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Containment of GM plant viruses being developed as gene technology vectors

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Containment of GM plant viruses being developed as gene technology vectors

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This project was carried out with a view to providing recommendations for good practice to ensure that genetically modified (GM) plant viruses engineered to produce novel proteins, such as vaccines, in plants remain contained within authorised research facilities and do not pose a risk of causing harm through accidental release (escape) into the wider environment. A literature review and experimental work were combined to identify the most commonly used plant virus vectors, the most likely causes of accidental virus dissemination and thus the real risks associated with the use of these viruses. Several plant viruses were identified as being regularly used as vectors for protein expression and gene silencing studies. Tobacco mosaic virus (TMV), Potato virus X (PVX) and Cowpea mosaic virus (CPMV) were selected for use in further experimental studies investigating risks of 'accidental' mechanical transmission for TMV and PVX as well as defining host and vector ranges for CPMV. The outputs of this report are recommendations for guidance on 'good practice' to be adopted in plant growth facilities to ensure appropriate levels of containment are achieved for the different virus systems involved.

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EXECUTIVE SUMMARY

The use of plant virus-vectors has proliferated over the last decade since they offer a number of advantages over the traditional transformation of plants, for the expression of foreign proteins. However, there are hazards associated with the use of virus vectors. For example, if a plant virus such as *Potato virus X* (PVX), modified to express a human toxin, infects an agricultural crop destined for human consumption. As the viruses are being used as tools, experiments are frequently being carried out by researchers who have a poor knowledge of basic virus biology. Therefore adequate containment procedures reducing the risks from using such virus vectors may not be identified and employed.

Any activity involving genetically modified (GM) plant pathogens is controlled in the UK by the Genetically Modified Organisms (Contained Use) Regulations 2000 (CU2000 Regs.) and the Plant Health (Great Britain) Order 1993 (PHO). Amongst other things, these require that a suitable and sufficient assessment be made of the potential risks posed by the work to both human health and the environment before the activity is started.

The main aim of this project was to complete a comprehensive literature review covering both the uses of virus vectors (protein expression and gene silencing) combined with the appropriate scientific experiments in order to provide a suitable output to underpin future regulation for the HSE. Identification of the risks should then allow selection of the appropriate containment measures.

A review of the literature revealed that the viruses most commonly used as vectors in the UK are PVX, *Tobacco rattle virus* (TRV), *Tobacco mosaic virus* (TMV) and *Cowpea mosaic virus* (CPMV). The different properties of the viruses will have different implications when considering the best containment strategy for each system. A review of the HSE guidance on the use of GM plant pathogens in containment highlighted the lack of specific guidance for mechanically transmissible viruses.

Experiments were undertaken to assess the extent of transmission of PVX and TMV during procedures such as watering and sampling. Wild type viruses were used to represent a worst-case scenario (as generally GM viruses are assumed to be less fit). The results demonstrated that TMV is more easily spread than PVX. In addition the results showed that both viruses could be spread through careless watering of plants and during sampling procedures. Thus one of the most likely means of accidental virus dissemination is via human error.

CPMV is an insect transmitted virus and is not indigenous to the UK. Experiments were undertaken to determine whether a range of commonly used greenhouse plants were hosts for CPMV and to assess whether insects commonly found in UK glasshouses could transmit CPMV from infected cowpea plants. The aphid species *Myzus persicae* and *Nasonovia ribisnigri*, the thrips species *Frankliniela occidentalis* and the biocontrol agent *Macrolophus caliginosus* were used in virus transmission experiments. Very few of the plants tested were found to act as a host for CPMV and none of the insects tested transmitted CPMV.

Thus the current guidance for working with genetically modified CPMV under contained use conditions was thought to be adequate. However, the production of additional guidance specific for activities with mechanically transmissible GM viruses is recommended. In particular this should focus on training staff working with such viruses and in addition minimising the number of people who have access to plants infected with mechanically transmissible GM viruses.

1 INTRODUCTION

1.1 BACKGROUND

As agents of disease, viruses have been the subjects of intense research. Viruses have been defined as intracellular parasites whose genetic material (consisting of either DNA or RNA), is packaged in a protective coat and transmitted from one host cell to another. They are relatively simple biological entities that are dependent on the biochemical pathways of their host cells. During the 1980's it was demonstrated that foreign genes could be inserted into a plant virus (CaMV; Brisson *et al*., 1984) and transiently expressed in the host cells. Therefore a great deal of research has been carried out on plant viruses not only because of their capacity to cause disease but also due to their potential use for foreign gene expression. More recently other properties of plant viruses have been identified which make them useful tools for molecular genetic analyses.

Thus, the use of plant viruses as vectors has become standard practice in many labs (Porta & Lomonossoff, 2002). In addition, genetically modified viruses have also been used for field scale trials (Turpen, 1999). Many of the researchers now handling GM viruses are not virologists and thus may not immediately appreciate the hazards associated with the natural spread of a plant virus. Furthermore, while some properties of viruses have been studied extensively, in many instances there is little data to support a full and comprehensive assessment of the novel properties which may arise from genetic modification of a virus.

The main hazard associated with the use of modified plant viruses is the possibility of virus strains, (with novel pathogenic properties or which are expressing a novel protein) escaping into the environment and, for example, propagating in a commercial crop. Even a limited spread of a GM pathogen from the lab into the environment would have major implications for the perception of the technology and in addition there would also be a profound effect on the agricultural community as they try to market produce perceived as being contaminated by a GM virus. In addition depending on the protein to be expressed there may also be effects on human health. For instance if a GM plant virus was being used to express proteins from the AIDS virus, accidental release of this virus into the environment could raise concerns over human health. It is also possible that an accidentally released GM virus could recombine with an indigenous virus thus resulting in further spread of viruses with novel properties.

The aim of this review is to identify the range of plant viruses currently being genetically modified and used for contained use research experiments in Britain. The characteristics of these viruses, including their most likely routes of dissemination will be discussed. This will allow the risks from such work to be identified. In addition this will highlight where further information should be collected to allow better risk assessment and selection of more appropriate containment measures.

2 RISK ASSESSMENT OF GENETICALLY MODIFIED PLANT VIRUSES

Within Britain, any activity involving the genetic modification of plant pathogens is controlled by the Genetically Modified Organisms (Contained Use) Regulations 2000 (CU2000 Regs.) and the Plant Health (Great Britain) Order 1993 (PHO). The aim of these regulations is to reduce the risks to human health, the environment and plant health. Under these regulations a risk assessment must be carried out which takes into consideration human health, environmental safety and plant health.

Most modifications of plant viruses are not likely to have an effect on human health and in fact people regularly consume fruit and vegetables which have been infected with unmodified viruses. Therefore risk assessment of projects involving genetic modification of plant viruses focuses mostly on the likely effects to the environment. However, the risks from the genetic modification will also depend on what is being inserted into the virus, for instance a project where a gene encoding a human toxin is inserted into PVX will have a higher risk to human health than when a gene encoding GFP is cloned into the same virus. Thus the assessment must not only consider the risks from the organism to be modified (for instance the transmission properties of a virus) but also the potentially harmful effects of the modification. It should be noted that when proteins prepared from plant material infected with viruses or viruses displaying peptides are used for injections into animal or human bloodstreams there could be an unexpected effect as the virus is being presented to cells and tissues in a form not previously encountered.

Following identification of the potential risks, suitable containment measures should then be selected to reduce and minimize the risks. Therefore, to carry out a sufficiently detailed risk assessment of the viruses to be genetically modified, comprehensive knowledge of the virus properties and logically reasoned arguments about the potential novel effects on plants are essential. Factors that must be considered as part of the risk assessment include; properties of the host virus to be modified, properties of the DNA sequence to be introduced into the virus, any potential hazards arising from modification of the virus itself together with the likely effect on the environment if the GM virus was accidentally released. Each of these risks will be explored in more detail to highlight the types of consideration that must be made for each risk assessment. As there are several different types of viruses with a wide range of properties it is not possible to carry out a generic risk assessment of genetically modified viruses and therefore for each new project/application a risk assessment on a case by case basis must be made. The risks to human health, the environment and plant health must be considered.

2.1 PROPERTIES TO BE ASSESSED

2.1.1 Properties of the parental virus

The properties of the host virus should be considered including whether the virus is indigenous to the UK and the means by which it is transmitted. In risk assessments for non-indigenous viruses it is frequently suggested that such viruses will not persist and spread if accidentally released. In some instances this may be true as there may not be a susceptible host plant species present. However, a non-indigenous virus may have the potential to spread if there are means by which it can be vectored, for instance if an invertebrate which can transmit the virus is found in the surrounding environment.

