subsequent appearance of new, distinct PepMV strains more recently, highlights the genuine risks associated with moving planting material between different countries and the role such movement can play in establishing new diseases.

Given this background, plant virologists are increasingly looking for new diagnostic methods, especially those that can permit the detection of a wider range of different viruses, including those often not previously associated with a particular host. They are also looking to develop generic technologies that have the potential to detect all required targets, thus avoiding the problems associated with parallel testing. Given these aims, array-based systems, including micro- and real-time arrays are being investigated. With the capacity to test for multiple targets simultaneously, arrays have the potential to be the ultimate generic technology.

In addition, there is also a need for more rapid detection methods, that allow the faster detection of pathogens, in particular those moving in international trade. In this area, the use of field-portable systems has the greatest potential; allowing testing to be moved out of centralised laboratories and into the hands of inspectors. The success of antibody-based lateral flow devices (LFDs) has demonstrated the power of this approach. However LFDs have limitations and the development of rapid, simple DNA-based detection systems would be a major advance. For this reason portable real-time PCR systems have been developed and are being validated. Recent advances include the development of ultra-rapid nucleic acid extraction and iso-thermal amplification.

Development of antibody-mediated resistance against Tomato Yellow Leaf Curl Virus

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Tomato Yellow Leaf Curl Virus (TYLCV) is the common name for a number of viruses causing severe tomato yield losses in tropical and sub-tropical areas worldwide. TYLCV causes a range of symptoms including leaf chlorosis, leaf cupping, plant stunting and flower abscission. This virus is a member of the geminivirus group characterized by twin icosahedral particles possessing a genome of one or two circular single-stranded DNA molecules. Because of its insect vector, *Bemisia tabaci*, it is difficult to control and all commercial tomato plant cultivars are susceptible, so obtaining tomato cultivars resistant to this virus is of considerable interest. For this purpose, several strategies such as pathogen-derived resistance (Brunetti *et al.*, 2001; Shivaprasad *et al.*, 2006) have been investigated. The lack of general applicability and potential risks associated with their use in plants, such as recombination and transcapsidation occurring between transgenes or transgene products and the incoming viruses, has limited the effectiveness of such strategies. An alternative approach to produce plants resistant to pathogens, lacking the aforementioned drawbacks, is the expression *in planta* of antibody fragments that could bind to essential functional components of pathogens, thereby inactivating pathogens within plant cells (Schillberg *et al.*, 2001). The first successful application of this strategy was the expression of specific single-chain variable fragments (scFv) against Artichoke Mottled Crinkle Virus (AMCV) (Tavladoraki *et al.*, 1993).

In this study, we exploited the expression of a specific scFv fragment in plant cells for suppression of disease caused by TYLCV. An scFv fragment (scFv-ScRep) interacting with the multifunctional replication initiator protein (Rep) was obtained through three rounds of affinity panning from a naïve scFv phage display library (Tomlinson I, MRC, Cambridge, UK). The selected scFv was expressed in the pHENHI bacterial expression vector and purified by immobilized metal affinity chromatography (IMAC). ELISA and Western blot analysis were used to analyze binding activities of bacterially expressed scFv. The observed high binding activity of bacterially expressed scFv-ScRep to both full length and C-terminal truncated Rep protein indicated scFv could successfully bind to the amino terminus of Rep. The amino-terminus of the Rep protein in geminiviruses contains critical cleavage, linkage, DNA binding and oligomerization domains which are indispensable for viral replication (Gutierrez, 1999).

The pTRAkt plant expression vector was used to clone the scFv-ScRep gene both individually and as an amino terminal fusion to GFP. In addition, the nuclear localization signal of simian 40 virus was used for nuclear targeting of scFv-ScRep and scFv-ScRep-GFP inside the cells. All constructs were used for transient transformation of tobacco plants via agrobacterium infiltration. Stable transformation of *N. benthamiana* plants was accomplished through leaf disc transformation. Plant expressed scFv was analyzed for its integrity and binding activities by ELISA and Western blotting. Expression and localization of scFv fused to GFP with and without the NLS tag was visualized by fluorescence microscopy.

Blotting analysis showed detectable amounts of scFv-ScRep and NLS-scFv-ScRep to be present in crude leaf extracts of transformed plants which have binding activity to recombinant Rep. This indicates the correct folding and integrity of scFv-ScRep despite the reducing conditions in the cytosol. Fluorescence microscopy results confirmed localization of scFv-ScRep-GFP fusion protein within the cytoplasm and nucleus.

