EXPRESSION OF POTATO VIRUS Y CYTOPLASMIC INCLUSION PROTEIN IN TOBACCO RESULTS IN DISORGANIZATION OF PARENCHYMA CELLS, DISTORTION OF EPIDERMAL CELLS, AND INDUCES MITOCHONDRIAL AND CHLOROPLAST ABNORMALITIES, FORMATION OF MEMBRANE WHORLS AND ATYPICAL LIPID ACCUMULATION

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ABSTRACT

Infection of plant cells by potyviruses induces the formation of cytoplasmic inclusions ranging in size from 200 – 1000 nm. To determine if the ability to form these ordered, insoluble structures is intrinsic to the potyviral cytoplasmic inclusion protein, we have expressed the cytoplasmic inclusion protein from Potato virus Y in tobacco under the control of the chrysanthemum ribulose-1,5-bisphosphate carboxylase small subunit promoter, a highly active, green tissue promoter. No cytoplasmic inclusions were observed in the leaves of transgenic tobacco using transmission electron microscopy, despite being able to clearly visualize these inclusions in Potato virus Y infected tobacco leaves under the same conditions. However, we did observe a wide range of tissue and sub-cellular abnormalities associated with the expression of the Potato virus Y cytoplasmic inclusion protein. These changes included the disruption of normal cell morphology and organization in leaves, mitochondrial and chloroplast internal reorganization, and the formation of atypical lipid accumulations. Despite these significant structural changes, however, transgenic tobacco plants were viable and the results are discussed in the context of potyviral cytoplasmic inclusion protein function.

KEYWORDS

Potyvirus, cytoplasmic inclusion, Potato virus Y

ABBREVIATIONS

Cytoplasmic inclusion protein (CIP), Cytoplasmic inclusion (CI), Cauliflower mosaic virus (CaMV), Ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS1*), Potato Virus Y (PVY), Plant tissue culture (PTC), Murashige and Skoog medium (MSO), Reverse transcriptase PCR (RT-PCR).

INTRODUCTION

The Potyviridae are the largest group of plant viruses and cause serious disease in a wide range of monocot and dicot crops, including potato, soybean and sugarcane. The genomes of potyviruses consist of a single strand of positive sense RNA (~10 kilobases) encoding a single large polyprotein that is processed into at least ten viral proteins; N-terminal protein (P1), helper component protease (HC-Pro), protein P3, 6KD protein (6K1), cytoplasmic inclusion protein (CIP), 6KD protein 2 (6K2), genome-linked protein (VPG), nuclear inclusion protein A (NIa), nuclear inclusion protein B (NIb) and coat protein (CP) (Riechmann et al., 1992). A characteristic of potyviral infection is aggregation of some of the non-structural proteins (CIP, HC-Pro, NIa, and NIb) into distinctive, stable structures (Edwardson, 1966; Knuhtsen et al., 1974; Martelli and Russo, 1976; Baunoch et al., 1990).

CIP accumulates as characteristic 'pinwheel'-shaped protein structures, known as cytoplasmic inclusions (CI), within the cytoplasm of potyvirus-infected plant cells (Fig. 1A) (Arbatova et al., 1998; Hammond, 1998; Shukla et al., 1994; Rouis et al., 2002; Hiebert and McDonald, 1973). All potyviruses produce pinwheel inclusions (Fig. 1A), although scroll (Fig. 1B) and laminated aggregate (Fig. 1C) inclusions are also produced by some potyviral species (Hammond, 1998; Shukla et al., 1994; Hiebert and McDonald, 1973; Ohshima et al., 1993). CIs, although insoluble, consist of fully folded, functional CIPs that have associated to form ordered, quaternary structures (Shukla et al., 1994).

Potyviral CIP functions as an RNA helicase, has ATPase activity and is involved in the cell-to-cell movement of potyviruses (Ohshima et al., 1993; Rodríguez-Cerezo et al., 1997; Roberts et al., 1998; López et al., 2001; Gómez de Cedrón et al., 2006; Fernández and García, 1996; Jiménez et al., 2006). This multiplicity of activity is reflected in the high amino acid sequence conservation between CIPs from different potyviral species and strains (Lee et al., 1996). CIs are found in close association with plasmodesmata early in infection and begin to form cytoplasmic aggregates in the later stages of infection, when the role of CIPs in RNA replication may become important (Rouis et al., 2002; Rodríguez-Cerezo et al., 1997; Roberts et al., 1998). The exact mechanism for potyviral CI formation has not been identified and it is not yet clear whether inclusions form spontaneously or if their assembly requires the activity of other viral/host proteins (Graybosch et al., 1989; Wittner et al., 1998). Studies have described the ability of CIPs to self interact via an amino-terminal protein domain in the absence of viral or plant factors (López et al., 2001; Gómez de Cedrón et al., 2006). However, CIs have also been found to associate with other proteins, although this may be due

to their involvement in virus movement and replication (Arbatova et al., 1998; Jiménez et al., 2006).

The expression of tobacco vein mottling virus CIP in *Nicotiana tabacum* demonstrated low level CIP accumulation was possible in the absence of viral infection (Graybosch et al., 1989). However, no CIs were observed and the authors suggest that the intracellular concentration of expressed recombinant protein, directed by the Cauliflower mosaic virus (CaMV) 35S promoter, was insufficient to induce inclusion body formation. Likewise, Maiti et al. (1993) describe the accumulation of recombinant tobacco vein mottling virus CIP in *N. tabacum* with expression directed by the CaMV 35S promoter. Finally, expression of plum pox virus CIP in *Nicotiana benthamiana* directed by the CaMV 35S promoter resulted in only low level accumulation of the recombinant protein and no CIs were observed (Wittner et al., 1998). In none of these three studies were electron micrographs of transgenic leaf tissues presented.