2.1.2 Properties of the inserted region

The properties of the sequence to be inserted should also be assessed including the length and the function of any protein encoded by the region to be inserted. Viruses tend to have compact genomes which do not tolerate large insertions and in risk assessments the point is often made that viruses with additional sequences will quite rapidly lose the insertion over a short period of time. However, few studies have been carried out to determine how the length of the additional sequence relates to the time taken for the insert to be lost, whether this is the same for all viruses or if some are more tolerant than others and if the actual sequence affects the time taken for it to be lost. Furthermore in order for an inserted sequence to encode a protein of interest a relatively large region (> 800 bp) of nucleic acid is used. In contrast for other applications of plant viral vector research, for instance virus induced gene silencing (VIGS), only short regions of nucleic acid will be used (< 300 bp) and therefore it may take a longer period of time before the insert is lost.

2.1.3 Properties of the recombinant virus

Identifying any potential hazards arising from the modification is difficult but is probably best illustrated by using specific examples; for instance a virus directing over-expression of a plant derived resistance gene so that a previously resistant plant species becomes susceptible to virus attack clearly has a higher risk, if this virus was accidentally released, than a virus engineered to express Green Fluorescent Protein (GFP) which allows the movement of the virus around the plant to be tracked. Similarly a virus vector which induces silencing of a plant encoded defence gene is also likely to be of higher risk than silencing genes involved in chromatin formation. For some proposed research it is difficult to accurately predict all the properties of a modified virus and therefore in these cases more containment measures may be required to ensure that a modified virus which is of higher risk than the parental one (for example more pathogenic) is safely contained.

2.1.4 Scale of the experiments to performed

The scale of the experiments to be done will also affect the risk. For example, mechanically inoculating less than twenty plants with a GM virus which is normally transmitted by nematodes but has had the sequences required for nematode transmission removed, will be of relatively low risk. However, in comparison a gene screening project which could require infection with several hundred GM virus isolates, using a highly mechanically transmissible virus would be of much higher risk.

2.2 RISK ASSESSMENT WITH QUANTITATIVE SUPPORTING DATA

While risk assessment in part requires expert judgment, it is also dependent on previously gathered scientific data. For instance, in order to be able to assess in the case of an accidental release whether a virus could infect plants in the surrounding environment, knowledge about the range of plants which is susceptible to that virus is required. It is well established that many plant viruses can infect more than one host species and therefore a virus which has been isolated from tomatoes (*Lycopersicon esculentum*) may not be restricted to only infecting other *Lycopersicon* species.

The necessity for detailed risk assessment of genetically modified organisms is a relatively new legal requirement. That is not to say that researchers planning and designing experiments did not take risk into consideration but until relatively recently it was not a legal requirement to carry out an in depth consideration of all possible risks. There are many different ways in which risk assessment can be carried out and various methods of risk assessment are being explored and developed. To date risk assessments of genetically modified plant viruses have mostly been carried out in a qualitative manner but it has become clear that quantitative

supporting data could allow more accurate assessments of risk. In addition this should be less subjective and thus lead to more consistent risk assessments being produced. However, often there is no data or insufficient data to allow the levels of uncertainty to be calculated. Lack of suitable supporting data is one of the major hindrances for detailed risk assessment. Furthermore as 'positive results' are generally required for publication of researchers findings, it may be difficult to find evidence of 'negative data' which will be important for accurate risk assessment. For instance in terms of risk assessment, the finding that a specific virus is not transmitted by a particular aphid species will be as informative as results from a study showing the specific aphid species that the virus is vectored by. In some cases it may be difficult to discern whether there is any information available about a specific issue or if studies have been carried out addressing a specific question from which the results were negative. In addition in order for 'negative data' to be statistically significant, a greater number of data sets will be required. Finally arguments can be made both for and against whether the responsibility for providing data for risk assessments should fall upon the researcher carrying out the experiments or the regulatory authorities.

Following identification of the risks, suitable containment measures must be selected to reduce the level of risk. It is essential that the regulatory authorities are in a position to accurately review the suitability and sufficiency of the risk assessments produced by the research centres, particularly as to whether the hazards have been accurately assessed and that the most appropriate containment measures have been applied. One of the aims of this review is to highlight where possible, more suitable containment measures could be used for GM plant virus vector work. Therefore, a brief overview of the containment measures currently used for research with GM plant virus vectors will be given.

3 CONTAINMENT STRATEGIES

Containment strategies can be subdivided into three areas which can be defined as physical containment measures including doors, filters and waste disposal, biological containment which covers the actual virus vector and host organism to be used and chemical containment which includes use of disinfectant(s) to inactivate any spilled material or treatment of a glasshouse compartment at the end of an experiment.

3.1 PHYSICAL CONTAINMENT

The physical containment measures that are required will depend on the properties of the virus vector and thus the risks identified in the risk assessment. These measures relate to the facilities and procedures used to carry out the research.

3.1.1 Access and security

In contained use research facilities for plants infected with GM plant viruses, it is important to limit the number of people with access to these facilities to as few as possible. One of the most likely means of accidental release of a GM plant virus vector is by transfer through people. Therefore reducing the number of people who have access to the material infected by GM viruses should reduce the risk of accidental release. Security is also an important consideration for reducing the opportunities of individuals who may wish to cause harm using GM viruses. Doors to growth room facilities are often locked or have some system by which access can be limited to specific researchers. In addition a 'double door' system should also be in place where it is not possible to open the final exit door until the inner door leading to the facilities is closed. Frequently exit through the double door system will involve a change of protective clothing so that if any GM material has become associated with the researchers protective clothing, it will remain within the building and be inactivated by washing or autoclaving.

3.1.2 Facilities

In terms of risk reduction, a contained use research facility to be used for plants infected by GM viruses or other plant pests should consist of a large building or greenhouse subdivided into a number of smaller, individually controlled compartments. In this way it would be possible to carry out experiments under a number of different conditions with smaller numbers of plants used by a few researchers. However, this is clearly more economically expensive than having a few larger compartments used by several researchers.

3.1.3 Filters

Regardless of the size of the compartment, it is not possible to maintain a specific temperature within a compartment without a means of temperature control and this is most frequently done by air input or output. However this necessitates the use of vents which is also a means by which viruses could escape or leave the compartment. Therefore, the input and output vents of the compartments should be filtered. In addition this should also prevent the entry of any other organisms including insects which could spread the GM virus. In contrast to human and animal viruses there are no known plant viruses which can be transmitted aerially. Therefore, the use of HEPA filters is not required in facilities used for growing plants incubated with GM plant viruses.

3.1.4 Drainage

Growing plants infected with GM viruses will require watering and any excess water could drain out of the compartment carrying GM virus infected plant material. The spread of virus via contaminated run-off water can be prevented by a number of different means. The drains in the compartment can be closed and the plants given minimal amounts of water so that any excess is

lost by evaporation. Alternatively the contaminated run-off water can be filtered, to remove large pieces of plant material and the water then treated either by heating or with chemicals to inactivate any GM viruses present.

3.1.5 Protective clothing

Suitable protective clothing should be used and in most instances gloves and lab coats dedicated to use within the contained use facilities should be in place. In some cases, for example when large volumes of Agrobacteria containing GM viruses are being used to inoculate plants, there may be a risk of spills and liquid containing active GM virus being transferred out of the facility by foot. For this purpose the use of overshoes is recommended or a footbath immediately outside the compartment containing a suitable disinfectant.

3.1.6 Control of disease vectors

As stated before many plant viruses are transmitted by insects. Therefore to prevent spread of GM viruses, any organism likely to vector the viruses should be controlled. As described above the ingress and egress of insects can be prevented by filters on the vents. Insect spread can also be controlled by the use of sticky traps, insect traps or insectocutors while spread of viruses by nematodes can be prevented by use of soil which has been sterilized. In some instances studies of plant viruses may investigate the transmission of viruses by invertebrate vectors. For such experiments dedicated facilities should be used so that all the potential invertebrate vectors are controlled.

3.1.7 Systems of work

As described above plant viruses can be transmitted by other organisms. Some viruses can also be transmitted through pollen or seeds. Therefore, for experiments with such viruses the flowering of infected plants and collection of seeds must be carefully controlled so that any material which could potentially carry GM virus is inactivated or securely contained. Any other plant material which could disseminate the GM virus should also be controlled.

