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The p23 Protein from *Citrus tristeza virus* is a Pathogenicity Determinant in Transgenic Citrus Hosts

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ABSTRACT

The 23 kDa protein (p23) coded by the 3′-terminal gene of *Citrus tristeza virus* (CTV), is a RNA-binding protein that contains a motif rich in cysteine and histidine residues in the core of a putative zinc-finger domain. Transgenic Mexican lime plants were generated carrying a p23 transgene, or a truncated version thereof, under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Ectopic expression of the *p23* gene from a severe (T36) strain of CTV induced phenotypic aberrations that resembled symptoms incited by CTV in non-transgenic lime plants, whereas transgenic plants expressing the *p23* truncated version were normal. The intensity of the CTV-like symptoms in *p23*-transgenic plants was associated with the p23 accumulation level. Besides, expressing the same gene from a mild strain (T-317) induced similar symptoms irrespective of the source CTV strain. Transformation of CTV-susceptible sweet and sour orange and CTV-resistant trifoliate orange with *p23*-T36 also led to CTV-like symptoms that did not develop when plants were transformed with a truncated *p23* version. In these transgenic citrus species, symptom intensity correlated with levels of *p23* transcripts, as protein accumulation was barely detectable. Conversely**,** transgenic expression of *p23* in CTV non-host *Nicotiana* spp. led to accumulation of p23 without phenotypic aberrations, indicating that p23 interferes with plant development only in *Citrus* species and relatives. This was the first case in which a protein encoded by a woody plant infecting RNA virus was identified as being directly involved in pathogenesis in its natural host. In this note, we summarize our results on the role of p23 as a pathogenicity determinant of CTV in citrus hosts.

Keywords: microRNA, phenotypic aberrations, post-transcriptional gene silencing, quick decline, RNA silencing, silencing suppressor, small interfering (si)-RNA

CONTENTS

INTRODUCTION

Citrus tristeza virus (CTV), a member of the genus *Closterovirus* within the family *Closteroviridae* (Karasev 2000), is the causal agent of the most economically important viral disease of citrus. The virus is naturally restricted to citrus species and relatives, and only occurs in phloem-associated tissues (Bar-Joseph *et al*. 1989). CTV strains often differ by their biological characteristics, particularly the type and intensity of symptoms induced in distinct hosts (Roistacher and Moreno 1991). Some strains are essentially symptomless in most citrus varieties, but most incite severe phenotypes that include i) decline and death of cultivars, except lemons (*Citrus limon* (L.) Burm.), when grafted on sour orange (*C. aurantium* L.) rootstock; ii) stunting, stem pitting, low yield, and poor fruit quality regardless of the rootstock used, and iii) leaf yellowing and growth cessation of sour orange, lemon, and grapefruit (*C. paradisi* Macf.) seedlings, a syndrome called seedling yellows (Fraser 1952).

Some species, such as Mexican lime (*C. aurantifolia* (Christm.) Swingle), are very sensitive and show symptoms upon infection with most CTV strains, whereas others, such as grapefruit and sweet orange (*C. sinensis* (L.) Osbeck), are affected only by severe strains. Although sour orange is sensitive as a rootstock, seedlings accumulate virus at low titer with most CTV isolates. General resistance to CTV has been observed in trifoliate orange (*Poncirus trifoliata* (L.) Raf.) (Yoshida 1996), and resistance to some isolates occurs in pummelo (*C. grandis* [L.] Osbeck) (Garnsey *et al*. 1996; Fang and Roose 1999) and kumquat (*Fortunella crassifolia* Swingle) (Mestre *et al*. 1997).

CTV virions are flexuous filaments of 2,000 × 10-12 nm in size, with two capsid proteins of 25 and 27 kDa (p25 (major coat protein) and p27 (minor coat protein), respec-