To assay for virus resistance, transgenic and non-transgenic *N. benthamiana* plants were agroinoculated with *Agrobacterium tumefaciens* harbouring a dimer of a fully infectious copy of TYLCV-Ir. Susceptibility or resistance of transgenic plants was monitored by scoring symptom development during three to seven weeks post inoculation (wpi). Virion accumulation and DNA replication were analyzed by PCR, TAS-ELISA and DNA hybridization methods. Infectivity assays indicated a range of resistance responses from susceptibility to immunity. While wild type and susceptible plants showed distinct symptoms 2-3 wpi, some transgenic lines were symptomless 7 wpi and others showed attenuation and delay in symptom development. Accumulation of viral DNA after agroinoculation by TYLCV was analyzed by Southern blotting. Plants lacking disease symptoms showed no viral DNA accumulation, while those with severe symptoms showed viral accumulation in an amount comparable to that of untransformed plants. PCR analysis confirmed the lack of viral DNA in symptomless plants. Further quantitative PCR analysis indicated that the amount of scFv transcripts in transgenic plants is directly related to the levels of resistance. This study presents the first successful approach to antibody mediated resistance against plant DNA viruses.

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Biological and molecular characterization of Lettuce Mosaic Virus from Iran

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Introduction

Lettuce Mosaic Virus (LMV) is one of the most destructive viruses in lettuce (*Lactuca sativa* L.) worldwide. It has flexuous filamentous particles of 750×13 nm (Revers *et al.*, 1999). The genome of LMV is a single, positive-sense 10,080 nt RNA molecule (Krause-Sakate *et al.*, 2002). Infected plants usually show vein clearing, yellow mottling, and stunted growth. Symptoms are quite variable and depend on the cultivar, the environmental conditions, and the developmental stage at which the plant became infected (Candresse *et al.*, 2002). LMV isolates have been classified into four pathotypes according to their virulence on lettuce varieties carrying the three resistance or tolerance genes *mol*^{1}, *mol*^{2} and *Mo*2, which have been identified in *Lactuca sativa* cultivars (Pink *et al.*, 1992a).

Sample collection and virus identification

Lettuce plants showing symptoms of LMV infection, including mosaic, mottling, dwarfing and leaf deformation were collected from fields throughout Tehran province. Samples were tested for LMV infection by immunoprinting.

Biological tests

Three samples that were positive in immunoprinting were collected and inoculated onto test plants. These isolates produced symptoms on susceptible lettuce cv. Terocadero and cv. Mantilia (containing the *mol*^{1} resistance gene), but did not produce symptoms on cv. Salinas 88, which contains the *mol*^{2} resistance gene. *C. quinoa* and *C. amaranticolor* plants developed chlorotic local lesions on inoculated leaves three to five days after inoculation, and systemic symptoms that included chlorotic lesions and leaf distortions two weeks after inoculation. *Nicotiana benthamiana* developed systemic symptoms and *Gomphrena globosa* developed necrotic local lesions two to three weeks after inoculation, and did not become systemic.

Serological tests

One of the virus isolates was propagated in *C. quinoa* and was purified from infected plants. Antiserum to LMV was produced in rabbit by a series of intravenous and intramuscular injections. The precipitin titre of this antiserum was 1:1024. ELISA, Gel double diffusion (GDDT) and western blotting tests were performed using this antiserum. The LMV antiserum reacted positively with purified LMV and LMV infected *C. quinoa*, and did not react with healthy *C. quinoa* in ELISA. In GDDT, precipitin bands appeared between antibody and infected plant sap and purified LMV, but not between antibody and healthy plant sap. In western blots, purified LMV and infected *C. quinoa* tissue reacted positively to LMV antiserum.

IC-RT-RCR

IC-RT-RCR was performed using the primer pair described by Zerbini *et al.* (1995) and LMV polyclonal antiserum. An approximately 1300 bp DNA fragment was amplified from LMV-infected plants by IC-RT-PCR with an LMV specific primer pair. No DNA fragment was amplified from uninfected plants.