3.1.8 Transfer of material

It may be necessary to transfer plant material infected with GM virus vector from the plant growth laboratories back to the laboratory for further analysis. Procedures should be drawn up to ensure that GM virus is not disseminated during transfer and can be arranged relatively simply for example, by placing infected plants into a plastic bag inside a box.

3.1.9 Waste disposal

Once the experiment has finished, all the infected material and any associated material, for instance uninfected plants which have been grown in the same compartment as plants infected with GM viruses, should be disposed of by validated means. Generally waste is inactivated by autoclaving and sent to land fill, incinerated or used for composting. Disposal of material infected with GM plant viruses via composting alone may not lead to complete inactivation of the viruses and therefore the risk would not be reduced.

3.1.10 Storage of GM viruses and plant material infected with GM viruses

GM plant viruses and infected plant material must be stored in a safe manner so that the GM plant virus is not accidentally disseminated.

3.1.11 Staff training

Staff training is essential particularly when plant virus vectors which are highly mechanically transmissible are being used. The risks from GM plant virus vectors can be effectively managed by training staff to have good working practices. Ensuring that staff have read and understood standing operating procedures as well as providing practical training are important for risk management.

3.2 BIOLOGICAL CONTAINMENT

The biological containment measures refer to the properties of the virus and modifications that can be made to reduce specific risks.

3.2.1 Vector transmission sequences

One of the most well characterized biological containment measures for plant virus vectors is removal of the sequences from the virus which lead to vector transmission. The presence of a conserved tripeptide, DAG in the coat protein of many different aphid transmissible potyviruses prompted the suggestion that this sequence is required for aphid transmissibility. In addition, Atreya *et al.* 1995 carried out a comprehensive study where the effect on aphid transmissibility of making amino acid substitutions within the tripeptide sequence, was determined. From these experiments predictions were made about how critical each residue of the tripeptide was. However, results from later studies indicated that the context of the tripeptide was also important and that the sequence alone did not confer aphid transmissibility (Lopez-Moya *et al.* 1999). Therefore caution should be exercised if mutation of this sequence is going to be used as biological containment method.

Similar studies have been carried out for viruses which are nematode transmissible of which TRV is one of the most well characterized. For TRV, it was frequently observed that laboratory strains of TRV were no longer nematode transmissible and in addition that serially passaging TRV by mechanical inoculation resulted in deletion of genes from RNA 2 and loss of nematode transmissibility (Hernández *et al.,* 1996). Further studies demonstrated that deletions or mutations in the non-structural 29.4K gene abolished nematode transmission but did not interfere with encapsidation or co-replication of RNA2 with RNA 1 (Hernández *et al.,* 1997). Therefore the risk of nematode transmission by modified TRV vectors can be removed by deleting this sequence.

As described previously the first experiments employing viruses as vectors used a gene replacement strategy whereby viral genes were replaced with the gene of interest. A modified version of TMV was produced where the coat protein gene was replaced by a gene encoding chloramphenicol acetyl transferase (CAT). This virus was able to move locally (cell-to-cell) but was not capable of inducing a systemic infection (Dawson *et al.* 1988, 1989). The coat protein was therefore thought to be important for long-distance movement. Although a virus vector which is not able to move systemically would not be suitable for some applications, such a vector could be used for expressing a protein of interest. Use of such a vector with a non-functional coat and or movement gene would be a form of biological containment as this virus would have limited spread if it was accidentally disseminated.

3.2.2 Preventing virus spread

Clearly the viruses being used as vectors can be modified or disabled to prevent them from spreading. However there are also other simple measures that can be used to ensure that in the unlikely event of accidental virus dissemination, the virus can not replicate and spread. These include the removal of any plants in the vicinity of the controlled environment room or glasshouse that could act as hosts for the virus or carrying out experiments at times of the year when host plants are not grown.

3.3 CHEMICAL CONTAINMENT

Chemical containment refers to the use of chemical barriers to prevent the accidental release of viruses to the environment and to avert harm to human health. Under the GMO (CU) 2000 Regs. specified disinfection procedures must be in place for all classes of work apart from low to negligible risk activities. These include selection of suitable disinfectants for routine disinfection and also for immediate use in the event of spillage. With respect to plant viruses, chemical containment measures used to reduce the risk of accidental dissemination include

footbaths in greenhouse compartments where GM plant viruses are being used and treatment of liquid waste prior to autoclaving.

3.3.1 Specified disinfection procedures

Appropriate disinfectants should be selected and procedures drawn up to inactivate any virus which is accidentally spread through spillage. Several disinfectants are available but not all are effective against plant viruses and in addition they may have other properties which could interfere with their effectiveness as a disinfectant, for example by causing metal oxidation and thus preventing virus inactivation on parts of a containment greenhouse. Although not done as part of this project, a parallel study has been carried out to identify important factors for selecting disinfectants to treat facilities where plant viruses have been used.

3.4 LIKELY METHODS OF SPREAD

By addressing the most likely methods of GM virus spread, advice can be targeted to areas of greater concern. If a risk assessment is made where incorrect containment measures have been selected, this could lead to the spread and dissemination of GM virus. However, as risk assessments are reviewed both locally by the Genetic Modification Safety Committee and also nationally by the Competent Authority, this should not be so likely to occur. The selection of incorrect containment measures could also be caused if there was an unanticipated means of spread. For instance, where greenhouse compartments are being shared by multiple researchers with several different plant species, a virus could be spread if it has a greater host range than previously known. Both these means of spread are at least in part, caused by human error.

Human error is one of the most likely causes of accidental dissemination of a GM plant virus vector, in particular for viruses that are highly mechanically transmissible. If researchers or workers taking care of virus infected plants are not aware that the virus can be easily spread simply by brushing between infected plants or touching infected plants when watering, GM virus can be accidentally disseminated. Therefore, training of all workers involved in working with GM virus infected plants is essential.

4 IDENTIFICATION OF PLANT VIRUSES COMMONLY USED FOR CONTAINED USE RESEARCH IN BRITAIN

One of the aims of this review was to highlight where there are gaps in terms of knowledge and therefore identify areas which require further research. Insufficient or misleading information can give rise to a poor or incorrect assessment of risk which as described above could lead to accidental dissemination of a GM virus. Providing more detailed information to fill the gaps in terms of knowledge will allow more accurate risk assessment and recommendations of specific containment measures to reduce the risk of accidental dissemination further. In order to do this, the four plant virus vectors which are most commonly used for contained use research experiments in Britain were determined and the information available for each virus highlighted. This established areas which required further research and the types of experiments to be carried out.

The four plant virus vectors which are most commonly used for contained use research experiments in the UK are PVX, TMV, CPMV and TRV. All of these are present in the UK with the exception of CPMV. PVX and TRV are used in several different research groups both for the expression of heterologous proteins and also for VIGS. TMV is used to direct expression of foreign proteins while CPMV is modified to direct the expression of peptides. A more detailed description of each of the four selected viruses is given and gaps in our knowledge highlighted.

4.1 *COWPEA MOSAIC COMOVIRUS* (SOCPHIR/EPPO: NOT PRESENT IN UK)

CPMV is a single stranded RNA virus which is transmitted by an insect vector. Virus particles are isometric (Figure 1), non-enveloped and are 20-24nm in diameter. Virions are found in the mesophyll of the host plant in the cytoplasm of infected cells. The genome is bipartite, each part of which encodes a polyprotein that is cleaved to produce functional proteins. Both replication of the genome and translation take place in the cytoplasm of the host cell. The proteins of RNA1 (B) are required for replication and the proteins of RNA2 (M) are structural proteins. It has been demonstrated that B-RNA inoculated to leaves cannot move to the surrounding cells in the absence of M-RNA (Rezelman *et al.*, 1982). The thermal inactivation point (lowest temperature at which the infectivity of sap is destroyed in 10 min) of CPMV is 55-65°C and longevity of the virus *in vitro* is 4-10 days.

CPMV was first reported in *Vigna unguiculata* from Nigeria (Chant, 1959). Spread of the disease has been recorded in Kenya, Nigeria, Togo, Mali, Republic of Benin, the Phillippines and Tanzania but CPMV is not present in the Americas. Under natural conditions symptoms include mosaic, vein yellowing and leaf and flower malformation. Symptoms can cause yield reductions as high as 95%.