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tively), coating 97 and 3% of the particle length, respectively (Febres *et al*. 1996; Satyanarayana *et al*. 2004). The genome is a single-stranded positive-sense RNA of approximately 20 kb that contains 12 open reading frames (ORFs) and 5′- and 3′-terminal untranslated regions (UTRs) (Karasev *et al*. 1995). The 2 5′-proximal ORFs encode replication-related proteins that are translated directly from the genomic RNA, whereas the 10 3′-proximal ORFs encode proteins p33, p6, p65, p61, p27, p25, p18, p13, p20, and p23, which are expressed via 3′-coterminal subgenomic (sg) RNAs (Hilf *et al*. 1995). The small hydrophobic p6 is proposed to have a role in virus movement (Satyanarayana *et al*. 2008); p65 is a homologue of HSP70 heat-shock proteins and, together with p61, p25, and p27, is involved in virion assembly (Satyanarayana *et al*. 2000). The minor coat protein p27 has been shown to initiate encapsidation from the 5′ end of the genomic RNA (Satyanarayana *et al*. 2004). The p20 protein accumulates in amorphous inclusion bodies of CTV-infected cells (Gowda *et al*. 2000) and it has silencing suppressor activity in *Nicotiana benthamiana* and *N. tabacum* (Reed *et al*. 2003; Lu *et al*. 2004). The function of p33, p13, and p18 presently is unknown. These three genes can be deleted from an infectious cDNA of CTV with little negative effect on the ability of the clonal strain to infect, multiply in and systemically invade some citrus genotypes (Satyanarayana *et al*. 2008).

The only CTV protein with no homologue in other closteroviruses is p23. In infected plants, p23 accumulates at moderate levels compared to other viral proteins (Pappu *et al*. 1997), but p23 sgRNA is the second most abundant viral mRNA in infected tissues or protoplasts (Hilf *et al*. 1995; Navas-Castillo *et al*. 1997). Furthermore, p23 sgRNA accumulates earlier than the other sgRNAs in infected protoplasts, suggesting an involvement of the p23 protein in early steps of viral replication or transcription (Navas-Castillo *et al*. 1997). Dolja *et al*. (1994) showed the presence of a cluster of positively charged amino acid residues in p23, and López *et al*. (1998) further characterized this conserved region which is rich in cysteine and histidine residues in the core of a putative zinc-finger domain. All these results suggested a regulatory function for p23, a view that was further supported by the finding that *in vitro*, p23 binds RNA in a sequence non-specific manner, and that mutations affecting the cysteine and histidine residues increase the dissociation constant of the p23–RNA complex (López *et al*. 2000). Additionally, p23 is involved in regulating the synthesis of plus and minus strands during RNA replication, with the zinc finger domain and an adjacent basic region being indispensable for asymmetrical accumulation of the plus strand (Satyanarayana *et al*. 2002).

p23-transgenic Mexican limes show CTV-like symptoms

Considering its regulatory role in CTV replication, we decided to explore whether over-expression in transgenic plants of p23 could affect the normal CTV infectious process. Mexican lime (*Citrus aurantifolia* (Christm) Swing.) was selected for genetic transformation because it is very sensitive to CTV and shows symptoms, such as vein clearing, leaf cupping, stunting and stem pitting, with most isolates of this virus (Roistacher 1991). The transformation vector was prepared by cloning the *p23* gene into the binary plasmid pBin19-s*gfp* under the doubly enhanced *Cauliflower mosaic virus* (CaMV) 35S promoter and the nopaline synthase terminator (*nos*-ter). This expression cassette was flanked by the selectable neomycin phosphotransferase II gene (*npt*II), between the *nos* promoter (*nos*-pro) and the *nos*-ter, and by the reporter gene of the green fluorescent protein (*gfp*) between the 35S promoter and the *nos*-ter. The transformation of internodal stem segments from lime seedlings was mediated by *Agrobacterium tumefaciens* (Ghorbel *et al.* 1999). Fifty independent transformants were obtained in three different transformation experiments. Expression of the green fluorescent protein (GFP), monitored under blue light, was observed in all the transformants. Integration of the *p23* transgene in all these plants was confirmed by Southern blotting analysis. In a parallel control experiment using the plasmid pBin19-s*gfp*, 10 transformants harbouring only the *npt*II and *gfp* genes were obtained. During the *in vitro* culture process, transgenic plants containing the *p23* gene were visually normal and indistinguishable from controls carrying only the marker genes.

Fig. 1 CTV-like symptoms exhibited by *p23*-transgenic limes. (**A**) Chlorotic pinpoints and vein clearing in leaves from a transgenic lime carrying the *p23*gene from CTV-T36. (**B**) Growth interruption in a transgenic lime carrying the *p23* gene from the mild CTV strain T317.