Discussion

The two closely linked or allelic genes, *mol*^{1} and *mol*^{2} are currently used worldwide to protect lettuce crops against the detrimental effects of LMV infection (Pink *et al.*, 1992b). These genes are recessive and do not provide immunity to viral infection by common LMV strains. Levels of resistance conferred by *mol*^{1} and *mol*^{2} genes can either be low (systemic virus accumulation but no symptoms, sometimes referred to as tolerance), or high (no virus multiplication), and this may depend on the virus isolate (Revers *et al.*, 1997).These genes restrict the long-distance movement of the virus or affect symptoms expression and/or viral accumulation (Bos *et al.*, 1994). New strains of the virus appear at intervals. Most seem to be restricted to certain areas or may disappear quickly. However, in recent years, several strains have persisted for longer periods of time, and at least two can overcome the resistance conferred by one or the other or both alleles (Ryder *et al.*, 2003). Based on biological tests LMV isolates from Iran overcame the *mol*^{1} resistance gene and infected cv. Mantilia, but did not produce symptoms on cv. Salinas 88, which contains the *mol*^{2} resistance gene.

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Occurrence and distribution of Bean Yellow Mosaic Virus in faba bean and *Gladiolus* fields of different provinces of Iran

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Introduction

Bean Yellow Mosaic Virus (BYMV) is a very variable virus, a characteristic shared by other *potyviruses* including Clover Yellow Vein Virus (ClYVV), Bean Common Mosaic Virus (BCMV) and Pea Seed Borne Mosaic Virus (PSbMV). Overlap exists among these potyviruses with respect to host ranges, symptoms and even serology, and a sort of continuum exists within the potyvirus genus. In faba bean, common strains cause a mild green mosaic or vein-banding often associated with some leaf distortion, and affected parts of the plant remain stunted. In *Gladiolus*, infection leads to overall stunting, colour break, flower distortion, reduced flowering and cormel production. The virus may also cause flower bud necrosis and ovule abortion. BYMV is transmissible mechanically in sap and non-persistently by aphids. The particles are usually flexuous, 750 nm long (but longer in the presence of divalent cations and when viewed in sap of *Chenopodium amaranticolor*) and 12- 15 nm wide. Particles are helically constructed (pitch 3.4 nm), but their axial canal is obscure. The particles sediment as one component at about 151S (varying from 140-166S) and contain 5.5- 6.0% nucleic acid and 94.0-94.5 % of one type of protein.

Materials and methods

A total of 331 faba bean and 150 *Gladiolus* samples with symptoms of viral infection were collected from Khuzestan, Golestan, Isfahan and Tehran provinces. For ACP-ELISA, leaf samples (100 mg) were ground in coating buffer (0.05M carbonate buffer pH 9.6) added to the ELISA plate. The plates were then incubated at 37°C for 4 h in a humid box and later washed with PBS-Tween (0.02M phosphate, 0.15M saline and 0.05% Tween-20 pH 7.5) for 3 min. The washing step was repeated three times. One hundred µl of 2% skimmed milk in PBS-Tween was added and the plates were incubated at 37°C for 30 min after which the blocking solution was removed by three washes in PBS-Tween. Subsequently antibodies specific to *potyviruses* (DSMZ AS057311) diluted at 1:1000 were added to each well (100 µl/well) and incubated at 37°C for four hours. Washing steps were repeated as described previously. In the next step 100 µl of conjugate (PBST+2% PVP+0.2% egg albumin) was added to each well at a dilution of 1:200. After incubation for two hours at room temperature, the wells were further subjected to washing with PBS-Tween. Finally 100 µl of substrate (p-nitrophenyl phosphate, 1 mg/ml) was added to each well and incubated for half an hour at room temperature. Positive and negative controls were included on the same plate. Absorbance at 405nm was measured using a flow ELISA micro plate reader. The reaction was considered positive for BYMV infection if absorbance was observed to be >0.1, which was at least three times the background mean of the healthy controls.

For DAS-ELISA, leaf samples (100 mg) were ground in coating buffer (0.05M carbonate buffer pH 9.6) added to the ELISA plate. The plates were then incubated at 37°C for four hours in a humid box and later washed with PBS-Tween (0.02M phosphate, 0.15M saline

and 0.05% Tween-20 pH 7.5) for three minutes. The washing step was repeated three times. Subsequently antibodies specific to BYMV (DSMZ AS0471) diluted at 1:1000 were added to each well (100 µl /well) and incubated at 37°C for four hours. Washing steps were repeated as described previously. In the next step 100 µl of conjugate (PBST+ 2%PVP+0.2% egg albumin) was added to each well in the dilution of 1:1000. After incubation for four hours at room temperature, the wells were further subjected to washing with PBS-Tween, p-nitrophenyl phosphate substrate added as described above and absorbance read at 405nm. The reaction was considered positive for BYMV infection if absorbance was observed to be >0.1, which was at least three times the background mean of healthy control.