CPMV has relatively few known hosts, of which most are legumes (*Vigna* spp). Hosts from other families include *Antirrhinum majus* (snapdragon) from the Scrophulariaceae, *Nicotiana clevelandii* and *N. tabacum* from the Solanaceae and two species from the Chenopodiaceae, *Chenopodium amaranticolor* and *C. quinoa* (Brunt *et al.* 1996). The latter two types of plant are host plants which are frequently used to test virus infection. Results from more recent experiments have demonstrated that 5 members of the Gramineae family are insusceptible to CPMV (Porta *et al.* 2003).

The virus is vectored by insect species including *Ootheca mutabilis, Paraluperodes quaternus* and *Nematocerus acerbus* (Whitney and Gilmer, 1974). *Cerotoma trifurcata, Diabrotica*

balteata, *D. undecimpunctata howardi*, *D. virgifera* and *Acalymma vittatum* have also been shown to act as vectors (Jansen and Staples, 1971). Reportedly, two thrips and two grasshopper species can also transmit CPMV (Whitney and Gilmer, 1974). In addition the virus can be transmitted by mechanical inoculation, grafting and at a low level via seeds.

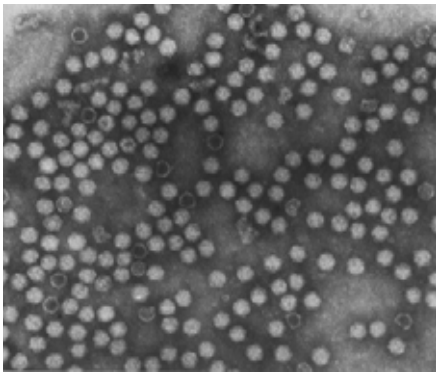

Figure 1 Electron micrograph of CPMV (Copyright 1994 Rothamsted Experimental Station)

Useful plant protection measures include the elimination of perennial hosts that serve as reservoir hosts and procurement of healthy, virus-free seed for production. Cowpea lines resistant to CPMV are recommended for high-risk areas.

The virus is not currently present in the UK (except under licence). Two of the key features for determining the risks from accidental dissemination of CPMV are (1) identifying if there are any potential hosts for the virus and (2) determining if there are any means by which the virus could be spread. If there are no means by which the virus can be transmitted there will be little risk from accidental dissemination of CPMV because the virus will not be able to spread even if there are host plants present which can support infection.

Cowpeas are not grown in the UK but there are several legume crops which have been shown to be susceptible to CPMV and these include *Phaseolus vulgaris*, *Pisium sativum* and *Vicia faba*. Although there have been a number of studies on the insect species that transmit CPMV, little work has been done to establish which insects do not transmit CPMV. An argument frequently made in risk assessments is that as the insect vectors which transmit the virus are not present in the UK, there is little or no risk of the virus being spread. However, unless evidence can be presented to demonstrate that the most common insect species indigenous to the UK cannot vector CPMV, it is not possible to be certain that the risk of the virus spreading, if it was accidentally disseminated, is low.

There are a number of potential vectors which are commonly found on *P. vulgaris*, *P. sativum* and *V. faba* and these include *Bruchus rufimanus*, *Aphis fabae* and *Frankiniella occidentalis*. Results from a study published in 2003 indicated that CPMV is not transmitted by the bean aphid, *Aphis fabae* (Porta *et al.* 2003). However, only a few of the experimental details were supplied for the transmission studies and it is not clear how many repetitions were made. This highlights an issue frequently encountered when selecting data for risk assessments. To have confidence, in statistical terms, about a data set, a certain number of repetitions must have been performed, particularly if the results are negative. Therefore, for CPMV experiments should be designed and performed to determine whether any of these insects can transmit CPMV to the host plants listed above within precise statistical confidence levels.

4.2 *TOBACCO RATTLE TOBRAVIRUS* (SOCPHIR/EPPO: PRESENT IN THE UK)

TRV is a single stranded RNA virus which is transmitted by a nematode vector. Virus particles are rod shaped (Figure 2) and comprise of long (180-197nm) and short rods (46-114nm). Both are required for the production of intact virus particles. Virions are found in all parts of the host plant in the cytoplasm of infected cells. The genome is bipartite with RNA1 encoding putative replicase and cell-to-cell movement proteins. The shorter RNA2 encodes the coat protein and an additional protein is required for nematode transmission. The thermal inactivation point of TRV is 80-85°C and longevity of the virus *in vitro* is 40-50 days.

TRV was first reported in *Nicotiana tabacum* from Germany (Böning, 1931). The disease has been found in Europe, North America, Japan and New Zealand. Under natural conditions, symptoms vary from necrotic local lesions, ringspots to mottling. Symptoms will persist with some strains; alternatively they will vary seasonally or disappear soon after infection.

The virus has a wide host range and can infect plants from several different families. In addition to tobacco, other economically important crops that are susceptible to TRV include *Beta vulgaris, Brassica oleracea* and *Solanum tuberosum.*

The virus is vectored by nematodes including *Paratrichodorus* spp. and Trichodoridae with considerable specificity between the strain of virus and vector species (Ploeg *et al.*, 1992a, 1992b). TRV can also be transmitted by mechanical inoculation, grafting and via seeds to various degrees.

General recommendations for control of the disease include weed control, soil treatment with nematicides and introduction of resistant plants into the crop rotation, which contributes to soil sanitation.

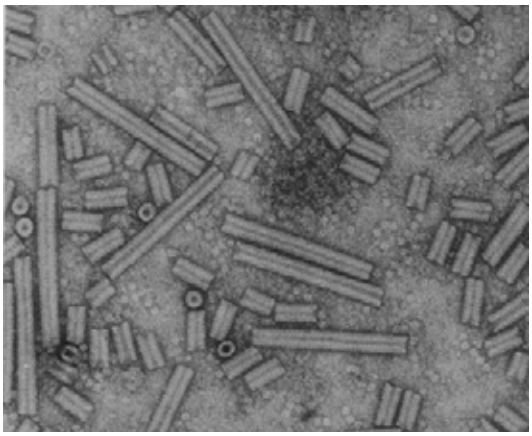

Figure 2 Electron micrograph of TRV (Copyright 1994 Rothamsted Experimental Station)

In considering the factors most likely to affect the spread of TRV, if the virus was accidentally disseminated it should be noted that TRV has a wide host range and is most commonly transmitted by nematodes. However as described in an earlier section, the regions of virus determining nematode transmission have been well studied and can be removed from TRV without affecting the properties of the virus for experimental purposes. TRV can also be transmitted by seeds although the frequency of transmission varies from species to species. Further experiments could be performed to determine the frequency at which TRV is transmitted by seeds in specific plant species. However, in experimental terms this is relatively easy to control and experiments can either be completed before the virus infected plants flower or flowers can be bagged to contain any seed produced. The seed can then be inactivated prior

to disposal. Therefore, the risk of potential TRV dissemination can be reduced by well defined experimental procedures and currently no further information is required.

4.2 *POTATO POTEXVIRUS X* (SOCPHIR/EPPO: PRESENT IN THE UK)

PVX was first reported in *Solanum tuberosum* in the UK in 1931 (Smith, 1931). Virions are filamentous, not enveloped, and usually flexuous (Figure 3) with a clear modal length of 515nm and 13nm wide. The genome consists of single stranded RNA (size 6.43Kb). Virions are generally found in the cytoplasm of mesophyll and epidermal cells. The thermal inactivation point is 68-76°C and longevity of the virus *in vitro* is 40-60 days. Symptoms range from necrotic streaks (in potato) to mild mosaic mottling, leaf distortion and plant stunting. PVX causes a mild disease and reduces yields from 10-20%, although mixed infections with PVA and PVY are particularly damaging.

PVX is probably distributed worldwide but is especially important as a pathogen of major solanaceous crops (notably potato, tomato, tobacco and peppers) although in some locations it also occurs occasionally in artichoke (*Scolymus* sp.), turnip *(Brassica rapa*), red clover (*Trifolium pratense*), grapevine (*Vitis vinifera*) and other crop species as well as a range of ornamental and weed species.

PVX has very wide natural and experimental host ranges. It is reported to occur naturally in at least 62 plant species of 27 families and to be transmissible experimentally to another 348 species in 33 families (Edwardson and Christie, 1997).

Transmission of the virus is mechanical e.g. by contact. It is not transmitted via seed or pollen. The virus retains infectivity when deproteinised with proteases, phenol or detergent.