Recombinant protein yield has been cited as the primary reason for a failure to provoke CI formation via heterologous potyvirus CIP expression *in planta* (Graybosch et al., 1989; Maiti et al., 1993; Wittner et al., 1998). The chrysanthemum ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS1*) promoter has been shown to direct 7- to 8-fold higher reporter protein accumulation *in planta* when compared to CaMV 35S (Outchkourov et al., 2003). *Nicotiana tabacum* (tobacco) is a host for the potyvirus Potato Virus Y (PVY) and infected tobacco leaf cells accumulate abundant CIs in their cytoplasm (Shukla et al., 1994; Lorenzen et al., 2006; Singh et al., 2003; McDonald and Kristjansson, 1993). Here, we describe the expression of the PVY CIP in tobacco driven by the chrysanthemum *rbcS1* promoter and the subsequent effects on tobacco leaf structure and sub-cellular morphology.

MATERIALS AND METHODS

Plant Material and Maintenance – Soil acclimatized wild type tobacco (*N. tabacum* cv. Samsun NN) and PVY infected tobacco were grown at 25 °C, 40% relative humidity and under a 12 hour photoperiod. Plant tissue culture (PTC) leaf disks and plants were grown on agar based media at 25 °C, under a 16 hour photoperiod. Relative humidity was not monitored.

Plasmid Construction - The PVY CIP coding region (1905 bp) was obtained by PCR using standard conditions. The primers used were P1 (5'-GGACCATGGCCTTAGACGATGTGATCAAGAAC-3') and P2 (5'-GGAGCGGCCGCTTATTGGTGATGAACGAACTGCAAAG-3') (Sigma-Proligo). The primers were designed to incorporate *Nco*I and *Not*I restriction endonuclease sites (underlined) at the 5' and 3' ends of the PCR product, respectively. pDH51-CI-Rep, a vector containing the PVY CIP coding region, was used as a template. As a result of this cloning strategy, a single nucleotide change was introduced that converted the second amino acid in PVY CIP from a serine to an alanine (Fig. 2A). The resulting PCR product was sub-cloned into the pGEM-T vector (Promega) to generate pKSMH-1.1 and sequenced using standard methods (Griffith University). The DNA fragment containing the PVY CIP coding region was released by restriction digestion with *Nco*I and *Not*I (Roche Diagnostics) and sub-cloned into the Impact 1.1 expression vector (Plant Research International, The Netherlands), which contains the chrysanthemum *rbcS1* promoter and terminator, thereby generating pKSMH-1.2. The PVY CIP expression cassette (containing the promoter, coding region and terminator) was excised from pKSMH-1.2 by restriction digestion with *AscI* and *PacI* (New England Biolabs) and sub-cloned into the super-binary vector, pBINPLUS (van Engelen et al., 1995), thereby generating pKSMH-1.3 (Fig. 2B).

Transformation of Tobacco Plants – Tobacco leaf disks were transformed by agroinfiltration using *Agrobacterium tumafaciens* (LBA4404 strain), based on the method described by Horsh et al. (1985), and media described by Murashige and Skoog (1962). Briefly, LBA4404 cells were transformed with pKSMH-1.3 by electroporation at 2800 V and cultured at 28 °C for 2 - 3 days on LB agar plates containing kanamycin (100 µg/ml) and rifampicin (25 µg/ml). The resulting colonies were grown for 1 – 2 days in liquid LB culture, plasmid DNA was isolated and the presence of intact vector confirmed by restriction endonuclease digestion. Fresh *Agrobacterium* cultures were diluted 1:10 with MSO liquid medium and mixed. Leaves from wild type tobacco grown in tissue culture were cut into ~1

cm^2 pieces and transformed by immersion in diluted LBA4404/vector culture. Leaf disks were blotted on sterile filter paper and placed with the upper leaf surface down onto MS104 agar without antibiotic selection. Plates were incubated at 25 °C for 2 - 3 days, before leaf disks were transferred onto MS104 antibiotic selection agar, containing kanamycin (200 µg/ml) and timentin (200 µg/ml). Plates were incubated at 25 °C and the selection media was changed every two weeks. Once well-defined stems were visible, twenty shoots were selected, excised and placed on MSO selection agar containing kanamycin (200 µg/ml) and timentin (200 µg/ml) to regenerate into whole plants.

Molecular Analysis of Transgenic Plants – Leaf tissues were ground in liquid nitrogen using a mortar and pestle, RNA was extracted using the RNAeasy Plant Mini Kit (QIAGEN) and eluted in 50 µl DEPC-treated H_2O. Reverse transcriptase PCR (RT-PCR) was performed using the Titan One Tube RT-PCR kit (Roche Diagnostics). For the identification of transgenic plants, CI specific primers P3 (5'-GAGTGCCATGTTCTAGATCCCTCC-3') and P4 (5'-GCACAAAGGTGTCATACAGTCTCG-3') were used (Sigma-Proligo). The primer annealing temperature was 50 °C and the size of the expected PCR product was 852 bp. pKSMH-1.2 was used as a positive control, with water and wild type tobacco providing the negative controls.

Inoculation of Tobacco with PVY - Wild type tobacco plants were mechanically inoculated as described by Smith (1977). Freeze dried plant material infected with PVY was ground in 0.1 M potassium phosphate buffer, pH 7.0. RT-PCR using the Titan One Tube RT-PCR kit (Roche Diagnostics) and the following degenerate primers specific to the potyviral coat protein were used to identify PVY infected samples: CN48 (forward); CN47, CN54, and CN55 (reverse) (Bateson and Dale, 1995). The primer annealing temperature was 45 °C and the size of the expected PCR product was 781 bp.