The 60 transgenic plantlets were side-grafted on vigorous rough lemon (*C. jambhiri* Lush) seedlings in the greenhouse. One month later, all grafts from the transgenic controls had sprouted, whereas 16 of the *p23*-containing transgenic grafts never sprouted. These grafts progressively showed chlorotic leaf spots and stem necrosis and died within a few weeks or months. The rest of the *p23*-containing grafts sprouted later than the controls and displayed severe vein clearing similar to that incited by CTV in both young and mature leaves; young leaves also developed chlorotic pinpoints (**Fig. 1A**). Six months after being transferred to the greenhouse, most plants exhibited leaf cupping. In the following growth, symptoms appeared again with variable intensity depending on the lines. These included leaf epinasty, apical necrosis, and growth interruption or stunting in the most severe cases. In general, the phenotype of the *p23*-containing transgenic plants strongly resembled the symptoms of non-transgenic Mexican lime inoculated with CTV, although vein clearing was less prominent in the latter. The aberrant phenotype observed in *p23*-containing transgenic plants could not be attributed to somaclonal variation or to epigenetic effects of the transformation/regeneration process, because none of the transgenic control lines displayed these alterations (Ghorbel *et al.* 2001). To corroborate that CTV-like symptoms were not caused by an accidental CTV infection, all transgenic lines were analysed by ELISA using monoclonal antibodies 3DF1 and 3CA5 against the CTV coat protein (Vela *et al*. 1986), with a negative reaction in all cases.

CTV-like symptoms usually developed in the first growth of the transgenic grafts, approximately 2 months after side grafting on the rough lemon rootstock. Similar results were observed when buds of the transgenic plants were propagated on the less vigorous citrus rootstocks sour orange (*C. aurantium* L.) and Carrizo citrange (*C. sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.). Phenotypic aberrations were particularly conspicuous in growing flushes, similar to that occurring with symptom development in a CTV infected Mexican lime (Roistacher 1991). Also, by analogy with CTV infection, stem pitting in transgenic plants was a late phenotypic effect that became evident only six months after grafting. Interestingly, 3 out of the 50 *p23*-

transgenic lines were visually normal and developed similarly to controls transformed with the empty vector or non-transformed plants. Later, it was revealed that this lack of developmental aberrations was correlated with high transgene loci number, transgene methylation, and accumulation of transgene-derived and specific small interfering RNAs (siRNAs), which are hallmarks of post-transcriptional gene silencing (PTGS) (Fagoaga *et al*. 2006).

p23 protein accumulation in transgenic Mexican lime is required for induction of CTV-like symptoms

To examine whether the CTV-like symptoms resulted from accumulation of the *p23* transcript itself or from its translation product, a modified *p23* truncation construct (tr-*p23*) was prepared containing a frameshift mutation in the *p23* ORF. This was produced by a deletion of two nucleotides that generated a stop codon after amino acid residue 14 of p23. A second set of transgenic Mexican limes was obtained with the tr-*p23* construct, using the wild-type *p23* gene construct as an internal control. Integration of the tr-*p23* transgene was confirmed by Southern blot analysis. As in the previous experiment, transgenic plants carrying the wild-type *p23* construct displayed CTV-like symptoms, with a proportion of the plants showing stem necrosis and collapse. However, all of the transgenic lines carrying the tr-*p23* construct grew normally and exhibited normal phenotypes. In Northern blot analysis with a *p23*-specific probe, the visually normal tr-*p23* transgenic plants expressed levels of the mutated *p23* transcript comparable to or higher than those found in the wild-type *p23*-transgenic plants showing CTV-like symptoms. The hybridization signals reflected the levels of *p23* or tr-*p23* transcripts, because all lanes were loaded with similar amounts of RNA. These results demonstrated that accumulation of the p23 protein, rather than that of the *p23* transcript incited the CTV-like symptoms in the transgenic Mexican lime plants.

The intensity of CTV-like symptoms parallels the accumulation level of the p23 protein

To analyse the accumulation of *p23* transcript in transgenic plants, the RNA fraction insoluble in 2 M LiCl was subjected to a Northern-blot analysis. The *p23*-specific probe hybridized with the corresponding transcript in *p23*-transgenic plants that was absent in RNA extracts from the transgenic controls carrying only the vector. Presence of the *p23* transcript in transgenic plants was therefore associated with the expression of CTV-like symptoms and, moreover, the intensity of these symptoms was directly correlated with the accumulation level of the transcript in young and adult leaves.