Control of the disease can be achieved by using virus-free stock and ensuring that the soil to be cultivated is free of residual tuber parts. It is also recommended that farm machinery is sterilised using chemicals such as formaldehyde, pyrolidone or slaked lime. Cultivars which are resistant to the virus are also effective in controlling virus spread.

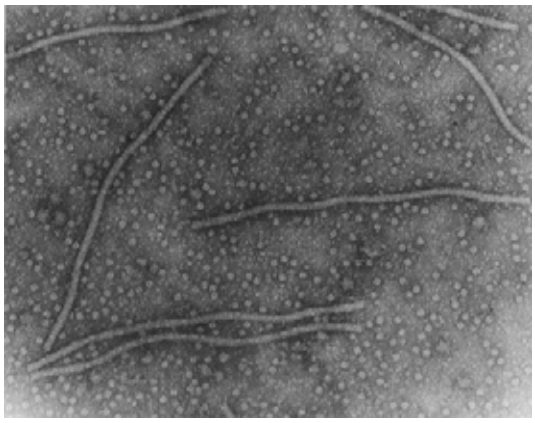

Figure 3 Electron micrograph showing PVX virions (flexuous rods) in the cytoplasm of a host plant cell (Brunt *et al.* 1996)

PVX is a highly mechanically transmissible virus but what does this mean in real terms ? In risk assessments some researchers state that spread of the virus between infected plants occurs relatively easily whereas others state that the risk of the virus spreading is quite low. The

researchers working with the virus will generally be aware of the means by which PVX is transmitted and adopt practices to prevent spread of the virus. One of the containment measures required for working with GM plant viruses is to limit access to material containing such pathogens to authorized personnel. This should reduce the risk of accidental dissemination because only researchers who are aware of the risks from working with GM plant viruses handle material containing these viruses. However, in practice this can be difficult to achieve because it is becoming commonplace for research establishments to build larger greenhouses with fewer compartments shared by multiple users. In addition, the reduction in the numbers of technical staff including gardeners and greenhouse personnel may mean that people who are unaware of the risks from GM plant viruses will be required to tend plants infected with such viruses. Therefore, experiments will be performed to determine how likely PVX is to be spread by brushing past plants, careless watering of plants or plant to plant contact.

4.3 *TOBACCO MOSAIC TOBAMOVIRUS* (SOCPHIR/EPPO PRESENT IN THE UK)

TMV is a single stranded RNA virus which is transmitted mechanically. Virus particles are rod shaped (Figure 4) and are 300nm long. Virions are found in all parts of the host plant in the cytoplasm, nuclei and possibly chloroplasts of infected cells. The unipartite genome is 6.395 Kb which includes 4 main ORFs. The thermal inactivation point is >90°C and its longevity *in vitro* is 3000 days. It is common for smokers to transmit the virus by contact with susceptible plants.

TMV was first reported in *Nicotiana tabacum* from Russia and the USA (Mayer, 1886). The disease is probably distributed worldwide. Under natural conditions symptoms include leaf mosaic and causes severe crop losses. On average TMV reduces yields by 30-35% and the market value is reduced by 50% as poor leaves can lead to a reduction in smoking quality.

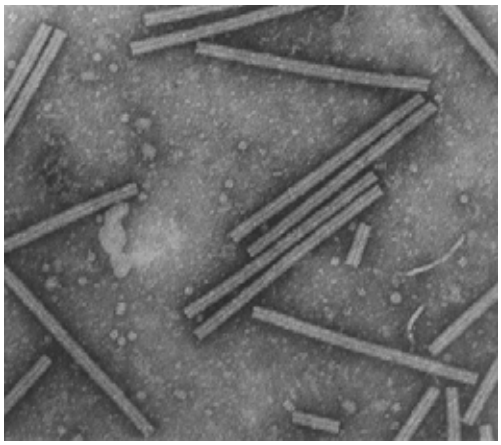

Figure 4 Electron micrograph showing TMV virions (rods) in the cytoplasm of a host plant cell (Brunt *et al.* 1996)

The virus is known to infect at least 199 species from 30 plant families (Shew and Lucas, 1991). Plants commonly infected with TMV include tomatoes, petunias, verbenas, peppers and potatoes (Anna Skelton, pers. comm.). *Digitalis*, *Datura* and *Nicotiana* species are also susceptible to infection by TMV.

TMV can be transmitted by mechanical inoculation, grafting and via seeds (via the testa, but not through the embryo).

Control of the virus can be quite difficult due to the marked stability of TMV. As with other viruses the best means of control is ensuring that clean, virus-free seed is planted, using soil that is free of tobacco, tomato and pepper residues, selecting fields that are far away from solanaceous acreages, crop rotation and prohibiting smoking during working. Cultivars that are resistant or tolerant to TMV are on the market.

With respect to the potential for accidental dissemination of GM TMV, as the virus is mechanically transmitted the problems are likely to be similar to those discussed previously for PVX. In addition, as TMV is known to be very stable, the potential for accidental spread of the virus through poor working practices could be greater than for PVX as TMV dispersed through droplets or dried material could retain infectivity. Therefore similar experiments to those outlined for PVX need to be performed.

5 EXPERIMENTAL WORK

5.1 COWPEA MOSAIC VIRUS – EXPERIMENTS TO ASSESS TRANSMISSION AND HOST RANGE

5.1.1 Introduction

Risk assessments for the use of modified CPMV often state that the work is likely to be of low risk because this virus is non-indigenous. In addition, the propagation host plant (cowpea, *Vigna unguiculata*) is not a crop in the UK and the specific crysomelid beetle vectors required for virus transmission are absent from the UK. However, while this is correct there is no supporting scientific evidence to demonstrate that CPMV cannot be transmitted via insects present in the UK and there is little data to show which other legumes may be infected by CPMV or to what degree. Therefore vector transmission studies were carried out using insects and plants that are commonly found in glasshouses and which could serve as vectors and hosts for CPMV.

A range of plant species commonly used as experimental hosts in glasshouses including *Vigna unguiculata, Vicia faba, Pisium sativum, Phaseolus vulgaris, Nicotiana tabacum, N. clevelandii, Datura stramonum, Chenopodium quinoa* and *C. amaranticolor* were mechanically inoculated with CPMV.

Insect transmission experiments were carried out using commonly found glasshouse pests including Western flower thrips (*Frankliniella occidentalis*), *Myzus persicae,* as well as the biocontrol agent *Macrolophus caliginosus. M. caliginosus* was used because in the absence of prey (e.g. whitefly and spider mites) the bug will feed on plant material and could act as a vector for CPMV. Thrips and aphid cultures are currently established at CSL and the biocontrol agent is commercially available. Infected and healthy plants were arranged in cages and pests introduced onto the plants. Virus movement was monitored by observing the healthy plants for symptoms and by testing the plants using ELISA.

5.1.2 Experimental procedures

Molecular methods

RNA was extracted from leaf samples as follows. The leaf material (100-200 mg) was placed in a grinding bag and frozen using liquid nitrogen. After removal from the liquid nitrogen the leaf was ground up and 1-2 ml of grinding buffer (2% CTAB, 100mM Tris-HCl pH 8.0, 20mM EDTA and 1.4M NaCl) was added. The extracted sap was decanted into a 1.5 ml microfuge tube and incubated for 15 min at 65°C. The sap was mixed with an equal volume of chloroform: IAA (24:1) and RNA was precipitated from the aqueous phase with isopropanol. After centrifugation the RNA pellet was washed with 70% ethanol, dried and resuspended in 100µl of sterile distilled water.

Plants were tested for virus infection two weeks post inoculation using reverse transcription and polymerase chain reaction (RT-PCR). A primer and probe set was designed using sequence from the CPMV RNA2 capsid protein sequence (van Wezenbeek, *et al*., 1983). A real-time PCR machine (ABI 7900HT) was used for the RT-PCR, the reactions were run for 40 cycles and appropriate positive and negative controls were used.

Virus cultures

CPMV virus inoculum was purchased from DSMZ GmbH. The isolate was mechanically inoculated onto cowpea (*Vigna unguiculata*) at the 2-leaf stage. Infected leaf material was

ground up in phosphate buffer and a small amount of Celite. The infected sap was then gently rubbed onto two of the leaves of the healthy plant. Systemic symptoms were observed 12-14 days post-infection. The isolate was sub-inoculated on to healthy *V. unguiculata* plants every 3-4 weeks. Leaves of infected plants were tested regularly for CPMV using RT-PCR.

