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Detection of citrus leaf blotch virus using digoxigenin-labeled cDNA probes and RT–PCR

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Abstract

Citrus leaf blotch virus (CLBV) was detected by dot-blot hybridization (DBH), and tissue print hybridization (TPH) and by one-step RT–PCR in citrus plants growing both in the greenhouse and in the field. DBH with digoxigenin-labeled cDNA probes allowed CLBV detection in dsRNA-rich and total RNA preparations equivalent to 5 and 0.1 mg of infected tissue, respectively. DBH gave intense signals with RNA extracts from young bark, tender shoots and young leaves, whereas the best hybridization signals with TPH were obtained using tender shoots and young leaf petioles. One-step RT–PCR was 10-fold more sensitive than DBH and amplification was obtained with all infected tissues. CLBV was readily detected in young leaves of infected Eureka lemon, Marsh grapefruit, Nules clementine, Navelina orange and Nagami kumquat in the greenhouse, using either hybridization or RT–PCR, but not in leaves of Pineapple sweet orange. Detection in field trees was less consistent and was only achieved by RT–PCR and DBH. CLBV was detected by DBH and RT–PCR in different citrus varieties from several geographic areas showing bud union crease on trifoliate rootstocks, but not in neighbor trees with the same symptoms or in other varieties showing bud union crease on those rootstocks. Failure to detect CLBV in trees with bud union crease could be due to low virus titer or uneven distribution within the plant. Alternatively, a different agent could be involved in causing bud union crease.

Abbreviations: CLBV – Citrus leaf blotch virus; DBH – dot-blot hybridization; DIG-probes – digoxigenin-labeled cDNA probes; TPH – tissue print hybridization.

Introduction

Declines associated with bud union disorders have been described in different countries affecting several citrus species and cultivars propagated on *Poncirus trifoliata* and its hybrids citrange (*Citrus sinensis* × *P. trifoliata*) or citrumelo (*Citrus paradisi* × *P. trifoliata*) rootstocks. Knowledge of these disorders is limited and data reported are often confusing. Some of them are probably of genetic origin (Nauriyal et al., 1958; Schneider, 1978), but others are known to be caused by pathogens (Calavan et al., 1963). In several cases, the nature of these bud union disorders has not been determined. In Corsica, two clones of Nagami kumquat (*Fortunella margarita*) showed bud union disorders when propagated on Troyer citrange (Vogel and Bové, 1988). In Israel, bud union crease was observed in some lines of Shamouti sweet orange (*C. sinensis*) on Troyer citrange or citrumelo, and in Nagami kumquat on Troyer citrange (Ashkenazi, 1988). Decline associated with bud union disorders on *P. trifoliata*, citrange or citrumelo rootstocks have also been reported in field trees of Navel sweet orange in California (Anonymous, 1989), Pera sweet orange in Brazil (Salibe, 1965),

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Marsh grapefruit (*C. paradisi*) and Roble sweet orange in Florida (Garnsey et al., 2001) and Nules clementine (*C. clementina*), Navelina and Navelate sweet orange in Spain (unpubl. observ.). Trifoliate rootstocks are important in many citrus growing areas of the world due to their tolerance of *Citrus tristeza virus*, and the high yield and fruit quality that they induce in different citrus varieties. Therefore, dispersal of a graft-transmissible pathogen causing bud union crease on these rootstocks could potentially cause important economic losses.

Recently, citrus leaf blotch virus (CLBV) was purified and characterized (Galipienso et al., 2000, 2001) from a Nagami kumquat, clone SRA-153, showing bud union crease when propagated on Troyer citrange. When plants of Nules clementine or Eureka lemon (*C. limon*) were graft inoculated with bark pieces from this clone, and buds of those plants were propagated on citrange seedlings in the greenhouse, some plants showed bud union crease (Galipienso et al., 2000). To assess the potential association of CLBV with the disorders observed on trifoliate rootstocks and to study the epidemiology of this pathogen, rapid and specific procedures for CLBV detection are necessary. Presently, the most reliable diagnostic method is biological indexing on greenhouse-grown Dweet tangor (*Citrus reticulata* × *C. sinensis*) seedlings, in which it induces chlorotic blotching in young leaves. However, biological indexing is a lengthy, costly process requiring greenhouse facilities and trained personnel and is unsuitable for large scale indexing. Trials to raise an antiserum specific to CLBV were unsuccessful due to the low virion concentration in purified extracts (Galipienso et al., 2001).

