

Origin and Inheritance of Dwarfing by the Citrus Rootstock *Poncirus trifoliata* 'Flying Dragon'

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Abstract. 'Flying Dragon' *Poncirus trifoliata* L. Raf. is a dwarfing rootstock for citrus. Inheritance of dwarfing ability was studied in a population of open-pollinated seedlings of 'Flying Dragon'. Molecular marker genotypes suggest that all seedlings originated from selfing. Progeny seedlings were budded with 'Cutter Valencia' orange and planted in the field to evaluate the dwarfing effect of the seedling rootstock. At 5 years after planting, rankit analysis of the frequency distributions of trunk cross-sectional area and canopy volume suggested the presence of two overlapping distributions of 34 dwarf trees and 7 nondwarf. This ratio is consistent with inheritance of rootstock dwarfing as a single dominant gene for which 'Flying Dragon' is heterozygous. Two morphological characteristics of 'Flying Dragon', curved thorns and twisted trunk growth, were closely linked to, or pleiotropic effects of, the dwarfing gene. Bulked segregant analysis was used to identify three RAPD markers linked to the dwarfing gene. 'Flying Dragon' was identical to nondwarfing cultivars of trifoliolate orange at 40 homozygous and heterozygous isozyme and RFLP markers; therefore, it is likely that 'Flying Dragon' originated as a mutant of a nondwarfing genotype and has not undergone sexual recombination since this event.

Citrus is an important crop grown throughout tropical and subtropical regions of the world. Dwarfing rootstocks reduce tree size and may reduce management costs and increase yield per unit area, although these advantages have yet to be documented in citrus (Wheaton et al., 1991). 'Flying Dragon' trifoliolate orange (*Poncirus trifoliata*) greatly reduces tree size when used as a rootstock for any citrus cultivar (Bitters et al., 1979; Roose, 1990). With most scions, mature trees on 'Flying Dragon' are no more than 2.5 m tall (M. L. Roose, unpublished data). In common with most other cultivars of trifoliolate orange, 'Flying Dragon' is resistant to citrus tristeza virus, phytophthora root rot, and citrus nematode, and trees budded on it produce high quality fruit. However, also like other cultivars of *P. trifoliata*, it is susceptible to iron chlorosis on calcareous soils. Unfortunately, little information is available on the genetic control of dwarfing by 'Flying Dragon', despite the potential importance of this knowledge in evaluating it as a breeding parent for new rootstock. Because citrus rootstock breeding is a costly long-term project, methods to increase the efficiency of selection for rootstock traits are particularly valuable.

Molecular markers are a valuable new tool for plant genetics (Paterson et al., 1991). Restriction fragment length polymorphisms (RFLPs) have been used to construct linkage maps in citrus (Durham et al., 1992; Jarrell et al., 1992) and other species (Barzen et al., 1992), and to tag useful genes (Ahn et al., 1992). They have also been used to analyze phylogenetic relationships in many plant species (Jarret et al., 1992; Miller and Tanksley, 1990), but their analysis is laborious and radioisotopes are usually used in the detection method. Recently, DNA markers detected with the polymerase chain reaction have been developed. Methods based on random primers, such as random simplified polymorphic DNA

(RAPDs) (Williams et al., 1990) and DNA amplification fingerprints (DAF) (Cactano-Anoiles et al., 1991) have been particularly useful for mapping and gene tagging because a large number of markers can be produced without prior knowledge of DNA sequence. However, RAPDs are usually diallelic, dominant markers in contrast to RFLPs and isozymes that are usually multiallelic and codominant. These characteristics reduce the information content of RAPDs for some applications. Michelmore et al. (1991) developed a method called bulked segregant analysis (BSA) to rapidly identify markers closely linked to specific target genes. BSA involves forming bulk DNA samples from individuals with different phenotypes for the trait of interest and then screening for markers that differ between these bulked DNA samples. Candidate markers are then tested in the whole segregating population. This method has been used to identify markers linked to a variety of genes including downy mildew resistance (Michelmore et al., 1991), oat stem rust (Penner et al., 1993), and bean rust (Miklas et al., 1993).

In this paper, we report the inheritance of dwarfing ability among zygotic progeny of 'Flying Dragon' identified using isozyme markers, identification of RAPD markers linked to the dwarfing gene, and evidence from RFLP analysis that 'Flying Dragon' originated as a mutant of nondwarfing form of trifoliolate orange.

Materials and Methods

Plant materials. Like many citrus taxa, 'Flying Dragon' produces polyembryonic seeds containing both sexual and apomictic embryos. The seedlings used in the study reported here derive from earlier research on the relative frequencies of nucellar and zygotic seedlings in three trifoliolate orange cultivars (Khan and Roose, 1988). Zygotic progeny from open-pollination of 'Flying Dragon', 'Rubidoux', and 'Pomeroy' were distinguished from apomictic seedlings by screening for genotypes at four heterozygous isozyme markers. Isozyme genotypes of all of these progeny were consistent with an origin by self-pollination rather than by outcrossing. The percentage of zygotic seedlings ranged from 4% to 76% in 10 samples of 'Flying Dragon' grown from seed produced by seed source trees in three locations and collected in 3 years. Randomly chosen zygotic seedlings identified by Khan and Roose were budded with virus- and viroid-free 'Cutter Valencia' orange scion and planted in five adjacent rows at Riverside in 1988 and 1989.