Host range study

A host range study was carried out with plants commonly used for experimental purposes in glasshouses including *Vigna unguiculata, Vicia faba, Pisium sativum, Phaseolus vulgaris, Nicotiana tabacum, N. clevelandii, Datura stramonium, Chenopodium quinoa* and *C. amaranticolor*. The test plants were mechanically inoculated at the 2-4 leaf stage and symptoms were assessed after 12-14 days. All plants were tested for CPMV using RT-PCR.

Insect transmission

Aphid transmission studies were conducted using two species of aphid, *Myzus persicae* (Sulzer) and *Nasonovia ribisnigri* (Mosely). Both alate and apterous aphids were used as well as juvenile and adult stages. Aphids were starved for 1h prior to being placed (using a small paintbrush) on cowpea plants infected with CPMV. Acquisition times were of two types, short periods of preliminary probing (monitored by observation), and long acquisition periods of two days. In both cases 100 aphids were transferred from infected cowpeas to healthy cowpeas (10 aphids per plant). Control plants were set up which were healthy cowpea plants that were touched with the paintbrush used to transfer the aphids. This was to ensure that any transmission detected was due to the insect and not the transfer procedure. The aphids were left to feed on the healthy plants for 2 days after which the plants were sprayed off using insecticide. The test plants were grown on for 14 days and were then tested for infection using RT-PCR.

Thrips transmission studies were conducted using the species *Frankliniela occidentalis.* At least 100 thrips (including juveniles and adults) were collected and placed on infected leaves in Tashiro cages and left for an acquisition period of 2 days. The thrips were then transferred to 10 healthy plants (10 thrips per plant) and the plants covered with a vented plastic cup. Control plants were set up as above. The thrips were left to feed on the infected plants for 2 days after which the plants were sprayed off using insecticide. The test plants were grown on for 14 days and were then tested for infection using RT-PCR.

Virus transmission via a biological control agent was assessed using *M. caliginosus* [now a synonym of *M. melanotoma* Costa (MIRIDAE)] which were supplied by Koppert UK Ltd as the pest control agent MIRICAL. At least 100 insects were released into an insect cage which contained a mixture of healthy cowpea plants and infected cowpea plants. A control experiment was also set up with a mixture of healthy and infected cowpea plants without insects. This was to ensure that any transmission detected was due to the insects feeding behaviour and not via mechanical transmission. The plants and insects were maintained inside the cage for 21 days. Leaf samples were taken from the healthy plants and tested for infection using RT-PCR.

5.1.3 Results

Host range study

A range of test plants (5 of each type) were inoculated with CPMV, assessed for visual symptoms and tested using RT-PCR 14 days post inoculation. The experiment was repeated twice. Cowpea plants exhibited chlorotic spots at the primary point of infection and mosaic symptoms on systemically infected leaves. *Chenopodium amaranticolor* and *C. quinoa* exhibited symptoms including necrotic local lesions and systemic chlorotic spots. None of the other plants had obvious symptoms. All plants were tested by RT-PCR with primers specific to CPMV. Positive results were obtained from cowpeas, *C. amaranticolor* and *C. quinoa*. In addition CPMV was detected in one of the *N. tabacum* plants. Previous reports suggested that

the majority of host plants for CPMV are members of the Leguminosae (Porta *et al*. 2003; Brunt *et al*., 1996). However, Brunt *et al.* 1996 stated that *Phaseolus vulgaris* cv. Pinto is a diagnostically susceptible host species for CPMV whereas Porta *et al.* 2003 did not detect infection of this host species but did find *P. vulgaris* cv. Black Valentine to be susceptible. This therefore indicates that there maybe a certain degree of variability between isolates of CPMV and susceptible plant species. The data presented here indicate that apart from *V. unguiculata* (cowpeas) and diagnostically susceptible host species such as *C. quinoa*, CPMV is not easily mechanically transmitted to other plants species commonly used in research facilities and therefore that the risk is very low.

Insect transmission

Experiments were conducted to examine whether insects that are commonly found in UK glasshouses would be capable of transmitting CPMV. Several different species were identified as being common pests in UK glasshouses including *M. persicae* (Sulzer), the lettuce aphid (*N. ribisnigri* (Mosely)) and the western flower thrip (WFT; *F. occidentalis*). A biocontrol agent MIRICAL (*M. caliginosus*), which is known to feed on plant material in the absence of insect prey, was also included in the study. The experiment involving the lettuce aphids did not progress beyond the first stage because it was impossible to get sufficient numbers of the aphids to survive on the test plants (cowpeas). None of the other tests resulted in the transmission of CPMV, indicating that the virus is not transmitted by these species.

Arguably the numbers of insects used in the study could have been much larger. However, if transmission is only detected when large populations of insects are used, it is likely that in contained use research facilities where the insect populations should be controlled, the risk of transmission will be negligible.

5.2 STUDIES ON TRANSMISSIBILITY OF MECHANICALLY INOCULATED VIRUSES

5.2.1 Introduction

The majority of plant viruses can be mechanically inoculated and some are naturally mechanically transmissible. However, the researchers carrying out the experiments with these vectors hold different opinions about how easy or difficult it is to accidentally spread viruses within the containment facilities. This could be an issue where the work is being carried out in facilities shared by a number of different researchers. Therefore, the purpose of this work was to investigate how procedural errors can contribute to the accidental spread of a modified virus within the containment facilities. Experiments were carried out to follow the spread of virus resulting from two different types of poor experimental practice. Experiments were done using PVX and TMV and 4 repetitions were carried out for each experiment. The two types of poor experimental practice were (i) brushing over the plants with a watering lance and (ii) taking leaf samples from several different plants in the compartment without changing gloves between plants. From the results a numerical estimate of the spread of these viruses was made. As the experiments were carried out under the same conditions, it was possible to compare the degree of spread for each virus.

5.2.2 Experimental procedures

Virus cultures

The isolates of TMV and PVX were from the cultures kept at CSL for diagnostic purposes. Virus isolates were maintained on *N. tabacum* and the plants were infected by mechanical inoculation. An infected leaf was ground up in phosphate buffer and a small amount of Celite. The infected sap was then gently rubbed onto two of the leaves of the healthy plant.

Enzyme-linked immunosorbent assay

Sap from the test plants was tested using enzyme-linked immunosorbent assay (ELISA; Clark & Adams, 1977). TMV was detected using a direct ELISA with antibodies from Loewe Biochemica GmbH. PVX was detected using an indirect ELISA using antibodies produced at CSL.

Transmission via watering

The experiment was conducted in a controlled environment (CE) room. Four plants infected with either TMV or PVX were arranged on a bench in the CE room along with 32 healthy *N. benthamiana* plants (Figure 5). The plants were watered 3 times per week. The plants were watered from above and no measures were taken to avoid brushing the leaves with the watering can. After 3 weeks the plants were scored for symptoms and then tested by ELISA. This experiment was repeated 4 times for each virus. In addition a control experiment was set up to assess virus spread through plant contact (e.g. leaves from infected and healthy plants touching). This involved the same arrangement of plants, but care was taken to water the plants from below to avoid contact with the leaves.

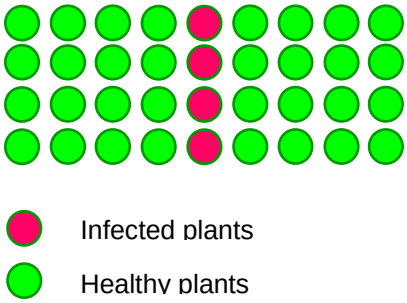

Figure 5. Experimental set up for mechanical transmission study.

Transmission during sampling

The experiment was carried out in a CE room. A leaf sample was taken from a plant infected with either TMV or PVX. Leaf samples were then taken from 15 other healthy *N. benthamiana* plants. Gloves were not changed between taking the infected sample and the healthy samples. The plants were maintained for 14 days and then scored for symptoms and tested for infection using ELISA. Each experiment was repeated 4 times.

5.2.3 Results

Transmission via watering

PVX and TMV are both mechanically transmissible viruses and there is the possibility that they could easily be spread during procedures such as watering plants. One week after the experiment was set up several of the healthy plants started to show symptoms of virus infection. An example of one of the TMV experiments 3 weeks after it was set up is shown in Figure 6. Results from the experiments using PVX showed that watering the plants so that the watering can brushed against the plants lead to an average virus spread of 2.9%. Results from the TMV experiments demonstrated an average virus spread of 16.6%. There were no additional infected plants in the control experiment where the plants were watered from below and the leaves were touching. An overview of all the results obtained is provided in Table 1.