Recently the complete nucleotide sequence of the CLBV genomic RNA (gRNA) has been determined and shown to contain three open reading frames (ORFs), encoding a polyprotein involved in replication, a potential movement protein and the capsid protein (Vives et al., 2001). In addition to the gRNA, infected plants contain two sets of 3′ and 5′ co-terminal subgenomic RNAs (Vives et al., 2002a). Availability of the gRNA sequence opened new possibilities for virus detection. In a preliminary paper we reported detection of CLBV by reverse transcription (RT) and PCR amplification using total RNA extracts from various tissues (Vives et al., 2002b). Here we report detection of CLBV by molecular hybridization using digoxigenin labeled cDNA probes (DIG-probes) and nucleic acid extracts or tissue prints, and compare this with the results of RT–PCR previously described, in greenhouse-grown plants and in field trees.

Materials and methods

Virus sources and hosts

The following sources of citrus tissue were used for analysis: (i) Nagami kumquat SRA-153 from Corsica, (ii) Washington navel, Navelina and Pineapple sweet orange, Eureka lemon, Marsh grapefruit and Nules clementine plants graft inoculated with bark pieces of kumquat SRA-153, to test if detection of CLBV was affected by host variety, (iii) freeze-dried tissue of Nagami kumquat from New South Wales, Australia (kindly provided by P. Barkley) and of Navel and Moro Blood sweet orange from California, USA (kindly provided by L. Marais), showing bud union crease on citrange, (iv) freeze-dried tissue of Navel and Roble sweet orange from several commercial orchards in Florida, USA (kindly provided by S.M. Garnsey), showing bud union crease on Swingle citrumelo and (v) field samples of different sweet orange varieties, showing bud union crease on citrange rootstocks, collected in various citrus growing areas of Spain. Sources in groups (iii)–(v) were used to check association between detection of CLBV and the presence of bud union crease on trifoliate rootstocks, and to test if CLBV was present in different geographic areas.

To optimize detection of CLBV in field trees, young leaves and tender shoots (when available) and old leaves were collected from two infected Navelina sweet orange plants grafted on Carrizo citrange in southern Valencia (Spain) in winter, spring and summer, and analyzed by hybridization and RT–PCR.

Nucleic acid extracts

Double stranded RNA (dsRNA) rich extracts were obtained from 10 g of young leaf tissue as described by Moreno et al. (1990), using 20 μg ml^{-1} of glycogen (Roche) as co-precipitant agent. Total RNA extracts were obtained from ~100 mg of fresh or 20 mg of freeze-dried leaf tissues using TRIzol® reagent (Invitrogen), following the manufacturer's instructions for samples with high sugar content. One microliter of serial dilutions of dsRNA-rich extracts (equivalent to 100, 50, 10, 5 and 1 mg of fresh tissue) and total RNA extracts (equivalent to 5, 1, 0.5, 0.1 and 0.05 mg of fresh tissue) from healthy and CLBV infected kumquat (isolate SRA-153), grown in the greenhouse, were used to optimize detection of CLBV by dot-blot hybridization (DBH). To determine the most appropriate tissue for

CLBV detection, 1 μl of total RNA extracts equivalent to 4 mg from tender shoots, young bark and young and old leaves from the same healthy or CLBV-infected kumquat plants, were subjected to DBH and RT–PCR analysis.