For the mechanical inoculation of plants, two isolates from faba bean and *Gladiolus* were inoculated onto faba bean, *Phaseolus vulgaris* cv. *Bountiful*, *Chenopodium quinoa*, *Chenopodium album*, *Chenopodium amaranticolor* and *Nicotiana benthamiana*. The selected samples were ground in phosphate buffer (pH 7.0) containing 2% PVP and indicator plants were inoculated by using Carborundum powder (300 meshes).

For IC-RT-PCR, the first steps of the method are the same as for DAS- ELISA. One hundred µl plant extract was submitted to immunocapture in PCR tubes precoated with 1µl/ml of anti-BYMV IgG at 37°C for four hours. After two washes with PBST and one wash with distilled water, 5µl of cDNA synthesis mix was added to 14µl H_2O, 1.5µl $MgCl_2$, 2.5µl 10x reaction buffer, 0.6µl Forward primer (5'- CT(AC) CA(AG) ATG GAG AA(CT) CC(CT) GC 3'), 0.5µl Reverse primer (5'- CCA AAG TTC CAA TCA CCA CC 3'), 0.5µl dNTP, 0.5µl Taq polymerase. PCR amplification was performed using a denaturation phase at 95°C for three min, followed by 35 cycles of amplification (94°C for one min, 58°C for one min, 72°C for two min) and a final extension at 72°C for 10 min.

Results

The plant sampling and serological assays were performed on different samples of *Vicia faba* and *Gladiolus*. Among 331 infected faba bean plants, the percentage of BYMV in the Khuzestan, Golestan, Isfahan and Tehran provinces were 69.8%, 57.7%, 72.8% and 11.1% respectively, and among 150 infected *Gladiolus* plants, the percentage of BYMV was 43.3% in Tehran province(Damavand) and 56.6% in Isfahan province (Mahallat). When sap from selected samples was mechanically inoculated onto faba bean, *Phaseolus vulgaris* cv. *Bountiful*, *Chenopodium quinoa*, *Chenopodium album*, *Chenopodium amaranticolor* and *Nicotiana benthamiana*, plants showed yellowing, stunting, local lesion and mosaic symptoms, and when IC-RT-PCR was performed on selected samples, a 970 bp fragment was amplified with the specific primers.

Discussion

This present study has revealed the widespread occurrence of BYMV in *Gladiolus* and faba bean which may cause serious damage to crops in subsequent years. It also suggests that plants negative for visual symptoms should not be taken directly for producing propagating material in bulk as they may carry latent infections, which may show symptoms in the next generation. Therefore, plants selected for producing propagating material by tissue culture should first be screened by ELISA for the presence of BYMV infection since this method can detect the latent infection in *Gladiolus* and faba bean. In addition, we conclude that the ELISA methods developed provide a quick, reliable and sensitive technique.

Use of the polymerase chain reaction for molecular analysis of Potato Leafroll Virus isolates in Tehran, Iran

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Abstract
Potato Leafroll Virus (PLRV) belongs to the genus *Polerovirus* and the family *Luteoviride*, a group of phloem limited, small isometric (23-25 nm diameter) plant viruses. Serological (TAS-ELISA) and molecular (IC-RT-PCR) tests were used to detect PLRV in 221 potato samples collected from different areas of Tehran province. Specific primers were used to detect PLRV in potato plants and PLRV-infected green peach aphids *Myzus persicae* sulzer.

The viral sequence encoding a portion of the coat protein gene (336 nt) was specifically amplified by RT-PCR in infected plants and aphids. One PCR product from plants and another from aphids were sent to MWG, Germany for sequencing to allow molecular characterization of the PLRV isolates in Tehran. Nucleotide and amino acid sequence comparisons of the CP gene of these isolates showed high sequence identity with other PLRV isolate sequences obtained from GenBank. We found that the Iranian isolate is strongly related (98-100%) to all the available strains in the database, supporting the idea that this part of the CP gene is a highly conserved sequence within the PLRV genome.