Figure 6. Transmission of TMV to *N. benthamiana* plants via watering. The infected plants are showing symptoms including stunting and severe chlorosis.

Transmission during sampling

This experiment was carried out investigate the degree of virus spread if gloves are not changed regularly when sampling plant material infected with either PVX or TMV. Results from the experiments using PVX showed that on average 6.67% of the plants became infected. Results from the TMV experiments showed a much greater degree of virus spread with 51.67% of the plants becoming infected. Interestingly the positive plants were not always adjacent to one another, as might have been expected from the way the experiment was set up.

Table 1. Experiments to assess the effect of careless watering and sampling on the spread of PVX and TMV. The results are expressed as number of infected plants out of the total number of plants in each replicate.

	N° Plants infected via watering		***N° Plants infected via sampling***	
Replicate	***PVX***	***TMV***	***PVX***	***TMV***
1	1/60	6/60	1/15	8/15
2	1/60	8/60	1/15	7/15
3	3/60	12/60	2/15	9/15
4	2/60	14/60	0/15	7/15
Average	1.75	10	1.0	7.75
% Spread	2.9	16.6	6.67	51.67

6 CONCLUSIONS AND RECOMMENDATIONS

CPMV

The results presented here show that CPMV does not have a wide host range within plant species commonly used for experimental purposes and is not transmitted by the most commonly found glasshouse pests in the UK. In addition there was no evidence that the bio-control agent *M. caliginosus* easily transmits CPMV. Therefore, the arguments that as there are few hosts and vectors for this virus in the UK, the risks from the use of CPMV are likely to be very low are valid. Thus there does not currently appear to be a need to update the guidance with respect to work with CPMV. Nevertheless the current containment measures and standards used for such work should still be maintained and risk assessments should be updated at regular intervals particularly in the event of any emerging additional information.

Mechanically transmissible plant viruses

Opinion on whether there was likely to be a real risk of virus transmission caused by human error appears to be quite divided. The results from the study presented here show that careless working practices can lead to virus transmission and spread. The extent of the spread is dependent on the virus and in this study TMV was more easily spread than PVX. Therefore, some researchers may underestimate the risk of accidental virus dissemination via human error and not use adequate containment measures to reduce this risk. However, this could be overcome by training all researchers who have to deal with virus infected plants so that they have a full understanding of the risks from dealing with this kind of material. Establishing working procedures such as watering virus infected plants from below and changing gloves during sampling should reduce the risk of accidental virus transmission. This should be done in parallel with training of staff to give them a better appreciation of how modifying the working practices will reduce the risk.

Currently in the GMO (CU) guidance the containment measure relating to the training of staff is listed under the containment measures for activities involving genetic modification of microorganisms in laboratories. The results of this project indicate that this measure should also be emphasised in the guidance for the activities involving genetic modification of plant viruses. This recommendation could be particularly important for researchers carrying out large-scale virus inoculations for the purposes of 'bio-pharming' (the production of commercially important proteins in plants).

Finally, the experiments using *M. caliginosus* indicated that this biological control agent did not transmit CPMV by either the classic vector-virus method or mechanical transmission via insect mouthparts. The latter was raised as an issue for mechanically transmissible viruses from a report detailing transmission of a Potexvirus (*Pepino Mosaic Virus*) via *M. caliginosus* under laboratory conditions in the absence of insect prey. However, in a commercial glasshouse, containing plants infected with PepMV where insect prey were available, *M. caliginosus* did not transmit the disease (Loomans *et al*., 2000). Given the fact that TMV is highly mechanically transmissible, further experiments to investigate transmission of TMV by *M. caliginosus* or other potential biological control agents should be performed before recommendations can be made about the use of such control on crops infected with modified TMV.

7 APPENDIX 1: USES OF PLANT VIRUS VECTORS

To provide a better understanding of the types of research studies using plant viral vectors, this appendix will give a brief overview of the various different uses of plant viruses. Several reviews have recently been published which provide a good background to the various different uses of viruses and therefore this appendix will not cover this subject in detail (see Matthews, 2002; Porta & Lomonossoff, 2002; Scholtoff *et al*., 1996; Lacomme *et al*., 2001).
The use of plant viruses as a biotechnology tool has proliferated over the last decade. A simple measure of this can be gained by examining the number of publications listed in the ISI database which have been produced from research studies using PVX as a vector. In the early 90's there was an average of 1 per year while in the years 2001-2003 there were on average 8 publications per year produced from laboratories worldwide. Viruses have been used for a wide range of applications including studying basic aspects of viral pathogenesis, presentation of heterologous peptides, as gene vectors and for elucidating gene functions via viral induced gene silencing (VIGS). However, although viruses have been used for many different aims, in simplistic terms the uses can be divided into two main areas where viruses have either been used to express genes of interest or to inhibit the expression of genes of interest.

Table 1 Examples of viruses used in research and the commercial sector

Research		***Commercial***	
Viral pathenogenesis	PVX, TMV, TEV	Protein production:	
		a) Novel vaccines	CPMV,PVX,TMV
Induced plant resistance	PVX	b) Therapeutic proteins	TMV, CaMV
Cell biology studies	PVX	c) Metabolic engineering	TMV
Gene silencing	PVX, TMV, TRV		
Gene function	PVX, TMV	Gene discovery	TMV

7.1 ADVANTAGES OF USING PLANT VIRUSES

The use of plant viruses has a number of advantages in comparison to approaches with similar aims using genetically modified (GM) plants.

7.1.1 Host range

Production of transgenic plants is dependent on the ability to transform the plant in question and currently only few plant species are transformable. In contrast, viruses can be used to inoculate a wide range of plants for a number of different purposes. Furthermore, many viruses can be used to inoculate plant species that are not their primary hosts.

7.1.2 High levels of protein expression

Use of plant virus vectors to direct the expression of proteins of interest will also overcome the problem of position effect which occurs in GM plants when the transgenes are integrated into different places within the plant genome. In addition, the problem of somaclonal variation which is the result of regenerating plants from undifferentiated, transformed plant cells and may lead to different levels of expression, will be avoided. Viruses induce transient gene expression where there are high levels of protein synthesis while in transgenic plants there is what may be considered a steady state situation, where the level of synthesis is in equilibrium with the level

of degradation. Therefore when maximum levels of protein expression are required, use of plant viruses for gene expression will be advantageous.

7.1.3 Flexibility

Viruses also allow a greater degree of flexibility than transgenic plants because virus inoculation is carried out on parts of mature plants. Therefore novel phenotypes can be observed which would not be detected using transgenic approaches particularly if the expressed gene product is toxic or silencing of the gene of interest is lethal. Use of virus particles purified from plants for antibody production has the added advantage that there are less likely to be contaminating proteins which are toxic or lead to disease in animals or humans. In contrast the presence of contaminating and or toxic proteins in antigen mixtures prepared from human or animal cells is now well established.

7.1.4 Speed

In addition, use of plant viruses is more rapid and allows a much larger number of genes to be screened. Recombinant virus vectors can be produced and used to inoculate plants within a matter of weeks whereas the generation of transgenic plants takes several months. For gene function studies, complete cDNA libraries can be cloned into viruses such as TMV and BSMV and then used to mechanically inoculate test plants. Following observation of a visible phenotype, the gene or fragment from the virus used to infect the plant can be isolated, sequenced and a putative function assigned. This type of approach is particularly useful when several genes involved in a specific function are being isolated, for example programmed cell death. It also allows screening on a scale which would not be possible with transgenic plants. It is likely that as this technique is refined, this type of screening will be more commonly used especially as a functional genomics approach to complement the information gathered from the many genome sequencing projects.

