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Protocols

Inoculation of plants with begomoviruses by particle bombardment without cloning: Using rolling circle amplification of total DNA from infected plants and whiteflies

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A B S T R A C T

A new system for inoculation of plants with begomoviral DNA without cloning or the use insect vectors is described. Total DNA extracted from begomovirus-infected plants was amplified by rolling circle amplification (RCA) using the bacteriophage phi29 DNA polymerase, and inoculated to plants by particle bombardment. Infection rates of up to 100% were obtained using this technique. This technique successfully inoculated all the begomoviruses evaluated: five bipartite (*Bean golden yellow mosaic virus*, *Cabbage leaf curl virus*, *Squash leaf curl virus*, *Tomato mottle virus, Watermelon chlorotic stunt virus*) as well as one monopartite (*Tomato yellow leaf curl virus*). The success of the technique was not dependent upon plant species. Four species from three plant families [*Phaseolus vulgaris* (bean), *Solanum lycopersicum* (tomato), *Cucurbita pepo* (squash), and *Citrullus lanatus* (watermelon)], could all be inoculated by this technique. The success of the method was not dependent upon either the type or the age of the source of virus. Infectious DNA was obtained successfully from fresh, freeze-dried or desiccated plant material, from squashes of plant leaves on FTA cards, as well as from the insect vector. Plant material collected and dried as long as 25 years ago yielded infectious DNA by this method. In summary, this method can be used to obtain infectious DNA of single-stranded circular DNA viruses that can be activated for purposes of completing Koch's postulates, for preservation of pure virus cultures, and for many other applications where infectious DNA is required.

1. Introduction

Koch's postulates are the corner stone of modern diagnosis of plant pathogens. According to Koch, following its identification, the new pathogen has to be inoculated to a test plant and the disease symptoms reproduced. This is challenging for diseases caused by viruses since they cannot be cultured, and virus inoculation is often complicated. In many cases the viruses cannot be readily transmitted mechanically but require a vector for transmission to new hosts. Such is the case for begomoviruses.

In the last decade, viruses belonging to the genus *Begomovirus* of the *Geminiviridae* family, have devastated production of important crops such as cassava, cotton, cucurbits, legumes, peppers and tomato (Lapidot and Friedmann, 2002; Mansoor et al., 2003; Jones, 2003). Geminiviruses are a family of plant viruses with circular single-stranded DNA genomes packaged within a small geminate particle. The *Geminiviridae* is divided into four genera based on plant host, genome organization, and insect vector (Varma and Malathi, 2003). *Begomovirus* is the largest of the four genera, containing over 110 recognized species (as of 2003) and is expending rapidly (Jones, 2003). Begomoviruses can be divided into two groups based on their genome organization; most have their genome split between two DNA molecules of approximately 2600 nt each, termed DNA-A and DNA-B (bipartite), while others have a single DNA-A-like genome of about 2800 nt (monopartite) (Fauquet et al., 2008). Begomoviruses are exclusively transmitted in a persistent circulative manner by the whitefly *Bemisia tabaci* (Gennadius).

Experimental transmission of begomoviruses is problematic. Mechanical inoculation from plant to plant is possible for some begomoviruses, but generally at low rates, and for others not at all. Graft transmission requires the presence of live infected plant and not all plants can be grafted easily. Most begomoviruses can

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be transmitted readily from plant to plant by the whitefly vector, but this also requires a live infected plant and extensive facilities to maintain the insect. In addition, none of these techniques allow for long term storage of virus culture or the manipulation of viral DNA prior to inoculations. For these reasons inoculation procedures such as agroinoculation and particle bombardment of cloned DNA as an inoculum were developed.

Agroinoculation uses *Agrobacterium tumefaciens* to deliver binary vectors containing a tandem repeat (dimer) of a cloned viral DNA into host cells (Grimsley et al., 1986, 1987). As a result, genome-sized viral DNA is produced, spreads systemically throughout the plant and induces disease symptoms. However, agroinoculation requires time-consuming subcloning procedures to introduce the viral DNA, which is more than one unit in length, into the binary vector. Also, another pathogen—*A. tumefaciens*—is also introduced into the host plant. It has been shown that in some cases, agroinoculation of cloned begomovirus DNA does not mimic whitefly transmission, probably due to the difficulties encountered with *A. tumefaciens* in the infection of some hosts (Buragohain et al., 1994).

Particle bombardment (or biolistic inoculation) is a well-known technique to introduce nucleic acids into plants (Klein et al., 1987; Sanford, 1988). In this technique microprojectiles are directed onto intact plants in a vacuum chamber, propelled by compressed gas, usually helium. The method is highly efficient, but requires considerable equipment such as a particle inflow gun, vacuum chamber and vacuum pump. This equipment is expensive, complex to build, and cumbersome. Another limitation is plant size—the inoculated plants have to be small enough to fit the vacuum chamber. Furthermore, the combination of bombardment using compressed gas and a vacuum takes a toll on the bombarded plants. Many times plants with soft tissue such as *Nicotiana benthamiana* cannot be used as test plants for viral inoculation by particle bombardment due to the extensive leaf damage. These issues were overcome by a simplified method of inoculating plants with a cloned cDNA of a potyvirus using a particle inflow gun without the use of a vacuum chamber (Gal-On et al., 1997). The microprojectiles are propelled into the plant tissue by compressed air (or gas) without vacuum, making it possible to inoculate soft plants and seedlings that otherwise do not survive particle bombardment (Gal-On et al., 1997). A hand-held version, termed the HandGun, was shown to be 10^5-fold more efficient than mechanical inoculation, albeit less efficient than "classical" particle bombardment using vacuum (Gal-On et al., 1997).