Reverse transcription and polymerase chain reaction amplification

For RT–PCR, two sets of primers were designed based on the nucleotide sequence of the CLBV gRNA (EMBL accession AJ318061): Primers KU-27 (5′ GATGCAAGCCAGGATGAATAC 3′, positions 5321–5350) and KU-15 (5′ CAGACACTCCAAGACCTTTCC 3′, positions 5776–5756), located in the RNA-dependent RNA polymerase (RdRp) conserved domains within ORF 1, and primers KU-18 (5′ TTAAGATTACAGACACGAAGG 3′, positions 7686–7706) and KU-19 (5′ CTGTTTTTGAATTTTGCTCG 3′, positions 8123–8104), containing the conserved C-terminal region of the coat protein gene (CP) (Vives et al., 2001, 2002c). For cDNA synthesis, 1 μl of dsRNA-rich or total RNA preparations and 0.2 μM of each primer were denatured at 85 °C for 5 min and chilled on ice. Then, one-step RT–PCR was performed in a 25 μl reaction volume containing 1 × PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 3 mM $MgCl_2$, 0.4 mM dNTPs, 4 units Rnase OUT™ ribonuclease inhibitor, 20 units SuperScript™ II Rnase H-reverse transcriptase and 1 unit *Taq* DNA polymerase (Invitrogene). The reaction mix was incubated at 42 °C for 45 min for RT and then the enzyme inactivated at 94 °C for 2 min. Thermocycling conditions were: 40 cycles of 20 s at 94 °C, 20 s at 50 °C (for KU-27 and KU-15 primers) or 45 °C (for KU-18 and KU-19 primers) and 30 s at 72 °C, followed by a final extension of 5 min at 72 °C. The PCR reaction products were separated by electrophoresis in 2% agarose gels in 1 × TAE buffer (40 mM Tris–acetate pH 8.3, 1 mM EDTA) and visualized on a UV-transilluminator after ethidium bromide staining.

Molecular hybridization

The cDNA obtained from CLBV isolate SRA-153 by RT–PCR using primers KU-27 and KU-15 (RdRp probe), or primers KU-18 and KU-19 (CP probe), was cloned in the pGEM-T plasmid vector (Sambrook et al., 1989). The cloned cDNA was DIG-labeled using the same primers and the PCR DIG-labeling and detection kit (Roche). Detection of CLBV was assayed by DBH and by tissue print hybridization (TPH). For DBH, 1 μl of either total RNA or dsRNA-rich extracts was denatured at 94 °C for 5 min in 50% formamide, chilled on ice, and then spotted onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech). For TPH, tender shoots or leaf petioles were transversally cut with a razor blade and the fresh cuts gently pressed on the membrane (Narváez et al., 2000). Usually four prints were prepared from each leaf or shoot. Hybridization essentially followed the protocol described by Narváez et al. (2000). Briefly, dot-blotted and tissue-printed membranes were air dried and UV-irradiated (50 mJ) in a cross-linking oven, prehybridized for 2 h at 55 °C in 0.02% SDS, 50% formamide, 5 × SSC (750 mM NaCl, 75 mM sodium citrate pH 7), 2% (w v^{-1}) blocking reagent (Roche) and 0.1% (w v^{-1}) N-lauroylsarcosine and then hybridized in the same solution at 55 °C for 16 h after adding the DIG-probe. After hybridization, membranes were washed twice in 2 × SSC and 0.1% (w v^{-1}) SDS at room temperature for 15 min, incubated for 30 min with anti-DIG antibodies conjugated with alkaline phosphatase, and washed twice with maleate buffer added with 0.3% Tween-20. The reaction was developed using CSPD chemiluminescent substrate (Roche) and Omat-S film (Kodak).

Results

Development of a hybridization procedure for CLBV detection

Positive hybridization by DBH was obtained with dsRNA-rich and total RNA preparations from CLBV infected kumquat equivalents to as little as 5 and 0.1 mg of fresh tissue, respectively, whereas no hybridization signal was observed with equivalent extracts from healthy plants (Figure 1). Detection of CLBV RNA by one-step RT–PCR was at least 10-fold more sensitive than molecular hybridization with either dsRNA-rich or total RNA extracts (data not shown). Similar results were obtained with probes RdRp and CP or with the primer sets KU-27/KU-15 and KU-18/KU-19 (data not show), therefore, only data obtained with probe RdRp and with primers KU-27/KU-15 will be presented.