The expression of the p23 protein in each transgenic line was examined by Western-blot analysis. Most *p23*-transgenic plants showed detectable amounts of the p23 protein, and its accumulation level also paralleled the intensity of the CTV-like symptoms.

For example, those lines that displayed very prominent CTV-like symptoms accumulated high levels of p23, whereas those ones that exhibited mild symptoms had low to moderate levels of p23. The Western-blot signals were a direct reflection of the levels of p23, because all lanes were loaded with similar amounts of protein. These results strongly suggest that expression of the CTV *p23* gene in transgenic Mexican lime causes an onset of symptoms similar to those produced by CTV in this host. Transgenic plants generally accumulated higher p23 levels than non-transformed controls infected with a severe CTV isolate, and similar observations were made when the experiments were repeated using tissues of different ages.

These results provided an explanation for the early and intense CTV-like symptoms that were observed in most transformants when compared with non-transgenic CTV-inoculated plants. Currently, we are generating transgenic Mexican lime plants carrying the *p23* transgene under the control of a phloem-specific promoter to assess whether the high intensity of CTV-like phenotypes in *35S::p23* transformants is also correlated with accumulation of the p23 protein in cells and tissues that are not infected by CTV.

The *p23* gene from a severe and a mild CTV strain incited similar CTV-like phenotypes and their intensity correlates with p23 accumulation irrespective of the source strain

To explore whether the *p23* source could influence symptom expression, Mexican limes were transformed with the *p23* gene from T317, a CTV strain that produces only mild vein clearing in young leaves. Transformation was performed with *Agrobacterium tumefaciens* harboring either an empty pBin19 vector or containing the *p23*-T317, *p23*-T36, or *p23*-tr versions. After 4 to 12 weeks, NPTII-resistant shoots positive for the expression of the green fluorescent protein (GFP) gene were regenerated. In all, 20 transgenic lines with the *p23*-T317, 20 with the p23-T36, 10 with the *p23*-tr, and 10 with the empty vector were selected. Integration of the *p23*-derived transgenes, or of the synthetic GFP *(sgfp)* transgene in the case of the empty vector, was confirmed by Southern blot analysis. Northern blot analysis showed variable transgene expression depending on the line, with an inverse correlation being observed between the transgene copy number and mRNA expression.

Soon after transfer to the greenhouse, five of the *p23*-T317 lines started showing severe developmental aberrations-leaf abscission, apical necrosis, and stem necrosis-and died several weeks later. The remaining *p23*-T317 lines showed CTV-like symptoms that ranged from mild vein clearing to leaf epinasty, apical necrosis, and stunting, except for two lines that were symptomless. These symptoms were essentially similar to those observed in *p23*-T36 transgenic limes (**Fig. 1A, 1B**). Extracts from the transgenic plants did not react with a mixture of the monoclonal antibodies 3CA5 + 3DF1 against the CTV major coat protein, thus discarding accidental infection with CTV.

The p23 protein accumulated in most transgenic limes carrying either *p23*-T36 or *p23*-T317, as revealed by reaction with the p23 antiserum in Western blots, but not in plants carrying *p23*-tr or the empty vector. More specifically, accumulation of p23 (and of its mRNA) was positively correlated with the intensity of the CTV-like symptoms exhibited by the transgenic limes, irrespective of the pathogenicity characteristics of the CTV strain (T36 or T317) from which the *p23* gene was obtained. Therefore, the accumulation of p23 rather than its origin seems to determine the intensity of the symptoms in transgenic limes.