Light Microscopy and Transmission Electron Microscopy (TEM) – Thin strips were cut from the mid section of wild type and transgenic tobacco leaves, and fixed in glutaraldehyde solution (3% v/v in 0.1M cacodylate buffer) overnight at 25 °C. After a buffer wash, samples were post-fixed with osmium tetroxide (1%), then uranyl acetate (1%), and dehydrated using 50%, 70% and 90% ethanol, followed by 90% and 100% acetone respectively. Samples were embedded in Spurr's epoxy and the resin polymerized overnight at 70 °C. Semi-thin leaf sections (1-2 microns in thickness) were cut from the resin-embedded samples, stained with toludine blue, and examined by light microscopy (Carl Zeiss Jenaval) and photographed with a Leica DFC480 digital camera (Leica Microsystems). Ultrathin sections (approximately 60nm) were cut, stained with uranyl acetate and lead

citrate, and examined and photographed with a JEOL JEM-1200EX transmission electron microscope operating at 80kV.

PVY virus particles in infected tobacco were detected by TEM using negative staining. Briefly, a drop of ammonium molybdate (1% aqueous) was applied to a small piece of leaf tissue and the tissue crushed. A drop of the sap/stain mixture was transferred to a celloidin and carbon coated copper grid, excess mixture was removed and the samples viewed with a JEOL JEM-1200EX transmission electron microscope.

RESULTS

Generation and Screening of Regenerated Tobacco Plants Expressing PVY CIP- Tobacco leaf disks were transformed using pKSMH-1.3 by agroinfiltration and placed on media containing antibiotic to select for regenerated plantlets containing the integrated transgene. The second residue in PVY CIP encoded by pKSMH-1.3 is an alanine, rather than the serine residue found in the wild type sequence. RT-PCR was used to screen each of the twenty selected putative transgenic plantlets for expression of the transgene and products of expected size were amplified in the majority of plants (Fig. 3). Results indicated that 19 out of 20 T_0 generation plants were expressing PVY CIP from pKSMH-1.3 (Fig. 3A). No bands were detected in the water controls or the wild type PTC tobacco control sample (Fig. 3B, lane 3). Five of the most advanced plants (pKSMH-1.3 #1, #2, #6, #9, and #13) were selected for further analysis. We noted considerable variation in the size of the plants, with pKSMH-1.3 #2 being the smallest, but none of the transgenic plantlets showed any other visible phenotypic abnormalities compared to wild type tobacco grown in tissue culture.

Expression of PVY CIP alters the arrangement of cells in tobacco leaves – Embedded samples for transmission electron microscopy were thinly sliced and their gross leaf structure assessed by light microscopy (Fig. 4). Leaves of wild type tobacco plants, taken 6 weeks after sub-culture in tissue culture, showed the typical arrangement of upper epidermis, palisade parenchyma, spongy parenchyma, and lower epidermal cells (Fig. 4A). A leaf sample from soil-acclimatized tobacco infected with PVY, taken 14 weeks post-infection (Fig. 4B), showed only a slight decrease in the cell density in the spongy parenchyma, but was otherwise normal.

Microscopic examination of leaves from transgenic plants revealed significant morphological variation between the control and transgenic plants, and also between transgenic plant lines. Analysis of transgenic leaf samples collected 3 weeks after the plantlets were regenerated from transformed leaf disks revealed a spectrum of morphologies, ranging from relatively normal (Fig. 4C), through an altered organization of both the palisade and spongy parenchyma layers (Fig. 4D), to a disorganized state in which the palisade and spongy parenchyma cell layers were severely affected (Fig. 4E). Of the five pKSMH-1.3 transgenic plants assessed, only pKSMH-1.3 #6 possessed defined cell layers comparable to wild type, while the remaining four showed evidence of structural rearrangement, with pKSMH-1.3 #2 being the worst affected (Fig. 4E). In addition to parenchyma rearrangements, upper and lower epidermal cells appeared distorted (Fig. 4D – E).

Interestingly, there appeared to be a correlation between loss of leaf structure and growth rate (i.e. plants with the most altered leaf structure were the smallest of the five transgenic plants in PTC).

Expression of PVY CIP under the control of the RuBisCO promoter in tobacco does not produce CIs but does induce cytological changes – TEM was used to assess transgenic leaf tissue for the formation of CIs. The leaves of wild type tobacco from tissue culture contained organelles of typical appearance (Fig. 5A).

No CIs were observed in any of the samples taken from transgenic plantlets for analysis by TEM. However, alterations to the structure of organelles and the presence of abnormal structures and accumulations were observed that were not present in the wild type control (Fig. 5). The most common feature was the enlargement of the mitochondria coupled with the loss of typical cristae arrangement and blebbing (Fig. 5B and 5C). Chloroplasts were also affected, and we observed considerable rearrangement of the thylakoid membranes (Fig. 5B, 5D and 5E). However, the nuclei of the transgenic cells appeared morphologically normal. Further, transgenic samples appeared to contain "accumulations" that were not observed in the control samples. The chloroplasts contained various amounts of accumulation, indicated by differing densities in the TEM images, and thylakoid membranes appeared to "separate", presumably to accommodate the build-up of this material (Fig. 5D). Membranous material was present between the chloroplasts and plant cell wall in the wild type controls; however, the abundance of this material increased dramatically in the transgenic samples. Further, this material appeared to "push" the chloroplasts away from the cell wall, causing them to distort (Fig. 5C). In addition, we observed lipid "whorls" budding off from the cytoplasm in pKSMH-1.3 #6 (Fig. 5F) that was not observed in any wild type samples. However, despite these obvious sub-cellular abnormalities, all transgenic plants grew in plant tissue culture and were sub-cultured successfully.