Introduction

Potato leafroll virus (PLRV) is the type species of the genus *Polerovirus*, family *Luteoviridae*; that infects potato crops worldwide causing widespread damage. Icosahedral particles of the virus contain ssRNA (5882nt) and infect phloem-associated tissues of their plant hosts. The RNA genome contains a VPg and six ORFs (0-5). The capsid protein (CP) encoded by ORF3 is about 23 KDa. The natural host range of PLRV is mainly restricted to a few solanaceous plants including *Physalis floridana*, *Datura stramonium*, and *Lycopersicum esculentum*, in addition to the potato (*Solanum tuberosum*). Similar to all *Luteoviruses*, PLRV can not be transmitted mechanically and infects plants only when delivered into phloem tissues by aphid vectors (in a circulative, persistent manner), grafting, or agroinfection.

Aphid transmission can be divided into four distinct processes:

- virus ingestion from the host plant into the lumen of the aphid's alimentary canal;
- acquisition of the virus through the aphid's gut;
- retention in the tissues and hemocoel;
- transmission through the salivary gland and into the phloem tissue of a host plant.

Material and methods

During the years 2005 & 2006, two hundred and twenty one potato samples with yellowing, leafrolling and stunting symptoms, which were suspected to have PLRV, were collected from the main potato fields in Tehran province. The presence of the virus was confirmed by serological tests (DAS & TAS-ELISA). For detecting the virus in infected plants and

aphids, IC-RT-PCR was performed using a specific primer pair which was designed according to a part of a coat protein gene of the virus and amplified a fragment of 336 bp.

For detecting the virus in aphids, 20-30 green peach aphids (*M. persicae*) were fed on PLRV infected plants for three days and then used in IC-RT-PCR. The amplified fragments, both from plants and aphids, were sent to MWG, Germany and sequenced. By using the computer programs: BLAST, Vector NTI and DNAMAN, these sequences were compared with other PLRV isolates in the GenBank database.

Results and discussion

Serological tests showed that PLRV infection in Tehran potato fields was low (about 10%). The IC-RT-PCR amplification of PLRV cDNA yielded the expected product of 336 bp. This product was detected in viruliferous aphids and in infected potato plants. 283 nucleotides of this product were sequenced by MWG. The percentage homology between the Tehran isolate from PLRV infected aphids and the isolate from infected potato plants was 100%. The sequence of this isolate was aligned with the seven sequences available in GenBank. Analysis confirmed the low variability of PLRV between the Iranian isolate and other isolates, as the nucleotide identity amongst these sequences were (98-100%). These results confirm that, similar to all PLRV isolates, the CP gene of the Tehran isolate is very stable without remarkable variability.

Location and identification of soil-borne viruses of sugar beet in Poland

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Introduction

Beet necrotic yellow vein virus (BNYVV) causes a damaging disease of sugar beet known as rhizomania. The virus is transmitted by zoospores of the soilborne plasmodiophorid *Polymyxa betae* Keskin. The disease was first described in Italy in the early 1950s and now has a world-wide distribution. Rhizomania is characterized by reduced taproot size and extensive proliferation of lateral rootlets that eventually become necrotic. BNYVV infections lead to reduction in root yield of 50% or more, and the sugar content can be badly affected.

BNYVV is a positive-strand RNA virus with rigid rod shaped virions and a multipartite genome. RNAs 1 and 2 encode the essential elements for replication, assembly, movement, and suppression of gene silencing, whereas RNAs 3 and 4 encode proteins more directly involved in pathogenesis and vector transmission. Additionally, some European and Japanese isolates also possess a genomic RNA 5 that encodes a single polypeptide and exacerbates symptom severity in synergy with RNA 3.

In the 1980s, another soil-borne virus, *Beet soil-borne virus* (BSBV), was found in sugar beet. First detected in the United Kingdom, BSBV was then reported in The Netherlands, Belgium, the United States, Sweden, Germany, France and Finland. Lesemann *et al.* (1989) described two serotypes for BSBV, namely, the Ahlum and Wierthe serotypes. Recent data has revealed that serotype Wierthe should be considered a distinct virus species named *Beet virus Q* (BVQ). These two pomoviruses are also transmitted by *Polymyxa betae* Keskin. Although their contribution to rhizomania remains a matter of debate, it is not uncommon to find them associated with rhizomania-infested fields.

Results and discussion

In 2005 and 2006, 70 samples of sugar beet with pale green leaves were collected from different parts of Poland. These sugar beets had dark brown bearded roots. Transverse cutting of the roots exhibited pale yellow-dark brown vascular discoloration.