In spite of the development of severe developmental aberrations, most transgenic lines were able to flower and fruit regularly. Seeds from two transgenic lines carrying *p23*-T36 and from two transgenic lines carrying *p23*-T317, all 4 showing highly conspicuous CTV symptom-like phe-

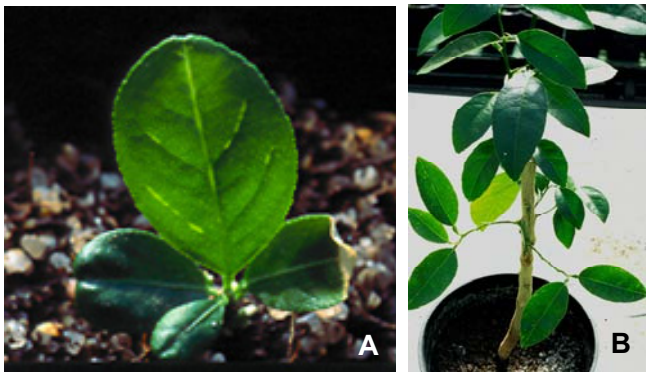

Fig. 2 (**A**) *p23*-T317 progeny seedling from a transgenic lime showing CTV-like vein clearing phenotype. (**B**) Non-symptomatic Mexican lime flush from a non-transformed bud grafted onto a *p23*-transgenic rootstock showing a CTV-like phenotype.

notypes, were sown in sterile soil to assess whether developmental abnormalities were transmitted to the progeny. Soon after germination all seedlings from the 4 transgenic lines started to show vein clearing and chlorotic pinpoints (**Fig. 2A**), indicating that the trait was not only inherited, but also expressed very early and throughout the entire life cycle of the transgenic plants.

To investigate whether p23-induced aberrations could be translocated from transgenic tissues to non-transformed cells, non-transgenic Mexican lime buds were grafted onto highly expressing *p23*-transgenic rootstocks. New non-transformed flushes sprouted normally without showing any sign of CTV-like phenotype, while transformed rootstock leaves were severely affected by distortion and vein clearing (**Fig. 2B**), suggesting that either the p23 transgenic protein could not move cell-to-cell through plasmodesmata, it could not enter into phloem tissue or it could not be unloaded from phloem into the non-transformed scion.

CTV susceptible and resistant citrus species and relatives transformed with *p23* gene, but not with a truncated version, also show CTV-like leaf symptoms

To further elucidate the role of p23 as a pathogenicity factor, citrus species and relatives with different susceptibility to CTV were transformed with the *p23* gene. Whereas Mexican lime is very sensitive to CTV, sweet orange is susceptible, but only shows symptoms when infected with severe strains, sour orange is susceptible to CTV strains that induce seedling yellows, and trifoliate orange is resistant to the virus because most CTV strains are unable to establish infections. Resistance of trifoliate orange cv. 'Burjasot' to CTV-T36 was confirmed by graft inoculation: DAS-ELISA and RT-PCR failed to detect the virus in samples taken at 6, 12, and 24 months after inoculation. Furthermore, simple sequence repeat (SSR) markers associated with the CTV-resistance locus (provided by M. Roose) (Yang *et al*. 2003) showed heterozygosity for this region in trifoliate orange cv. 'Burjasot'.

Internodal stem segments of sweet orange cv. 'Pineapple' and sour orange cv. 'Sevillano' and epicotyl segments of trifoliate orange cv. 'Burjasot' were transformed with *A. tumefaciens* carrying either the *p23*-T36 or the *p23*-tr version. Regeneration of transgenic shoots from sweet and sour orange and trifoliate orange usually occurred with low efficiency, particularly with *p23*-T36, strongly suggesting that *p23* integration and expression were toxic for these three species. The early effect of p23 during the *in vitro* culture stage was more prominent than that previously observed with Mexican lime.

In the greenhouse, after grafting transgenic plantlets onto vigorous rough lemon (*C. jambhiri* Lush) seedlings, about half of the sweet orange, sour orange, and trifoliate orange lines carrying the *p23* transgene showed severe abnormalities: they did not sprout, became necrotic, and died several weeks later. Shoots from the surviving grafts exhibited CTV-like leaf symptoms and developmental aberrations of different severity depending on the transgenic line and citrus type. The CTV-like phenotype observed in transgenic sour and sweet orange plants generally included vein clearing, epinasty, and stunting, which are produced in these species only by very severe CTV strains. Other aberrations, such as leaf distortion, apical necrosis, and leaf abscission, were also found (**Fig. 3A, 3B**). Interestingly, most *p23*-transgenic plants showed either aberrations or normal phenotype in alternate growing flushes, suggesting a complex relationship between the transgene expression and the developmental stage. Two *p23*-sweet orange plants exhibiting the most severe necrosis, leaf distortion, and stunting died after 2 years in the greenhouse. Transgenic *p23*-trifoliate orange plants showed chlorosis, leaf abscission, stem necrosis, stunting, and apical necrosis, which caused the death of one 1-year-old plant. On the other hand, the *p23*-tr transgenic plants of the three species were phenotypically normal and developed as the non-transgenic controls (**Fig. 3C**).