DISCUSSION

The identification of the transgenic tobacco lines generated in this study is based on the RT-PCR detection of PVY CIP transcript. Tobacco has a tetraploid genome, and *Agrobacterium*-mediated transformation typically results in the insertion of a single copy of the transgene in the tobacco genome (Przetakiewicz et al., 2004). Therefore, the transgenic tobacco plants in this study are highly likely to be heterozygous for the CIP gene and will need to be taken through multiple generations in order to generate homozygous plants. Further, quantitative PCR will be used to correlate the level of CIP transcript with the observed phenotypes.

The absence of CIs in the transgenic tobacco plants analyzed in this study may have been due to a number of factors. Previous studies have suggested that there was insufficient recombinant CIP accumulated in the transgenic plants to provoke CI formation (Graybosch et al., 1989; Wittner et al., 1998). The absolute amount of expressed PVY CIP in the transgenic plants in the present study has not been determined. Therefore, purification of CIs using the method established by Hiebert et al. for antibody production and subsequent Western blotting will be an important tool in establishing both the presence of PVY CIP and its abundance (Hiebert and McDonald, 1973; Purcifull et al., 1973). In addition, expression of PVY CIP from a high-level expression system based on the rolling circle replication mechanism of single stranded DNA virus developed in our laboratory (known as INPACT) will be assessed (Dale et al., WIPO Patent Application Number 72996A1).

The involvement of host cell or viral factors in CI formation cannot be determined from the current study because none of the transgenic plants assessed produced visible CIs. Future studies will examine the co-expression of viral proteins with PVY CIP to investigate their possible role in CI formation in tobacco leaf. Immunoelectron microscopy has suggested that PVY CIP/P1 (Arbatova et al., 1998), and tobacco vein mottling virus CIP/P3 could interact (Rodríguez-Cerezo et al., 1993). Further, papaya ringspot virus amorphous inclusions (composed primarily of HC-Pro) and beet mosaic virus induced nuclear inclusions ("satellite bodies" composed primarily of NIa and NIb) have been shown to contain CIP using the same technique (Riedel et al., 1998). These viral proteins would be the obvious choice for co-expression experiments. However, no interaction was detected between either full length plum pox virus (PPV) CIP or its sub-domains with PPV P3/6K1, NIa, NIb or CP in the yeast two hybrid system (López et al., 2001). Therefore, future co-expression studies may involve the pairwise co-expression of PVY CIP with each of the remaining PVY structural and non-

structural proteins. Alternatively, In addition, translational fusion of the recombinant protein with green fluorescent protein will be used to provide an insight both into the abundance of the recombinant protein and its sub-cellular localization. This approach has been used successfully for the visualization of potyviral movement proteins (Rodríguez-Cerezo et al., 1997; Roberts et al., 1998; Vogel et al., 2007).

The detection of abnormal cellular structures and cytopathic effects associated with PVY CIP expression requires further investigation. Leaf structural rearrangement and organelle disruption, especially to the mitochondria, was surprising since most of the transgenic plants generated in this study did not appear to be visibly different to the wild type controls, with the exception of those plants with a reduced growth rate. Cytopathic effects observed in plant cells infected with potyviruses include abnormal enlargement and vesiculation of mitochondria, abnormalities in chloroplasts, starch/lipid accumulation and membrane disorganization in chloroplasts, and the formation of membrane masses in the cytoplasm (Shukla et al., 1994). No such observations had been previously made in CIP over-expression experiments in tobacco (Graybosch et al., 1989; Wittner et al., 1998; Maiti et al., 1993). Currently, the exact molecular determinants of these effects are unknown and it is not clear if they arise from the expression of a potyviral protein or proteins, or as a general "side-effect" of potyviral infection (Shukla et al., 1994; Dardick, 2007). Intriguingly, however, these cytopathologies are similar to those we observed in transgenic plants expressing PVY CIP and may indicate a causal correlation. Future studies will involve the expression of other potyviral proteins to determine if these cellular changes are specifically induced by CIP or are as a result of recombinant protein expression in general.

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REFERENCES

Arbatova, J., Lehto, K., Pehu, E., Pehu, T., 1998. Localization of the P1 protein of potato Y potyvirus in association with cytoplasmic inclusion bodies and in the cytoplasm of infected cells. Journal of General Virology 79, 2319-2323.

Bateson, M., Dale, J.L., 1995. Banana bract mosaic virus: characterization using potyvirus specific degenerate PCR primers. Archives of Virology 140, 515-527.

Baunoch, D.A., Das, P., Hari, V., 1990. Potato virus Y helper component protein is associated with amorphous inclusions. Journal of General Virology 71, 2479-2482.

Dardick, C., 2007. Comparative expression profiling of *Nicotiana benthamiana* leaves systemically infected with three fruit tree viruses. Molecular Plant-Microbe Interactions 20, 1004-1017.

Edwardson, J.R., 1966. Cylindrical inclusions in the cytoplasm of leaf cells infected with tobacco etch virus. Science 153, 883-884.

van Engelen, F.A., Molthoff, J.W., Conner, A.J., Nap, J.P., Pereira, A., Stiekema, W.J., 1995. pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Research 4, 288-290.

Fernández, A., García, J.A., 1996. The RNA helicase CI from plum pox potyvirus has two regions involved in binding to RNA. FEBS Letters 388, 206-210.

Gómez de Cedrón, M., Osaba, L., López, L., García, J.A., 2006. Genetic analysis of the function of the plum pox virus CI RNA helicase in virus movement. Virus Research 116, 136-145.

Graybosch, R., Hellmann, G.M., Shaw, J.G., Rhoads, R.E., Hunt, A.G., 1989. Expression of a potyvirus non-structural protein in transgenic tobacco. Biochemical and Biophysical Research Communications 160, 425-432.

Hammond, J., 1998. Serological relationships between the cylindrical inclusion proteins of potyviruses. Phytopathology 88, 965-971.