7.2 PLANT VIRUSES AS EXPRESSION VECTORS

As described above the use of plant viruses for protein expression offers a number of advantages over plant transformation methods. There are four main strategies for expression of foreign genes using plant viruses (Scholtof *et al*., 1996). These are a) gene replacement, b) gene insertion, c) epitope presentation and d) complementation. In order to understand these strategies, a brief description of virus gene structure will be given. Although there are a wide variety of plant viruses, a common feature of all viruses is that the entire viral genome is contained within a relatively short region of nucleic acid. Virus stability and viability is strongly influenced by viral genome size. Thus the size of a plant virus is a limiting factor when considering insertion of additional material. All plant viruses contain a basic set of genes which encode proteins involved in replication (polymerase/replicase) and intercellular transport (movement proteins) which are essential for pathogenicity. In addition some proteins encoded by virus genes can have more than one function, for example in PVX where the coat protein (CP) is also required for cell-to cell movement. Other virus encoded gene products which may be considered as peripheral, at least in certain gene technology applications, include the proteins involved in the suppression of gene silencing and proteins required for transmission via vectors e.g. nematode transmission proteins encoded by RNA2 of TRV.

7.2.1 Gene Replacement

The main advantage of this approach is that the effects of increased genome size are avoided by the replacement of existing viral genes with foreign genes. Of course this approach requires the deleted viral gene to be non-essential. Therefore genes necessary for virus replication and movement are not good candidates for replacement, but genes for functions such as insect transmission or even possibly the coat protein could be dispensable. However, as viruses have

evolved to have a specific gene structure it is quite likely that deletion of a virus gene or replacement with another gene will affect the stability of the resulting recombinant virus.

7.2.2 Gene Insertion

In this strategy none of the viral genes are removed and the additional sequence is inserted into the complete virus genome. This is used when a gene replacement strategy can not be employed for instance when all of the viral genes are essential for the overall fitness of the virus. Depending on the packaging (encapsidation) some viruses will be more tolerant of larger insertions than others.

7.2.3 Epitope presentation

This approach involves the translational fusion of small foreign peptides to viral proteins. The proteins are expressed in such a way that the peptides in the mature protein are projected outwards at the surface of the virus particle. The virus particles can then be isolated relatively easily from the infected plant. As the peptides are displayed on the surface the purified particles can then be used directly and this has commonly been done with the aim of raising antibodies. Alternatively if purified peptides are required they can be isolated from the particles via some relatively simple protein purification steps.

7.2.4 Complementation systems

With this strategy the genome of the virus is 'disarmed' through replacement of an essential gene with a foreign gene. The virus vector is then inoculated onto a transgenic plant that expresses the 'missing' virus gene for complementation of the disarmed vector. Expression of the deleted viral gene can also be provided by co-infection with a helper virus. This approach overcomes the loss of function by replacing viral genes with the gene of interest or the instability caused by inserting the gene of interest. However, one of the frequent problems with this type of approach is that the virus may recombine in order to re-gain the deleted gene and in the process lose the gene of interest.

7.3 PLANT VIRUSES AND FUNCTIONAL GENOMICS

Discoveries concerning plant viruses and gene silencing indicated that virus-based technology would be a useful complement to existing functional genomics tools. A good overview of this approach is provided in David Baulcombe's paper (1999). Basically it is thought that VIGS is caused by a natural RNA-mediated defense (RMD) mechanism which is induced in plants by viruses. This mechanism works in a sequence specific manner which is thought to be activated as the virus accumulates in the plant. The RMD mechanism functions at a post-transcriptional level and thus results in post-transcriptional gene silencing (PTGS). Researchers have found that this mechanism can be manipulated by inserting regions which are homologous to a gene in the host plant into a genetically modified virus. The RMD will then target both the viral RNA and the corresponding mRNA from the endogenous gene leading to viral induced gene silencing. In addition, VIGS can be mediated by relatively short regions of RNA (100-300 nt) and does not require expression of a functional protein from the gene of interest. Therefore, virus instability caused by insertion of large regions of nucleic acid or deletion of viral genes should not be a major problem for plant virus vectors modified to induce VIGS.

As an additional complication, some viruses have evolved a strategy to overcome or suppress the PTGS. Viral proteins have been identified that are suppressors of PTGS including the Hc-protease (HcPro) encoded by plant viruses from the Potyvirus genus and the 2b protein from cucumber mosaic virus (CMV) (Voinnet *et al.* 1999). Clearly this has implications for virus vector design and application. When viral vectors are used with the aim of inhibiting gene expression via VIGS, it will be beneficial to use a vector which does not express a suppressor protein and therefore will be a strong inducer of silencing. Conversely when high levels of

protein expression are required, use of a viral vector which encodes a suppressor should be advantageous. There is also some evidence to show that experimental gene silencing is limited to a few specific hosts although researchers are actively trying to expand the range of plants in which this technique functions (Brigneti *et al.* 2004, Liu *et al.* 2002). Therefore selection of a specific host/virus combination will be important for each practical application.

7.4 COMMERCIAL USES OF PLANT VIRUS VECTORS

Much international interest has been focused on the use of GM plant virus vectors for a variety of different uses including the development of orally administered vaccines and the large scale expression of proteins for commercial applications (examples of which are listed in Table 2). Several of the approaches described above in Section 7.2 have been used for this purpose.

Table 2 Examples of some of the proteins which have been expressed using GM plant virus vectors

Proteins expressed for vaccine production	***References***
Foot-and-mouth disease virus epitopes	Wu *et al.* 2003
Antigenic determinants from rabies virus proteins	Yusibov *et al.* 2002
Amyloid-beta peptide, Abeta, (which accumulates during the onset of Alzheimers disease)	Szabo *et al.* 2004
Rotavirus major inner capsid protein, VP6, (which causes severe diarrhoea in children)	O'Brien *et al.* 2000
Other proteins expressed from GM plant virus vectors	***References***
Major birch pollen allergen Bet v 1	Krebitz *et al.* 2000
Hev b 1 and Hev b3, two latex allergens which are associated with spina-bifida	Breiteneder, *et al.* 2001
Mal d2 thaumatin-like, apple allergen	Krebitz, *et al.* 2003
Bovine follicle stimulating hormone	Dirnberger, *et al.* 2001
Nocistatin, a neuropeptide	Lim *et al*, 2002

Many of these research articles can be viewed as feasibility studies to determine whether the expressed protein is biologically active and produced in sufficient quantities for relatively simple purification. However some of the studies looking at the production of proteins for vaccines have advanced to the stage where the efficacy of the protein expressed in viruses, has been tested in trials with animals and humans (Wu *et al.* 2003; Yusibov *et al.* 2002). Initially these studies were set up with a view to producing sufficient quantities of protein to allow purification followed by immunization. Further experiments have also been carried out to analyse the effect of the protein when administered as an oral vaccine, i.e. by feeding with plant material containing the expressed protein of interest. The initial results indicate that although oral administration does provide some protection against subsequent viral attack, it is not as effective as when the expressed protein is injected. Nevertheless this area of research has the potential to be explored further with a view to developing vaccines that could be administered orally to people in third world countries. The majority of these experiments have been carried out using virus infected plants kept under contained use conditions. It is frequently argued that these experiments do not present any major risks due to the fact that the modified viruses

rapidly lose the insert and if accidentally released, would be out-competed by the more fit, wild-type viruses. However, it is clear that if such GM viruses were accidentally released they could pose a significant economic threat by 'contaminating' agriculturally important crops. In addition, studies using virus vectors to direct the expression of major allergen proteins could pose a serious hazard to human health.

Some field trials with genetically modified TMV have been carried out in the USA by the Biosource Technologies, Inc. company (Turpen, 1999) which subsequently merged with and took the name of the Large Scale Biology Corporation (LSBC). The practices normally adopted for the growth of tobacco and management of TMV were used. These included 'disking' into the soil of any weed species found growing within the trial area and the planting of a non-host crop in the trial site in the following year. There was no evidence to suggest infection of weed species and when tobacco plants were grown in the following year at one trial site, there was no "carry-over" and infection from previous years (Larry Grill, LSBC pers. comm.). The LSBC is now a large company involved in a number of different applications several of which use genetically modified plant viruses. It is involved in the production of a wide range of therapeutic proteins including Aprotinin, Interferon, personalised antibodies to treat non-Hogkins lymphomas, Alpha-galactosidase A for Fabry disease enzyme replacement therapy and Lysosomal Acid Lipase (LAL) for cardiovascular therapy.

LSBC have also established collaborative links with a number of research institutes with a view to improving the currently used viral vectors and developing new ones. In addition, a German based company, Icon Genetics, (which has links with LSBC) has also developed a GM plant viral vector system for the production of proteins (Marillonnet *et al.* 2004). It therefore seems probable that the use of GM plant viral vectors is likely to increase in the future at both a commercial and academic level. As further modified versions of these vectors are developed, it is essential that efforts are made to accurately assess the risks from use of such vectors.

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