Total RNA extracts equivalent to 4 mg of infected kumquat tissue, obtained from tender shoots, young bark and young leaves yielded intense hybridization signals by DBH, whereas only mild or faint signals were obtained with RNA extracts from old leaves

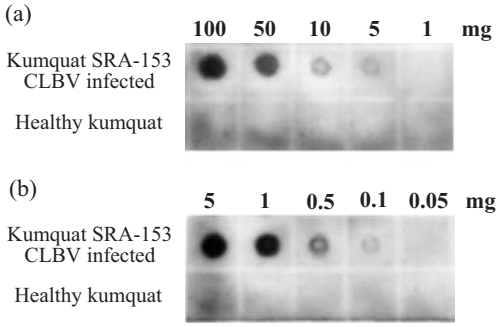

Figure 1. DBH of the RdRp probe with serial dilutions of dsRNA-rich (a) or total RNA (b) preparations of healthy or CLBV-infected (isolate SRA-153) kumquat, equivalent to the indicated weight of fresh tissue.

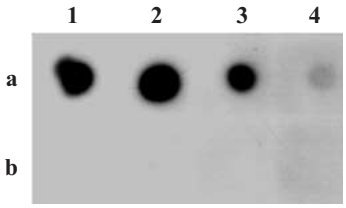

Figure 2. DBH of the RdRp probe with total RNA extracts equivalent to 4 mg of fresh tissue from young bark (1), tender shoot (2), young leaf (3) and old leaf (4) of kumquat infected with CLBV isolate SRA-153 (a) or healthy (b).

(Figure 2). RT–PCR amplification from all tissue sources was similar. No hybridization signal or PCR amplification were observed with equivalent tissues from healthy kumquat (data not shown). Since leaves are easier to handle, this tissue was used in subsequent experiments.

In TPH analyses, a strong hybridization signal was observed with tissue prints from tender shoots and young leaf petioles, a weak signal with prints from old leaf petioles, and no signal with prints from rolled young or old leaf blades. No hybridization was observed in equivalent tissue prints from healthy plants (Figure 3).

Six young leaves and six old leaves from single plants of Navelina and Pineapple sweet orange, Eureka lemon, Marsh grapefruit and Nules clementine graft inoculated with the CLBV isolate SRA-153 were analyzed by DBH and RT–PCR, and six tender shoots were analyzed by TPH. A Nagami kumquat plant infected with CLBV SRA-153 was used as positive control and a healthy kumquat as negative control. CLBV RNA was readily detected by all methods assayed in all

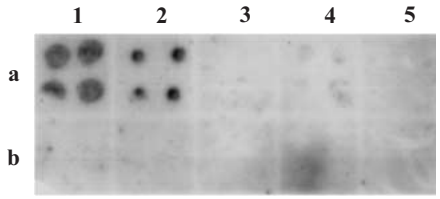

Figure 3. TPH of the RdRp probe with imprints from tender shoots (1), young leaf petioles (2), rolled young leaf blades (3), old leaf petioles (4) and rolled old leaf blades (5) of kumquat infected with CLBV isolate SRA-153 (a) or healthy (b).

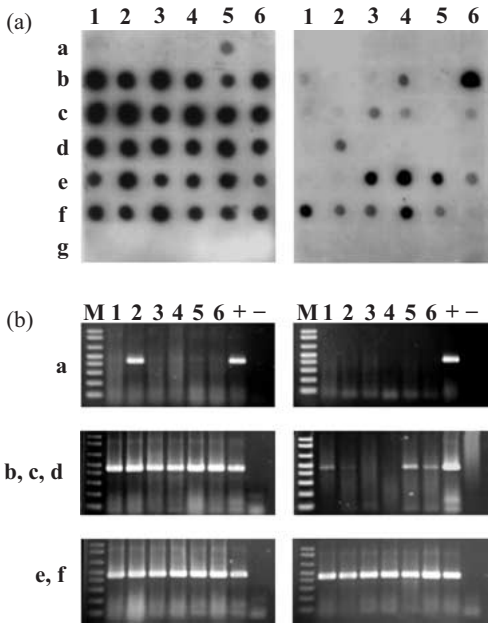

Figure 4. Detection of CLBV by DBH (a) or RT–PCR (b) using total RNA extracts from young leaves (left panels) or old leaves (right panels) of the following citrus varieties: Pineapple sweet orange (a), Eureka lemon (b), Marsh grapefruit (c), Nules clementine (d), Navelina sweet orange (e) and Nagami kumquat infected with CLBV (f) or healthy (g). Numbers on the top correspond to different samples of the same plant (1–6). Nagami kumquat infected with CLBV isolate SRA-153 was used to graft inoculate the other citrus varieties, and was also included as positive (+) control for RT–PCR. Healthy kumquat was used as negative (−) control. The RdRp probe and primers KU-27/KU-15 were used for DBH and RT–PCR, respectively. M, 1 Kb Plus DNA Ladder (Invitrogen).

young leaves and tender shoots of Nagami kumquat, Navelina sweet orange, Eureka lemon, Marsh grapefruit and Nules clementine, but only one out of six Pineapple sweet orange leaves reacted positively by some of the procedures used (Figures 4 and 5).