Hiebert, E., McDonald, J.G., 1973. Characterization of some proteins associated with viruses in the Potato Y group. Virology 56, 349-361.

Horsh, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers, S.G., Fraley, R.T., 1985. A simple and general method for transferring genes into plants. Science 227, 1229-1231.

Jiménez, I., López, L., Alamillo, J.M., Valli, A., García, J.A., 2006. Identification of a plum pox virus CI-interacting protein from chloroplast that has a negative effect in virus infection. Molecular Plant-Microbe Interactions 19, 350-358.

Knuhtsen, H., Hiebert, E., Purcifull, D.E., 1974. Partial purification and some properties of tobacco etch virus induced intranuclear inclusions. Virology 61, 200-209.

Lee, K.C., Mahtani, P.H., Chng, C.G., Wong, S.M., 1996. Sequence and phylogenetic analysis of the cytoplasmic inclusion protein gene of zucchini yellow mosaic potyvirus: its role in classification of the *Potyviridae*. Virus Genes 14, 41-53.

López, L., Urzainqui, A., Domínguez, E., García, J.A., 2001. Identification of an N-terminal domain of the plum pox potyvirus CI RNA helicase involved in self-interaction in a yeast two-hybrid system. Journal of General Virology 82: 677-686.

Lorenzen, J.H., Meacham, T., Berger, P.H., Shiel, P.J., Crosslin, J.M., Hamm, P.B., Kopp, H., 2006. Whole genome characterization of *Potato virus Y* isolates collected in the western USA and their comparison to isolates from Europe and Canada. Archives of Virology 151, 1055-1074.

Maiti, I.B., Murphy, J.F., Shaw, J.G., Hunt, A.G., 1993. Plants that express a potyvirus proteinase gene are resistant to virus infection. Proceedings of the National Academy of Sciences USA 90, 6110-6114.

Martelli, G.P., Russo, M., 1976. Unusual cytoplasmic inclusions induced by watermelon mosaic virus. Virology 72, 352-362.

McDonald, J.G., Kristjansson, G.T., 1993. Properties of strains of potato virus Y^N in North America. Plant Disease 77, 87-89.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

Ohshima, K., Inoue, A.K., Shikata, E., 1993. Molecular cloning, sequencing, and expression in *Escherichia coli* of the potato virus Y cytoplasmic inclusion gene. Archives of Virology 128, 15-28.

Outchkourov, N.S., Peters, J., de Jong, J., Rademakers, W., Jongsma, M.A., 2003. The promoter-terminator of chrysanthemum *rbcS1* directs very high expression levels in plants. Planta 216, 1003-1012.

Przetakiewicz, A., Karaś, A., Orczyk, W., Nadolska-Orczyk, A., 2004. *Agrobacterium*-mediated transformation of polyploid cereals. The efficiency of selection and transgene expression in wheat. Cellular and Molecular Biology Letters 9, 903-917.

Purcifull, D.E., Hiebert, E., McDonald, J.G., 1973. Immunochemical specificity of cytoplasmic inclusions induced by viruses in the Potato Y group. Virology 55, 275-279.

Riechmann, J.L., Laín, S., García, J.A., 1992. Highlights and prospects of potyvirus molecular biology. Journal of General Virology 73, 1-16.

Roberts, I.R., Wang, D., Findlay, K., Maule, A.J., 1998. Ultrastructural and temporal observations of the potyvirus cylindrical inclusions (CIs) show that the CI protein acts transiently in aiding virus movement. Virology 245, 173-181.

Rodríguez-Cerezo, E., Ammar, E.D., Pirone, T.P., Shaw, J.G., 1993. Association of the non-structural P3 viral protein with cylindrical inclusions in potyvirus-infected cells. Journal of General Virology 74, 1945-1949.

Rodríguez-Cerezo, E., Findlay, K., Shaw, J.G., Lomonossoff, G.P., Qiu, S.G., Linstead, P., Shanks, M., Risco, C., 1997. The coat and cylindrical inclusion proteins of a potyvirus are associated with connections between plant cells. Virology 236, 296-306.

Rouis, S., Ayadi, H., Bouaziz, S., Lakhoua, L., Gargouri, R., 2002. *In situ* immunocytochemical detection of potyviral proteins in plant cells. Biotechnic & Histochemistry 77, 111-115.

Shukla, D.D., Ward, C.W., Brunt, A.A., 1994. The *Potyviridae*. CAB International, Cambridge.

Singh, R.P., McLaren, D.L., Nie, X., Singh, M., 2003. Possible escape of a recombinant isolate of *Potato virus Y* by serological indexing and methods of its detection. Plant Disease 87, 679-685.

Smith, K.M., 1977. Plant Viruses. Chapman and Hall, London.

Vogel, F., Hofius, D., Sonnewald, U., 2007. Intracellular trafficking of Potato leafroll virus movement protein in transgenic Arabidopsis. Traffic 8, 1205-1214.

Wittner, A., Palkovics, L., Balázs, E., 1998. *Nicotiana benthamiana* plants transformed with the plum pox virus helicase gene are resistant to virus infection. Virus Research 53, 97-103.

FIGURE LEGENDS

Fig. 1. Transmission Electron Micrographs of Cytoplasmic Inclusions in the Cytoplasm of Potyvirus-Infected Tobacco. Tobacco (*Nicotiana tabacum* cv. Samsun NN) were mechanically inoculated with the potyvirus PVY, screened for infection by PCR and prepared for electron microscopy. Pinwheel (A), scroll (B) and laminated aggregate (C) cytoplasmic inclusions are clearly visible in PVY-infected tobacco leaves but are not detectable in uninfected tobacco (D). Scale bars represent 200nm.

