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Production of mandarin + pummelo somatic hybrid citrus rootstocks with potential for improved tolerance/resistance to sting nematode

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Abstract

Sting nematode (*Belonolaimus longicaudatus* Rau) has become a primary factor limiting citrus production in localized regions of the central Florida sandridge citrus production area, making the development of resistant rootstocks a new breeding objective. In efforts to develop a replacement rootstock for the widely adapted sour orange, our focus has been on somatic hybridization of selected mandarin + pummelo combinations [Grosser, J.W., Gmitter, Jr., F.G., 1990. Protoplast fusion and citrus improvement. Plant Breed. Rev. 8, 339–374; Ananthakrishnan, G., Calovic, M., Serrano, P., Grosser, J.W., 2006. Production of additional allotetraploid somatic hybrids combining mandarins and sweet oranges with pre-selected pummelos as potential candidates to replace sour orange rootstock. In Vitro Cell. Dev.: Plant 42, 367–371], since sour orange is probably an introgression hybrid of mandarin and pummelo as suggested by molecular marker analyses [Nicolosi, E., Deng, Z.N., Gentile, A., La Malfa, S., Tribulato, E., 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theor. Appl. Genet. 100, 1155–1166; Gulsen, O., Roose, M.L., 2001. Lemons: diversity and relationships with selected Citrus genotypes as measured with nuclear genome markers. J. Am. Soc. Hort. Sci. 126, 309–317]. Somatic hybrid plants were produced from four new mandarin (*C. reticulata* Blanco) + pummelo (*C. grandis* L. Osbeck) parental combinations by fusing embryogenic suspension culture-derived protoplasts isolated from selected mandarins with leaf protoplasts of pummelo seedlings previously selected for tolerance/resistance to the sting nematode (*B. longicaudatus* Rau) as follows: Amblycarpa mandarin + 'Liang Ping Yau' (seedling) pummelo seedling SN7; Amblycarpa mandarin + 'Hirado Buntan Pink' (HBP) pummelo seedling SN3; Murcott tangor + pummelo seedling SN3; and Shekwasha mandarin + pummelo seedling SN3. Somatic hybridization was verified by ploidy analysis (via flow cytometry) and RAPD analyses. Mandarin parents were selected for wide soil-adaptation and ability to produce friable embryogenic callus lines. Pummelo seedlings used as leaf parents were identified from a previous screen of large seed populations (200 each) from four pummelos for resistance to sting nematode as follows: 'Hirado Buntan Pink'; 'Red Shaddock'; 'Large Pink Pummelo' and a seedling pummelo of 'Liang Ping Yau'. Ten resistant/tolerant pummelo seedlings were selected from the 800 pummelo seeds planted in the screen for further study. The four new somatic hybrids have been propagated to evaluate their horticultural performance and resistance to the sting nematode. These potential somatic hybrid rootstocks should also have potential to control tree size due to polyploidy.

Keywords: Citrus tissue culture; Protoplast fusion; Tetraploid; Tree size control

1. Introduction

The sting nematode, *Belonolaimus longicaudatus* Rau, is widely distributed in sandy soils throughout the southeastern and central United States and is a serious pathogen of numerous agronomic and some horticultural crops including citrus (Koenning et al., 1999). All vermiform stages of the nematode feed at root tips, killing the meristematic cells. Root systems of heavily infested citrus trees can be almost devoid of fibrous roots and those that remain are stubby and often swollen. Population density of this relatively large nematode is directly related to the sand content of the soil (Mashela et al., 1991, 1992). The nematode is nearly ubiquitous in the deep sandy soils of Florida's central ridge and can generally be detected in the shallower soils of the flatwoods if the sand content is high. Citrus trees that are planted in heavily infested soil become stunted and even die (Kaplan, 1985; Duncan et al., 1996). Intraspecific variability of virulence on tested plant species is high (Abou-Garbeih and Perry, 1970) and *B. longicaudatus* is actually a species complex, based on numerous molecular autapomorphies (Gozel et al., 2006).

Sting nematodes became a widespread problem in Florida's citrus industry following a series of freezes during 1983–1990

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when up to half of the orchards on the central ridge were killed and replanted (Duncan et al., 1996). Heavily infested young trees may remain severely stunted for several years until the root mass increases enough that transpiration can periodically dry the surface soil, forcing nematodes into deeper soil horizons. Nematicides are used to reduce sting nematode numbers below damage thresholds until trees are large enough to tolerate the nematode, but they are expensive and pose significant health and safety hazards. Although use of resistant rootstock germplasm offers the best solution, all commercial rootstocks are highly susceptible to damage by sting nematode, and there are no known sources of resistant germplasm (Kaplan, 1985). In efforts to develop a replacement rootstock for widely adapted sour orange, our focus has been on somatic hybridization of selected mandarin + pummelo combinations (Grosser et al., 2004; Ananthakrishnan et al., 2006), since sour orange is probably an introgression hybrid of mandarin and pummelo as suggested by molecular marker analysis (Nicolosi et al., 2000; Gulsen and Roose, 2001). Somatic hybrid rootstocks also have good potential for tree size control due to polyploidy (Grosser et al., 1995, 2000). The focus of this study is to produce somatic hybrid rootstock candidates that combine mandarins with pummelos pre-screened for tolerance/resistance to sting nematode.