All samples were analyzed by a double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) with commercial antiserum against BNYVV (BIO-RAD), and then the same samples were tested using a triple antibody sandwich (TAS)-ELISA with commercial antisera against BSBV/BVQ and BSBV (DSMZ). Total RNA extracted from roots of the symptomatic plants was tested in a multiplex reverse transcriptase polymerase chain reaction (mRT-PCR) assay with specific primers designed to amplify a fragment of the RNA 1 for BSBV and RNA 2 for BNYVV and BVQ. Three mRT-PCR products were amplified with the primers specific to BNYVV, BSBV and BVQ and sequenced. The Polish isolate of BSBV had 98% nucleotide and amino acid sequence identity with the German BSBV isolate. Sequence analysis of the Polish isolate of BVQ indicated 97%

nucleotide and 94% amino acid sequence identity with the previously published sequence of BVQ. This was the first report of BSBV and BVQ in Poland.

During the fall of 2005, five plants of a cultivar susceptible to rhizomania (cv. Alyssa) and five resistant to rhizomania (cv. Henrietta) were collected from fields in Western Poland where BSBV, BVQ and BNYVV had been previously identified. Rhizomania was identified only in sugar beet samples of the susceptible variety. However, nine of the sugar beet plants gave positive reactions with antiserum against BSBV/BVQ. Using mRT-PCR and specific primers, the presence of BSBV and BVQ was confirmed in the resistant sugar beets. During the summer of 2006, we identified a lot of samples in which BSBV was associated with BVQ in the North and Central parts of Poland. Out of 70 beet samples analysed, 20 gave positive reactions for BNYVV and 29 gave a positive reaction for BSBV and BVQ. In Poland, BSBV and BVQ were more often identified than BNYVV.

Results obtained worldwide have revealed the existence of three strain groups of BNYVV in Europe. The A type is found mainly in Southern Europe, but also in the North West, especially in the Netherlands, whereas the B type is dominant in Germany and France. The third type named P is present in a small area around the French town of Pithiviers and also in the UK. This type P seems to be more aggressive than the A and the B types.

Up to 2005, we knew about BNYVV type A in Poland. In 2006, mRT-PCR with two sets of primers was used to distinguish A and B types of BNYVV. This showed that type A is still predominant in Poland. Type A was detected in 50 BNYVV-infected samples of sugar beet plants from 18 fields in Poland while type B was found in seven samples from two fields (one in northern and one in southern Poland). On the basis of phylogenetic analyses we showed that Polish isolates of BNYVV are very similar to others deposited in the GenBank database. For the type B isolates, a 178 nt region of the PCR product had 100% nucleotide and amino acid sequence identity with all BNYVV type B sequences. For type A isolates, a 324 nt region of the PCR product had 100% nucleotide and amino acid sequences identity with all BNYVV type A sequences.

The occurrence of three different viruses, transmitted by the same vector, within a single sugar beet raises questions regarding the epidemiology of rhizomania syndrome. Now, we would like to compare different varieties of sugar beet resistant to rhizomania for their susceptibility to BSBV and BVQ, using a highly sensitive detection method - Real time PCR.

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Characterization of a tymoviruses causing disease in diascia ornamental plants

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Most of the diascia and nemesia ornamental plants purchased from nurseries around the Washington State and Oregon showed symptoms of vein yellowing and mottling on infected leaves. Infected plants also displayed reduced vigour. *Diascia* x *hybrida* 'Sun chimes coral' developed virus-like symptoms of mild mottling and yellowing. RNA was extracted from infected tissues following the Dunn & Hitchborn (1965) procedure, and the entire genome was cloned and sequenced.

A sequence consistent with a member of the genus *Tymovirus* was obtained. The genome consists of a positive sense, single stranded RNA molecule and is 6,290 nucleotides (nt) long. Three potential reading frames (ORF) were identified. The 5'-untranslated region (UTR) is 154 nucleotides long and an RNA that can fold into four hairpin structures was predicted in this region of the genome. The putative products of ORF I, II ad III are 637, 1,790 and 192 amino acids, respectively. ORF I overlaps the 5'-terminus of ORF II and its predicted product is similar to the movement protein of tymoviruses. The putative product of ORF II is a viral replicase. The smallest ORF, ORF III, encodes the putative coat protein. Secondary structure analysis of the 3'-terminus showed that the RNA can form a transfer-like RNA structure that has an anticodon specific for histidine. The 3'-terminus and the 5'-terminus can potentially form secondary structures similar to those formed by *Nemesia ring necrosis virus* isolated from *Nemesia fruticans* (NeRNV-Nf) (Koenig *et al.*, 2005a).