Fig. 2. Schematic Representation of the PVY CIP amino acid sequence and the Super-Binary Construct for Tobacco Transformation. (A) Amino acid sequence of PVY CIP. The mutation of second codon is indicated in bold and underlined. The boxed region is the DEXDc domain, belonging to the DEAD-like helicase superfamily; a diverse group of proteins involved in ATP-dependent RNA or DNA unwinding. This domain contains the ATP-binding region. The ATP-binding region is indicated in bold with hash symbols above the residues. The putative Mg^{2+}-binding site is indicated in bold with star symbols above the residues. (B) Schematic illustration of the PVY CIP expression cassette in binary vector pKSMH-1.3. The genetic elements within the left and right borders are inserted into the tobacco genomic DNA during *Agrobacterium*-mediated transformation.

Fig. 3. RT-PCR Analysis of Transgenic Tobacco. RNA was isolated from regenerated plantlets, used as a template for a reverse-transcriptase reaction to generate single-stranded DNA and then PVY CIP specific primers P3 and P4 were used to detect the presence or absence of the CIP coding region. *Lanes 1, 15, 16, and 27*: molecular weight marker X (Roche Diagnostics). *Lane 2*: PCR water only negative control. *Lane 3*: PCR wild type tobacco negative control. *Lane 4*: pKSMH-1.2 vector positive control. *Lanes 5-14 and 17-26*; samples from T_0 generation regenerated tobacco plantlets transformed with pKSMH-1.3 in ascending order from 1 to 20.

Fig. 4. Analysis of Wild Type and Transgenic Tobacco using Light Microscopy. (A) Wild type tobacco from plant tissue culture. Upper epidermal cells (1), palisade parenchyma (2), and a vascular bundle within the spongy parenchyma layer (3) are indicated by arrows. (B) Soil-acclimatized PVY-infected tobacco leaf. Transgenic plants showing varying degrees

of abnormalities are presented in the following panels. (C) pKSMH-1.3 #6. (D) pKSMH-1.3 #9. (E) pKSMH-1.3 #2. All scale bars represent 100 μm.

Fig. 5. Transmission Electron Micrographs of Transgenic Tobacco Expressing PVY CIP and Wild Type Tobacco. (A) Wild type tobacco in plant tissue culture, showing normal organelle morphology (c, chloroplasts; m, mitochondria; n, nucleus; v, vacuole). Electron micrographs of transgenic plants expressing PVY CIP showing morphological abnormalities are presented in the following panels. (B) pKSMH-1.3 #1. Mitochondria lacking normal cristae arrangement are indicated by arrows. (C) pKSMH-1.3 #13. A large accumulation of membranous material, indicated by the arrow, between the chloroplast and cell wall is evident (cw, cell wall). (D) pKSMH-1.3 #6. Separation of thylakoid membranes within the chloroplasts is indicated by arrows. (E) pKSMH-1.3 #1. Severe morphological changes in the arrangement of thylakoid membranes in the chloroplast are evident. (F) pKSMH-1.3 #6. Unusual lipid whorls protruding into the vacuole are evident. Scale bars on panels A and C represent 500 nm. Scale bars on panels B and E represent 200 nm. Scale bars on panels D and F represent 1 μm.

DISCLOSURE STATEMENT

The authors have nothing to disclose.

Figure 1
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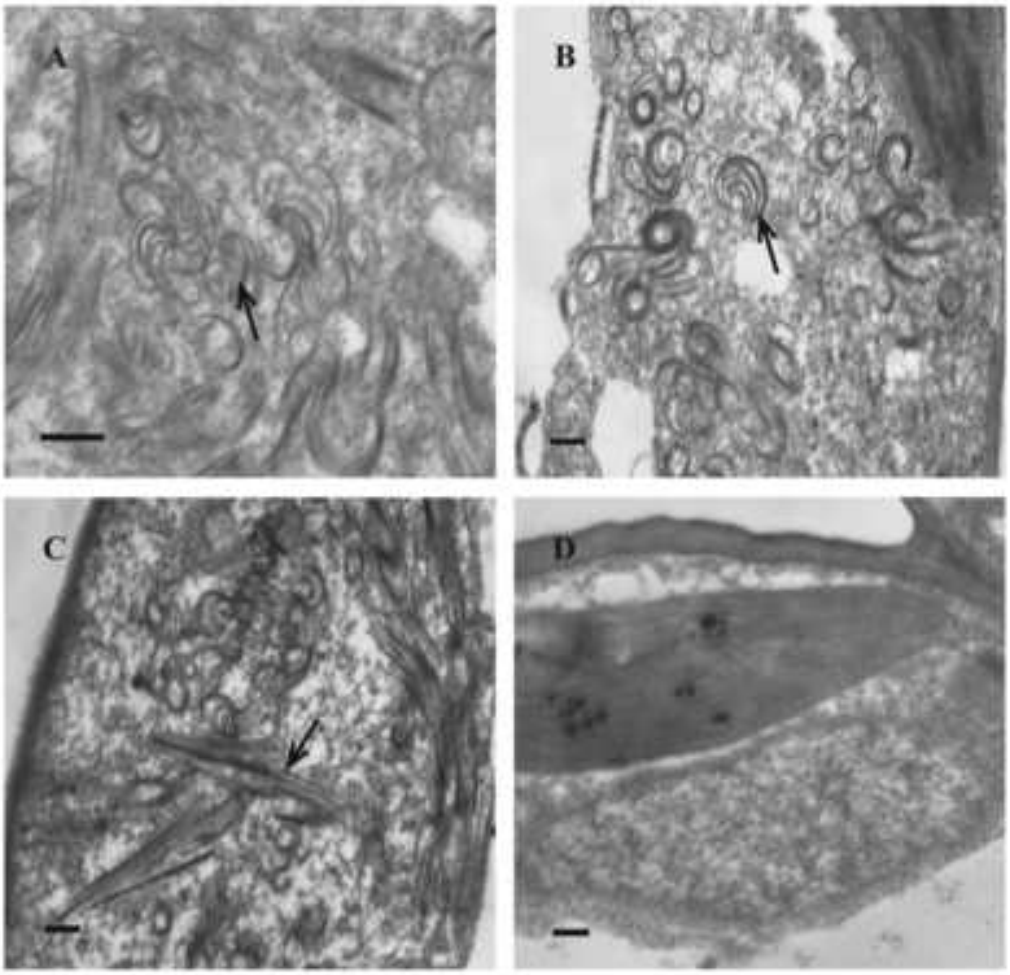