Particle bombardment has also been used to inoculate plants with cloned begomovirus DNA (Gilbertson et al., 1991; Garzon-Tiznado et al., 1993). A high inoculation efficiency was achieved using biolistic inoculation of either unit-length (monomer) or tandem repeats (dimer) of cloned begomovirus DNA, thus eliminating the need for elaborate DNA manipulations and facilitating genetic analysis of begomoviruses (Bonilla-Ramirez et al., 1997). Although biolistic inoculation of a viral DNA monomer in the cloning plasmid was possible, higher inoculation rates were achieved following the excision of the viral clone from the plasmid prior to biolistic inoculation (Bonilla-Ramirez et al., 1997).

Begomovirus genomic DNAs usually accumulate to low levels in infected plants. Hence, to clone a begomovirus genome one needs either to isolate the low level viral DNAs or use polymerase chain reaction (PCR) combined with restriction enzymes. Recently, Inou-Nagata et al. (2004) demonstrated that high levels of begomoviral genomic DNAs can be obtained from infected plants by rolling circle amplification (RCA) using the bacteriophage phi29 DNA polymerase. The begomoviral genome is circular, and therefore can serve as a template for the phi29 DNA polymerase (see Johne et al., 2009). Combining the use of phi29 DNA polymerase with restriction fragment length polymorphism for begomovirus detection and discrimination has been demonstrated (Haible et al., 2006). Viral DNA has been cloned successfully from samples containing low level of viral DNA without any prior knowledge of viral sequence, without the need for specific primers (as is the case with PCR) and can serve as an excellent assay system for cloning and identifying viruses unknown previously (Haible et al., 2006; Schubert et al., 2007). The combination of RCA and partial digestion by restriction enzyme was used to develop a rapid and simple method for cloning begomoviruses dimers directly into *Agrobacterium* vectors, thus constructing of "agroinfectious" begomovirus clones (Wu et al., 2008; Ferreira et al., 2008). Furthermore, cloned genome components (DNA-A and DNA-B) of a begomovirus were excised from their plasmids, ligated and amplified by RCA. The amplification products were inoculated biolistically into plants and shown to be very effective for inducing infection (Knierim and Maiss, 2007). Undigested RCA products were also used successfully to inoculate biolistically plants, showing that the RCA products are viable (Haible et al., 2006). Recently, direct sequencing of rolling circle amplified total DNA has been demonstrated (Jeske et al., 2010).

A combination of RCA with FTA© Classic Cards has been demonstrated to detect efficiently and clone geminiviral genomes from large numbers of samples (Owor et al., 2007; Shepherd et al., 2008). FTA is a paper-based system designed to fix and store nucleic acids directly from fresh tissue pressed (or blotted) into it. FTA©Classic Cards (Whatman) have been shown to be useful for sampling and retrieval of both DNA and RNA viruses from plant tissues (Ndunguru et al., 2005).

This manuscript describes the development of a new method for inoculation of begomoviruses by particle bombardment of test plants with RCA-amplified begomoviral genomes directly from total DNA extracted from infected plants and viruliferous whiteflies. This method requires no cloning or separation of the viral DNA prior to inoculation, nor does it require prior sequence knowledge of the virus to be inoculated. The versatility of this approach with regard to different begomoviruses, sources of nucleic acid and inoculated plants is demonstrated.

2. Materials and methods

2.1. Viruses and plants

The following plants were used as test plants in this study—watermelon cv. Malali [*Citrullus lanatus* (Thunb.) Matsum. & Nakai], squash cv. Ma'yan (*Cucurbita pepo* L.), tomato cvs. Lanai and R13 (*Solanum lycopersicum* L.), and common bean cv. Topcrop (*Phaseolus vulgaris* L.).

Viruses were maintained in infected plants grown in isolation in a greenhouse. *Squash leaf curl virus* (SLCV) was maintained in squash plants, *Watermelon chlorotic stunt virus* (WmCSV) in watermelon plants, *Cabbage leaf curl virus* (CabLCV) in cabbage plants (*Brassica oleracea* var. *capitata* 'Earliana') and *Bean golden yellow mosaic virus* (BGYMV) in common bean, *Tomato mottle virus* (ToMoV) and *Tomato yellow leaf curl virus* (TYLCV) in tomato.

2.2. Sources of begomoviral DNA

Total DNA was extracted according to Dellaporta et al. (1983) from a variety of sources.

Fresh tissue—plants inoculated with viruses using whiteflies (*Bemisia tabaci*) were maintained in growth chambers or greenhouses. Whiteflies were removed after the inoculation period using appropriate insecticides. Samples for testing were collected from these plants three to six weeks after inoculation.

FTA Cards—one young symptomatic leaf was removed from an infected plant and placed on a single FTA© Classic Card (Whatman