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Seedlings from different cultivars were not randomized among rows. Each seedling was also budded onto 'Troyer' rootstock and planted in the field as a source tree. In December 1993, the effect of each progeny genotype on tree size when used as a rootstock was determined by measuring scion wood trunk circumference ≈ 6 cm above the bud union to estimate trunk cross-sectional area and by measuring tree height and width to estimate canopy volume, assuming that tree shape is a prolate sphere (Turrell, 1946).

DNA extraction. The DNA extraction procedure was based on that of Webb and Knapp (1990) modified so that only 1.5-ml centrifuge tubes were used. About 0.1 g fresh leaf tissue (young or mature) from each source tree was ground with 0.70 ml extraction buffer using a mortar and pestle. The rest of the published procedure was followed without modification.

RFLP analysis. DNA samples of *P. trifoliata* 'Flying Dragon', 'Pomeroy', and 'Rubidoux' were digested with HindIII, EcoRI, and XbaI and analyzed with 29-cDNA probes as described by Jarrell et al., 1992.

PCR amplification. The PCR reactions were carried out in 16- μ l volumes containing 10 mM Tris-HCl, pH 8.3; 5 mM MgCl₂; and 20 mM of each dNTP; 1.0 unit of Taq polymerase (Promega Biotech), 30 ng primer (decamers, Operon), and 25 ng genomic DNA. Amplification was performed in an Ericomp Thermal Cycler programmed for 45 cycles of 5 sec at 94C, 30 sec at 36C, and 60 sec at 72C. Amplification products were separated on 1.5% agarose gels. After staining with ethidium bromide, gels were

observed and photographed under ultraviolet light.

Primer screening and data analysis. To screen for RAPD markers linked to the dwarfing gene, bulked segregant analysis was conducted using two DNA bulks. One bulk consisted of DNA from nine 'Flying Dragon' progeny plants that dwarfed a 'Valencia' scion, while the other bulk contained DNA from five 'Flying Dragon' progeny that did not dwarf a 'Valencia' scion. Primers that amplified a product with the DNA from dwarfing progeny but not from the nondwarfing progeny were used to amplify DNA from each plant in the population. GMendel 3.0 (Holloway and Knapp, 1994) was used to construct the linkage map. The population was analyzed as an F₂ with a minimum LOD score of 3.0. The Kosambi mapping function was used to translate recombination frequencies into map distances. Details of cDNA clones and RAPD primers used are available on request to the corresponding author.

Results

Inheritance of dwarfing. Among all 41 'Cutter Valencia' trees grafted onto zygotic 'Flying Dragon' seedling rootstock (ignoring variation in thorn morphology, which is discussed separately below), the frequency distribution of trunk cross-sectional area (TCSA) showed a substantial departure from the normal distribution. A plot of rankits (normal scores) for TCSA vs. TCSA is clearly not linear, but appears composed of two separate linear