Fig. 3 CTV-like vein clearing phenotypes of leaves from sweet orange (**A**) and sour orange (**B**) transformants carrying the *p23* gene from CTV. (**C**) A *p23*-trifoliate orange plant showing developmental aberrations that include leaf abscission and stunting (right) compared with a *p23*-tr control plant (left).

Intensity of CTV-like leaf symptoms in citrus species other than Mexican lime correlates with *p23* expression

Integration and expression of *p23* transgenes were confirmed in the three species by Southern and Northern blot analyses, respectively. The number of transgene loci was variable, ranging from one to eight, with levels of *p23*-T36 and *p23*-tr transcripts generally showing an inverse correlation with the number of copies. In plants harboring one or two copies of the transgene, the severity of the CTV-like leaf symptoms generally correlated with the accumulation of *p23*-transcripts. However, Western blot analysis only revealed the presence of p23 in a few sweet and sour orange plants carrying one or two copies of *p23*-T36.

To test whether the low or undetectable levels of p23 in transgenic plants paralleled those in non-transgenic CTV-infected plants, several citrus species with different susceptibility to CTV were inoculated with the severe strains T36 or T305. Whereas sensitive hosts, such as *C. macrophylla*, Mexican lime, and grapefruit, showed high to moderate accumulation of p23, the protein was barely detectable in sweet and sour orange. These results were reproduced with leaves at different developmental stages. The parallelism found in the accumulation of p23 between *p23*-transgenic and CTV-infected non-transgenic Mexican lime, and sweet and sour orange, suggests that the latter two species cannot accumulate p23 at high levels and that even minimal amounts of this protein cause deleterious effects in these two species.

It is assumed that in virus-infected plants, symptoms are caused by metabolic changes induced by the virus and different viral proteins have been identified as pathogenicity determinants. A strategy to test the role of certain viral proteins as pathogenicity factors has been to make these proteins disfunctional by mutation, to alleviate their expression from the viral genome, or to substitute them with a homologue from a related virus (Li *et al*. 1999; Kasschau and Carrington 2001; Yelina *et al*. 2002; Havelda *et al*. 2003; Himber *et al*. 2003). Unfortunately, this cannot be done with p23 because it is CTV specific and essential for virus replication (Satyanarayana *et al*. 1999). To circumvent this limitation, we have compared the ectopic expression of p23 in citrus species and relatives with different CTV susceptibility: sweet, sour, and trifoliate orange. Although CTV-T36 causes symptomless infection in sweet orange, transgenic expression of *p23*-T36 induced leaf symptoms resembling those produced by very severe CTV strains in this host. Similarly, CTV-T36 incites in sour orange seedling yellows and stunting, but not the vein clearing and epinasty observed in transgenic plants expressing p23-T36. Finally, morphological alterations caused by the ectopic expression of p23 in trifoliate orange were somehow unexpected

because this species is resistant to CTV infection. However, CTV is able to replicate in trifoliate orange protoplasts as efficiently as in sweet orange protoplasts, suggesting that resistance results from a defect in virus movement (Albiach-Marti *et al*. 2004). Interestingly, trifoliate orange genotypes lacking the markers associated with the CTV resistance locus can be infected systemically by CTV and display leaf symptoms similar to those here observed in the *p23*-transgenic trifoliate orange (Broadbent *et al*. 2000; P. Broadbent and D. Hailstones, pers. comm.).

Although *p23* overexpression occasionally led to necrosis and plant death in Mexican lime, this deleterious effect was more generalized in sweet, sour, and trifoliate orange, in which high levels of the transcript, but not of the protein, were observed. Reduced accumulation in transgenic plants of other viral pathogenicity factors has been reported before (Van der Wilk *et al*. 1997; Silhavy *et al*. 2002) and interpreted as the consequence of rapid degradation of these proteins due to their toxicity.