Figure 1

Figure 2
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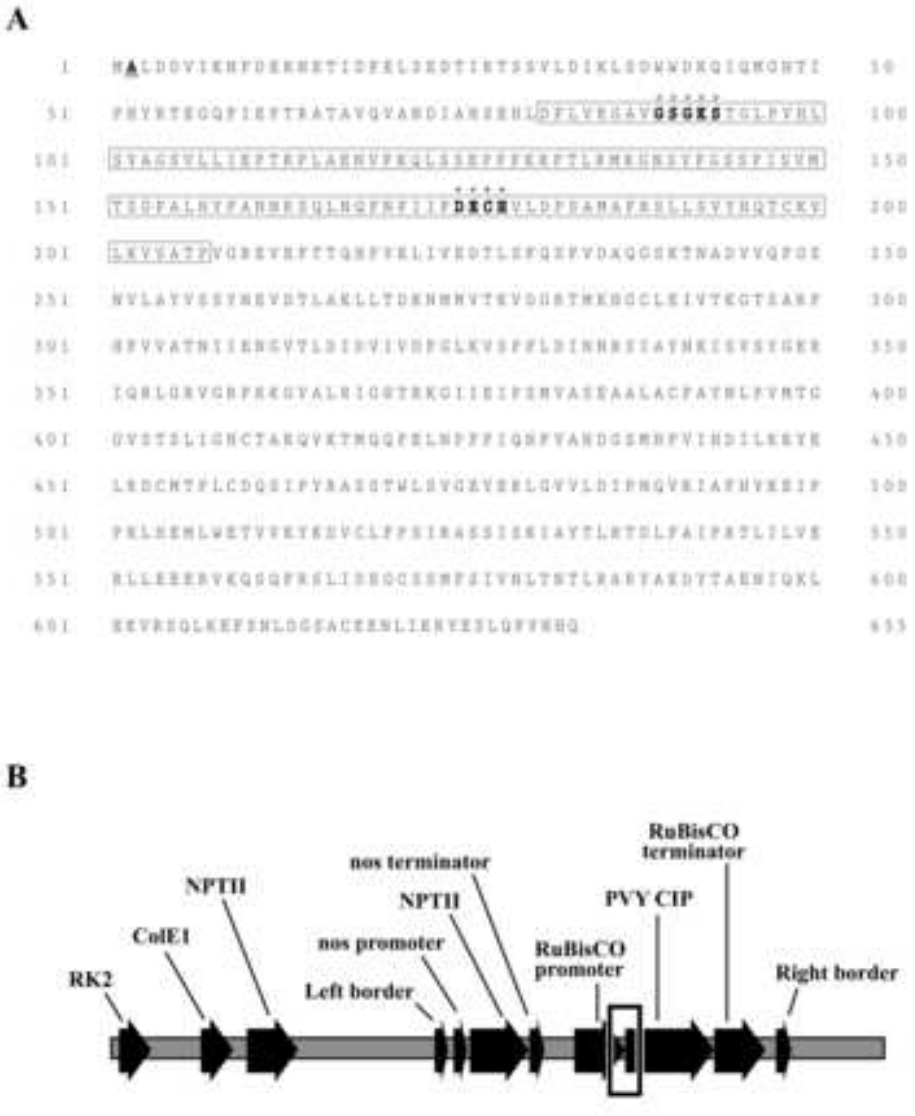

Figure 2

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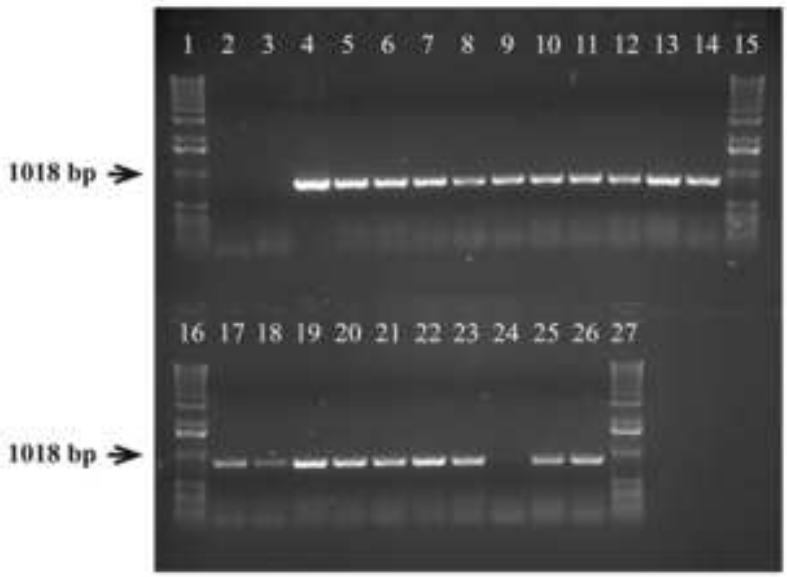