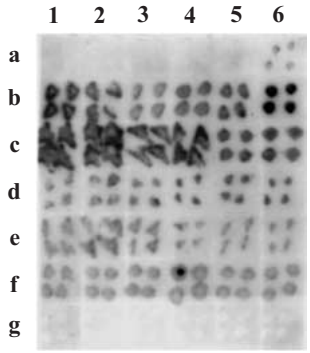

Figure 5. TPH of the RdRp probe with imprints of young shoots from Pineapple sweet orange (a), Eureka lemon (b), Marsh grapefruit (c), Nules clementine (d), Navelina sweet orange (e) and Nagami kumquat infected with CLBV isolate SRA-153 (f) or healthy (g). Numbers on the top (1–6) correspond to different shoots. Nagami kumquat infected with CLBV isolate SRA-153 was used to graft inoculate the other citrus varieties.

CLBV detection in old leaves by DBH, TPH or RT–PCR amplification was inconsistent in some varieties, and the virus was never detected in old Pineapple sweet orange leaves (Figure 4).

Detection of CLBV in field trees

Detection of CLBV was less consistent in field trees than in greenhouse-grown plants, and the best results were obtained in spring and summer. In these seasons, positive reactions were obtained by RT–PCR and DBH in one to four out of six young leaves sampled from each Navelina tree, but differences were observed in the intensity of the hybridization signals or the PCR-amplified DNA bands (Figure 6). Tissue prints from tender shoots usually gave weak hybridization signals and detection was not consistent (data not shown). In winter, zero to one out of six samples from each tree reacted positively by DBH and zero to three samples reacted positively by RT–PCR. To assess reliability of these procedures for routine detection of CLBV, 67 trees were analyzed from an orchard of Navelina sweet orange grafted on Carrizo citrange, in which some trees showed bud union crease. Six young leaves were sampled in the spring from each tree, pooled and total RNA used for DBH and RT–PCR analysis. Six of 67 trees gave a weak hybridization signal and 14 yielded a RT–PCR amplification product. All trees that tested CLBV positive showed severe bud union crease and none of the trees with normal bud union indexed

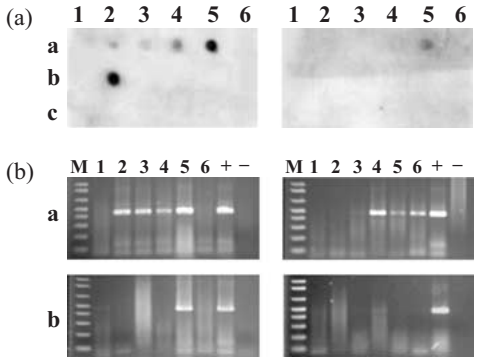

Figure 6. Detection of CLBV by DBH (a) or RT–PCR (b) using total RNA extracts from young leaves (left panels) and old leaves (right panels) of two CLBV-infected Navelina sweet orange field trees (a,b) and a healthy greenhouse-grown Navelina plant (c). Numbers on the top (1–6) correspond to different samples of each plant. Nagami kumquat infected with CLBV isolate SRA-153 (+) and healthy kumquat (−) were used as positive and negative controls for RT–PCR. The RdRp probe and primers KU-27/KU-15 were used for DBH and RT–PCR, respectively. M, 1 Kb Plus DNA ladder (Invitrogen).

positive for CLBV. Only one tree reacting positively by DBH was negative by RT–PCR amplification.

Detection of CLBV in citrus plants from different geographic origins

CLBV was detected by DBH and RT–PCR in a Nagami kumquat from New South Wales (Australia), and in three Roble sweet orange trees grafted on Swingle citrumelo from two different commercial orchards at Haines City and Lake Wales, Florida (USA). However, CLBV was not detected in some trees from the same orchards in Florida, neither in different Navel and Moro sweet orange sources from Florida and California (USA), which also showed bud union crease.