Transgenic expression of p23 in CTV non-host *Nicotiana* spp. does not induce phenotypic aberrations

To investigate whether transgenic expression of *p23* was sufficient to produce developmental aberrations in other plants irrespective of their CTV host or nonhost condition, tobacco (*Nicotiana tabacum*) and *N. benthamiana* plants were transformed with the *p23*-T36 and *p23*-tr constructs. *Nicotiana* spp. are non-host for CTV; however, whereas *N. benthamiana* protoplasts support virus replication, protoplasts of *N. tabacum* do not (Navas-Castillo *et al*. 1997; Albiach-Marti *et al*. 2004). Transgenic plants of *N. tabacum* and *N. benthamiana* were generated and grown in the greenhouse. Western blot analysis showed the accumulation of p23 in all *Nicotiana* plants transformed with *p23*-T36 that exhibited normal phenotypes and developed as non-transformed controls. This indicated that p23 interferes with plant development only in citrus species and relatives.

CONCLUSIONS AND FUTURE DIRECTIONS

Important clues about the underlying mechanism of viral pathogenesis in plants have come from the discovery of RNA silencing pathways that function as inducible host defense reactions against viruses. RNA silencing is a sequence-specific mechanism of inhibition of gene expression evolutionarily conserved in most eukaryotes and relies on the perception of double-stranded RNA (dsRNA) as the trigger of a series of core reactions that start with dsRNA cleavage by RNase III-like enzymes (DICER-like, DCL) into RNA duplexes of 21-25 nt called small interfering RNAs (siRNAs) (Hamilton and Baulcombe 1999; Bernstein *et al*. 2001). Upon unwinding, one of the siRNA strands is incorporated into the RNA-induced silencing complex (RISC) and programs it to degrade complementary single-stranded RNA (Hammond *et al*. 2000). Another class of host-encoded small RNAs are the microRNAs (miRNAs), which consist of short (21–24 nt), single-stranded RNA molecules derived from transcripts of endogenous plant loci that are also incorporated into RISC, and target endogenous plant transcripts for degradation or translational repression in a sequence-specific manner (Llave *et al*. 2002; Aukerman and Sakai 2003). MicroRNAs (miRNAs) play vital roles in regulating plant development (Kidner and Martienssen 2005).

Virus-infected plants also produce virus-derived siRNAs presumably arising from dsRNA replicative intermediates and from viral single-stranded RNA (ssRNA) with extensive secondary structure, or after being converted into dsRNA by a host RNA-dependent-RNA polymerase (Hamilton and Baulcombe 1999; Dalmay *et al*. 2000; Molnar *et al*. 2005). These observations have led to propose that RNA silencing arose as a defense mechanism against viruses in primitive eukaryotes (Baulcombe 2004). To counteract this defense mechanism, many plant viruses encode specific silencing suppressors, which allow the viruses to overcome RNA silencing and proliferate in their specific hosts. The importance of these suppressors for viral infections is reflected by the fact that many suppressors have been previously identified as viral cell-to-cell or long-distance movement proteins, essential for spread in their hosts (Voinnet 2005; Xie and Guo 2006). Virus mutants that lack functional suppressors accumulate at low levels and often are restricted to inoculated cells or leaves (Voinnet *et al.* 1999; Kasschau and Carrington 2001; Qiu *et al*. 2002; Silhavy *et al*. 2002; Yelina *et al*. 2002; Havelda *et al*. 2003; Himber *et al*. 2003). Silencing suppressor proteins encoded by unrelated RNA and DNA viruses bear no similarity to each other in either coding sequence or protein structure, suggesting separate origins and variable functional mechanisms for each suppressor type. In recent years, the interactions of different silencing suppressors with the RNA silencing pathways have been studied intensively. It has been found that the silencing suppressor activities of viral proteins, such as p19 of *Tomato bushy stunt virus* (TBSV, *Tombusvirus*), p21 of *Beet yellows virus* (BYV, *Closterovirus*) and HC-Pro of *Turnip mosaic virus* (TuMV, *Potyvirus*) each inhibit the intermediate step of RNA silencing via binding to 21-nucleotide siRNAs. On the other hand, P25 cell-to-cell movement protein of *Potato virus X* (PVX, *Potexvirus*), P38 coat protein of *Turnip crinkle virus* (TCV, *Carmovirus*), and P50 movement protein (MP) of the *Apple chlorotic leaf spot virus* (ACLSV, *Trichovirus*) primarily prevent the short- or long-distance spread of the RNA silencing signal within the plant (Dunoyer *et al.* 2004; Bayne *et al*. 2005; Deleris *et al*. 2006; Yaegashi *et al*. 2007). Interestingly, overexpression of some of these suppressors in transgenic plants has led to the generation of more or less disturbed phenotypes depending on the host plant and the specific suppressor tested, suggesting that they were interfering with RNA silencing pathways. More recently, it has been shown that viral suppressors (e.g., P1/HcPro, P19, P15 of *Peanut clump virus* [IPCV, *Pecluvirus*), and P21), interfered with miRNA biosynthesis in transgenic *Arabidopsis* plants that either inhibited or enhanced the cleavage of target mRNAs involved in plant development (Ray *et al*. 1996; Jacobsen *et al*. 1999; Llave *et al*. 2002; Mallory *et al*. 2002; Park *et al*. 2002; Kasschau *et al*. 2003; Chapman *et al*. 2004; Chen *et al.* 2004; Dunoyer *et al*. 2004; Mallory *et al*. 2004; Vazquez *et al*. 2004; Chellapan *et al*. 2005; Millar and Gubler 2005; Alvarez *et al*. 2006). The phenotype caused by overexpression of the 2b protein of *Cucumber mosaic virus* (CMV, *Cucumovirus*) in transgenic *Arabidopsis* varied from no symptom expression to severe, depending on the mild or aggressive nature, respectively, of the viral strain from which the transgene was isolated (Lewsey *et al*. 2007). All this suggested that disruption of miRNA metabolism is an effect of many plant silencing suppressor molecules and that it may be one of the most common mechanisms underlying viral symptom induction (Voinnet 2005).