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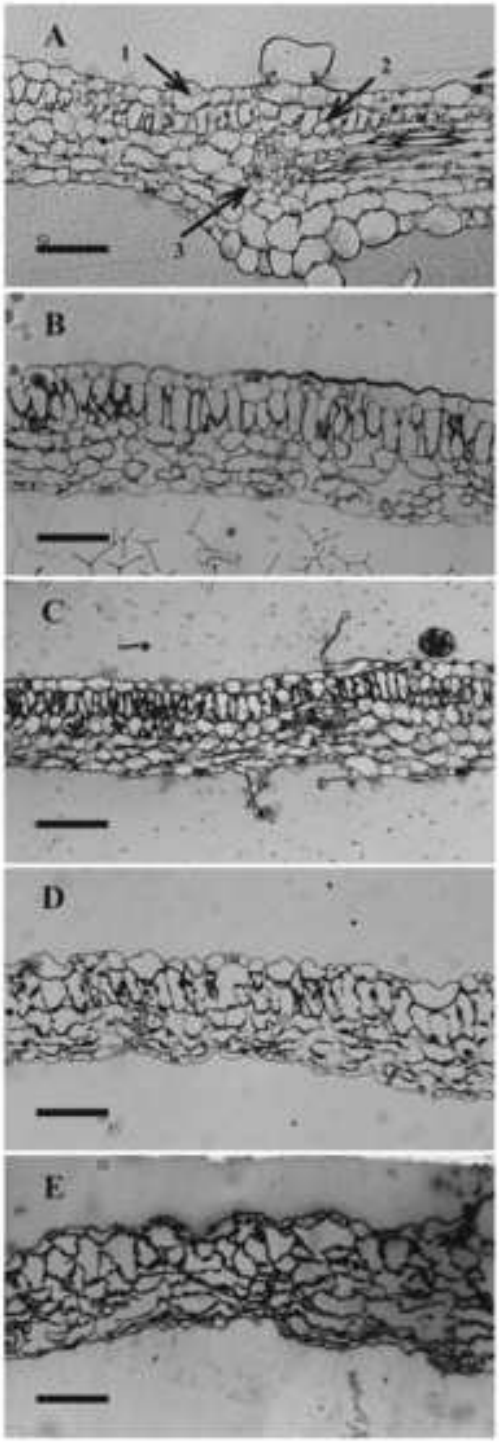

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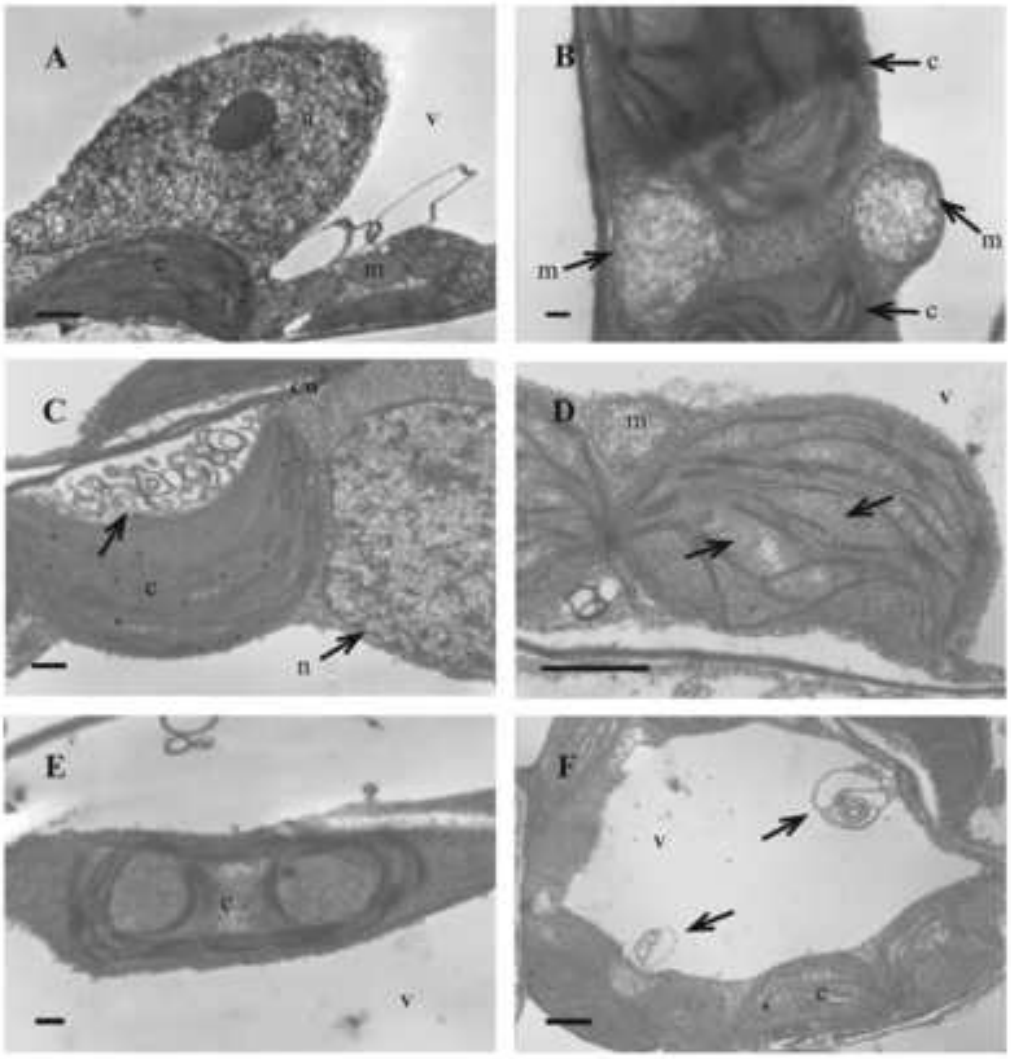

Figure 5