When the entire genome was considered, it showed 77.8% nucleotide sequence identity to NeRNV-Nf isolate sequenced by Koenig *et al.* (2005b). Sequence alignments from cloned reverse transcription-polymerase chain reaction (RT-PCR) products using primers for the coat protein region revealed the presence of a mixture of two tymovirus-like sequences. The two isolates were denoted as Nemesia Ring Necrosis Virus – Washington State strain (NeRNV-WA) and Diascia Yellow Mottle Virus (DiaYMV). The product of ORF II from DiaYMV shares 54.0% amino acid sequence identity with ORF II of NeRNV-Nf. Analysis of the predicted coat protein amino acid sequence of NeRNV-WA showed 99.5% sequence identity to NeRNV-Nf while DiaYMV showed 94.5% sequence identity to NeRNV-Nf. The name DiaYMV is therefore proposed for this isolate based on the sequences differences of the ORF II. The movement protein is not being considered as an option for species demarcation in tymoviruses, but its contribution may be accessed differently in the future as more complete viral sequences of plant viruses become available. Experimental and supportive work aimed at differentiating DiaYMV and NeRNV-WA is ongoing.

Enzyme linked immunosorbent assay (ELISA) was used to test plants with the commercial *Nemesia ring necrosis virus* (NeRNV) antiserum and the monoclonal antibody produced against the DiaYMV. Natural infections with NeRNV and DiaYMV were confirmed in six accessions of *Nemesia* x *hybrida* and seven accessions of *Diascia* x *hybrida*. The only diascia plant that was not naturally infected with either tymovirus was *Diascia* x *hybrida*

'Coral belle'. Susceptible hosts to DiaYMV include *Nicotiana benthamiana*, *Nicotiana clevelandii* and *Nicotiana occidentalis* and several species and cultivars in the genus *Nemesia* and *Diascia*. Systemic infection was not detected in mechanically inoculated *Nicotiana alata* 'Nicki red', *Nicotiana tabacum* 'Bright yellow', *Chenopodium quinoa*, *Vicia faba* L. 'Broad Windsor' and *Datura stramonium*. Failure to detect *Scrophularia mottle virus* (ScrMV) with the monoclonal antibody was supporting evidence that DiaYMV is not a strain of ScrMV. This study also confirmed the occurrence on NeRNV in Northern America as reported by Mathews & Dodds (2006).

Criteria for species demarcation in the genus *Tymovirus* show that DiaYMV is a variant (strain) of NeRNV based on ORF III (CP) amino acid identity (> 90%); and the 3'-UTR is more closely related to NeRNV-Nf than other viruses in the OYMV cluster. Other factors used in species demarcation of tymoviruses include the host range and serological relatedness of the viruses. In the host range studies, it has been shown that DiaYMV and NeRNV share similar hosts and the range of symptoms are variable within hosts; hence this factor cannot be used as a reliable indicator. A wider host range still needs to be explored. The DiaYMV monoclonal antibody produced in the study however did not differentiate between the NeRNV and DiaYMV suggesting the two viruses could be serologically related, a feature that has been observed with viruses in the *Ononis yellow mosaic virus* (OYMV) cluster (Koenig *et al.*, 2005b). Tymoviruses have been divided into four serological groups, and members within a group very often cannot be differentiated into species based on serology although amino acid sequences of the CP regions suggest that they are distinct species. Molecular criteria favour DiaYMV and NeRNV as same species, while other data could go both ways and yet support DiaYMV as a distinct species.

In conclusion, diascia and nemesia in nurseries in the Pacific Northwest are frequently infected with tymoviruses. A newly described virus, DiaYMV, and a strain of NeRNV were identified. The two viruses often occur as mixed infections. Because of the similarities in their coat protein sequences, the molecular and serological assays developed in this study do not permit conclusions on the relative frequencies of the two viruses. These hosts degrade the quality of ornamental hosts, but they pose little threat to agronomic crops since tymoviruses typically have a limited host range. Until further studies are completed, it should be considered that wild members of the family *Scrophulariaceae* could be impacted by the introduction and widespread distribution of these viruses.

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