The similarity between CTV symptoms and the alterations induced by ectopic expression of CTV *p23* in citrus hosts, the recent characterization of p23 as RNA silencing suppressor in *N. benthamiana* and *N. tabacum* (Lu *et al*. 2004), and its RNA-binding properties (López *et al*. 2000) suggest that p23 could also exert its effects, at least in part, through a parallel miRNA-mediated mechanism in CTV woody hosts. Despite being a strong silencing suppressor in *N. benthamiana* and *N. tabacum* (Lu *et al*. 2004), p23 accumulates efficiently in transgenic *Nicotiana* spp. without causing any developmental aberration, likely indicating that the miRNA-mediated development and the siRNA-mediated defense pathways could be only partially overlapping, as previously described in *Arabidopsis* (Dunoyer *et al*. 2004; Lewsey *et al*. 2007). These results also discard that p23 may follow a molecular mechanism similar to that of the p19 from Tombusviruses, because this silencing suppressor operates through siRNA sequestration and, therefore, exerts its effects in a broad range of organisms, including

human and *Drosophila* cells (Dunoyer *et al*. 2004; Lakatos *et al*. 2004). The particular toxicity of p23 for citrus and relatives suggests the involvement of a citrus-specific factor that is absent in *Nicotiana* spp. Tandem affinity purification experiments could help in locating p23-citrus proteome specific interactions that would explain CTV-induced symptomatology in citrus hosts.

One of the most economically important CTV effects is the decline of the majority of citrus cultivars grafted on sour orange, which has caused the death of millions of trees propagated on this rootstock (Bar-Joseph *et al*. 1989). Virus infection in the scion causes phloem necrosis on the sour orange rootstock immediately below the bud union, leading successively to girdling, depletion of starch reserves in the rootstock, and tree decline (Lee and Bar-Joseph 2000). We have proposed that this could result from p23 downloading from the CTV-infected scion into the sour orange rootstock, which is highly sensitive to the accumulation of this protein. Interestingly, sweet orange is also highly sensitive to p23 accumulation but, when used as a rootstock, infected trees do not decline. However, whereas CTV usually reaches high titers in sweet orange, most virus strains are barely detectable and unevenly distributed in sour orange. Mapping of p23 regions involved in CTV pathogenesis through the generation of transgenic citrus plants expressing p23-derived fragments, and working out the host developmental genes that likely become overexpressed or downregulated in *p23*-transgenic citrus plants through microarray analysis of the citrus transcriptome, may shed light on the molecular basis of symptom expression and help in developing strategies to control CTV-induced damage.

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