2. Materials and methods

2.1. Selection of sting nematode tolerant/resistant pummelo seedlings

Two hundred open-pollinated seed were extracted from fruit of each of the following pummelos: 'Liang Ping Yau' sdlg. (China); 'Large Pink Pummelo' (SE Asia); 'Hirado Buntan Pink' sdlg. (HBP) (Japan); and 'Red Shaddock' (SE Asia), obtained from The Florida Citrus Arboretum, Florida Department of Agriculture & Consumer Services, Division of Plant Industry, Winter Haven, Florida. Flats containing Candler sand were inoculated with commercial Bermuda grass seed obtained from a local Feed store (planted in rows). Seed were allowed to germinate and grow 4 weeks in the greenhouse. Bermuda grass is capable of hosting large populations of sting nematode. Sting nematodes from a local University of Florida's Citrus Research and Education Center (CREC) field plot were extracted by sucrose centrifugation collected over 10/325 mesh sieves and nematodes were hand-selected for inoculation. Each flat was inoculated with 50–65 females and 10 males. After 6 weeks, 200 open-pollinated seed from each of the four pummelo selections (one selection per flat) were planted directly into the flats between the rows of established Bermuda grass and allowed to grow along with the grass and sting nematodes. Sting nematode population development over the next 3 months was in the greenhouse with a temperature range of 21–31 °C, irrigation as needed, and fertilization biweekly with Peters liquid 20-20-20. After 3 months, the seedlings were removed from the flats and the 10 best surviving healthy seedlings (designated SN1–10) were repotted into commercial potting soil for further study.

2.2. Plant material

Embryogenic suspension cultures of Amblycarpa and Shekwasha mandarins and 'Murcott' tangor were initiated from friable embryogenic callus cultures maintained in the citrus embryogenic callus collection of the CREC. Suspension-derived protoplasts were obtained from approximately 1-year-old suspension cultures that were continuously maintained in H + H medium on a 2-week subculture cycle, with protoplasts isolated during days 4–12 (Grosser and Gmitter, 1990). Grafted plants of 'Liang Ping Yau' (seedling) pummelo seedling SN7; 'Hirado Buntan Pink' seedling SN3 were maintained in small pots in a low-light (via double shadecloth) greenhouse. Tender, fully expanded leaves from these plants were used to obtain leaf-derived protoplasts.

2.3. Protoplast isolation and fusion

Protoplasts were isolated from the Amblycarpa, Shekwasha, and 'Murcott' suspension cultures in a 2.5:1.5 (v:v) mixture of 0.7 M BH3 protoplast culture medium and enzyme solution according to Grosser and Gmitter (1990). Prior to protoplast isolation, selected leaves from greenhouse parental plants were decontaminated by immersion in 1 N HCl for 5 s, followed by immersion in 20% commercial bleach for 15 min, and rinsed with sterile distilled water for 10 min. Sterile leaves were feathered and incubated overnight (including a 25-min vacuum infiltration) in a 8:3 (v:v) mixture of 0.6 BH3 protoplast culture medium and enzyme solution (Grosser and Gmitter, 1990). Protoplasts from both sources were purified by passage through a 45 μm stainless steel mesh screen and then centrifugation on a 25% sucrose/13% mannitol gradient (Grosser and Gmitter, 1990).

The standard method of fusing embryogenic culture-derived protoplasts of one parent with leaf-derived protoplasts of the second parent was utilized in all experiments (Grosser et al., 2000). Fusions were conducted using the PEG (40% polyethylene glycol) method (Grosser and Gmitter, 1990). Following fusion, protoplasts were cultured initially in a 1:1 (v:v) mixture of 0.6 M BH3 and 0.6 M EME protoplast culture media (Grosser and Gmitter, 1990), and maintained in plastic boxes under low light.

2.4. Plant recovery and ploidy analysis

Regenerating calli were transferred to solid EME medium containing 50 g/l sucrose or maltose (Perez et al., 1998) for somatic embryo induction according to Grosser and Gmitter (1990). Most, but not all of regenerated embryoids were cultured over 0.22 μm cellulose acetate membrane filters placed on fresh plates of EME-maltose solid medium to normalize and enlarge the embryoids (Niedz et al., 2002). Large somatic embryos, usually exhibiting abnormal shapes, were screened for ploidy level using a Partec flow cytometer (Model D-48161, Münster, Germany). Only confirmed tetraploid embryos were transferred directly to DBA3 medium for shoot induction (Deng et al., 1992). Developing shoots were rooted on RMAN medium (Grosser and