Discussion

These results showed that CLBV can be specifically detected by DBH, TPH and one-step RT–PCR in different citrus species and varieties. One-step RT–PCR allowed reliable detection of CLBV in all types of tissue from greenhouse-grown plants, however, selection of tissue was critical for detection of CLBV by TPH or DBH. CLBV was readily detected by DBH or TPH in tender shoots or young leaves, but detection in old hardened leaves was inconsistent and did not allow a reliable diagnosis.

Routine diagnosis of CLBV by RT–PCR has some disadvantages compared with hybridization: (i) hybridization, particularly TPH, allows simultaneous diagnosis of many plants, while only a limited number of samples can be processed at the same time by RT–PCR, (ii) RT–PCR is prone to give false positives due to cross-contamination, and negative reactions sometimes are due to inhibition of reverse transcriptase and/or *Taq* DNA polymerase by the presence of polyphenolic compounds in plant tissues (Thomson and Dietzgen, 1995; Singh, 1998) and (iii) primer annealing for RT–PCR is more sensitive to nucleotide variations than hybridization with large sized probes, therefore, RT–PCR might not detect some CLBV variants, although we have shown recently that genetic variability of CLBV isolates is very low (Vives et al., 2002c).

Inoculation of CLBV to various hosts showed that citrus varieties may differ in their pattern of viral accumulation. CLBV was readily detected by either DBH or TPH in young shoots and leaves of Nagami kumquat, Nules Clementine, Eureka lemon, Marsh grapefruit or Navelina sweet orange, but detection in Pineapple sweet orange was difficult in young leaves and never achieved in old leaves, even using RT–PCR. This finding indicates that CLBV detection in Pineapple sweet orange, and perhaps in other varieties not tested, might require the analysis of at least 10 individual samples per tree to reduce the risk of false negatives. These results agree with previous data on CLBV detection by biological indexing in which it was observed that graft inoculation from infected kumquat caused intense foliar symptoms in most Dweet tangor and Etrog citron receptor plants, whereas transmission of CLBV from other citrus species, particularly from Pineapple sweet orange, to these indicator plants was more erratic and generally caused milder symptoms (Galipienso et al., 2000).

CLBV detection was less consistent in field trees than in greenhouse-grown plants, possibly due to lower virus accumulation and/or uneven virus distribution within infected trees. This hypothesis is supported by the finding that RNA extracts from young leaves of Navelina sweet orange grown in the greenhouse always gave a strong hybridization signal by DBH and TPH, whereas similar extracts from field trees often yielded a low intensity signal and CLBV was detected in only one to three out of six young leaves. Detection in old leaves was achieved in four out of six samples from greenhouse-grown plants, but in none taken from field trees. Also, in some field trees that reacted negatively by RT–PCR using an RNA extract from six pooled leaves, CLBV was detected in extracts from some individual leaves (Vives et al., 2002b).

Detection of CLBV in citrus varieties from different geographic regions confirms that this pathogen may be widespread in many citrus growing areas. Finding that all CLBV field sources showed bud union crease on trifoliate rootstocks, but the virus could not be detected in other trees with similar symptoms, sometimes growing in their close vicinity, could be due to (i) low virus titer and/or uneven distribution of the virus within the plant or (ii) bud union crease would be caused by a different pathogen or by an interaction between CLBV and other biotic or abiotic factors.

Up to now, the only diagnostic procedure for CLBV was biological indexing on Dweet tangor seedlings using at least six indicator plants to avoid false negatives (Galipienso et al., 2000). Results presented in this paper indicate that quick detection of CLBV in sanitation, quarantine and certification programmes can be performed by molecular hybridization and/or by RT–PCR selecting the appropriate tissue as RNA source. DBH could be also helpful in massive surveys or epidemiological studies using at least six young leaves or shoots sampled around the tree. A major advantage of these diagnostic procedures over biological indexing is that imprinted membranes or desiccated tissue can be shipped to other labs for analysis, without the risk of spreading this pathogen in areas where it has not yet been detected, thus allowing safe international cooperation.

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