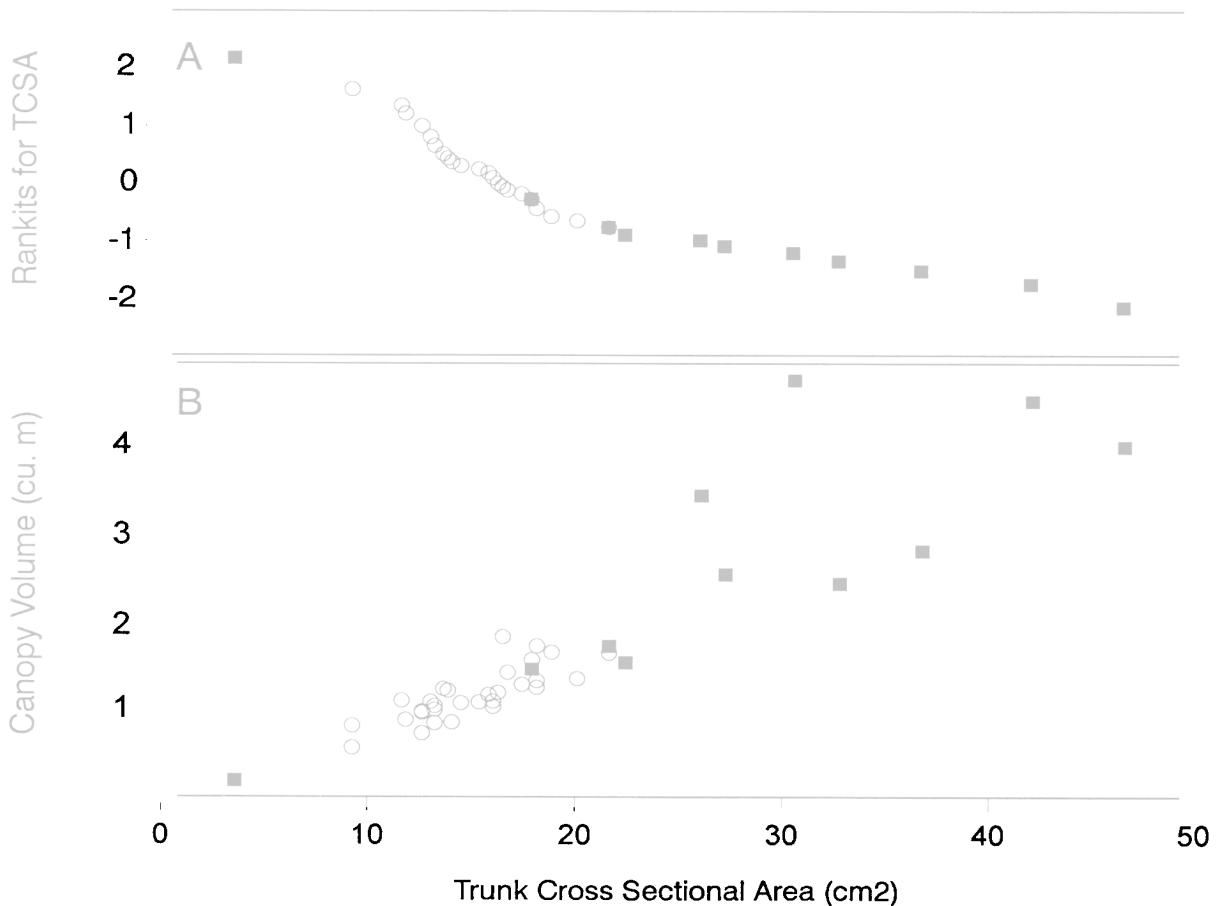


Fig. 1. (A) Plot of rankits (normal scores) for trunk cross-sectional area of 4- and 5-year-old Valencia orange trees budded on self-pollinated progeny of 'Flying Dragon' trifoliolate orange. Open circles represent progeny trees with curved thorns and trunk, and filled squares represent trees with straight thorns and trunk. (B) Relationship between canopy volume and trunk cross-sectional area of 4- and 5-year-old Valencia orange trees budded on self-pollinated progeny of 'Flying Dragon' trifoliolate orange.

components (Fig. 1 a) suggesting two overlapping normal distributions (Sokol and Rohlf, 1981). Progeny genotypes were divided into two classes, dwarf and nondwarf, at a value of 24 cm² (the most obvious gap in the frequency distribution). The observed ratio of 34 dwarf : 7 nondwarf is consistent ($\chi^2 = 2.30, P > 0.10$) with the 3:1 ratio expected for control of dwarfing by a single dominant gene for which 'Flying Dragon' is heterozygous. Similar results were obtained when canopy volume of trees was analyzed, and the two traits were strongly correlated with $R^2 = 0.81$ (Fig. 1 b).

'Cutter Valencia' trees on zygotic progeny of *P. trifoliata* 'Rubidoux' and 'Pomeroy' were significantly larger than adjacent trees on 'Flying Dragon' progeny (Table 1). The statistical analy-

sis shown in Table 1 is not strictly valid because seedlings from different cultivars were not randomized, but the field is unlikely to contain the large environmental gradients that would be required to explain the observed differences in tree size. Neither of these cultivars produced any progeny that were classed as dwarfing rootstocks using the criteria applied to the 'Flying Dragon' progeny.

Morphological markers. 'Flying Dragon' and some of its progeny display two unusual morphological characters, having curved thorns and trunks in contrast to the straight thorns and trunks of normal trifoliolate orange (Fig. 2). There were 30 progeny with curved thorns and trunks and 11 progeny with straight thorns and trunks, consistent with a 3: 1 ratio ($\chi^2 = 0.07, P > 0.50$). Thus,

Table 1. Canopy volumes and trunk cross-sectional area (TCSA) of Valencia orange trees budded on nucellar and zygotic seedlings of trifoliolate orange cultivars.

Cultivar	Genotype	n	Canopy vol (m ³)	TCSA (cm ²)
Flying Dragon [†]	Nucellar	3	1.6	17.1
Flying Dragon	Zygotic-curved	30	1.2 c [‡]	15.1 d
Flying Dragon	Zygotic-straight	11	2.7 b	28.0 c
Pomeroy	Zygotic	53	4.1 a	43.0 b
Rubidoux	Zygotic	9	3.9 a	50.4 a

[†]Not included in statistical analysis.

[‡]Mean separation by LSD test using $P < 0.05$.



Fig. 2. Progeny trees of self-pollinated 'Flying Dragon' trifoliolate orange showing (a) curved thorn and trunk and (b) straight thorn and trunk.

curved thorn and trunk were dominant, and straight thorn and trunk were recessive. No recombinants were found between the trunk and thorn characters.

All 'Flying Dragon' progeny with curved thorns and trunks produced dwarf trees when used as rootstocks (Fig. 1). Of the eleven progeny with straight thorns and trunks, seven were clearly nondwarfing as rootstocks, three produced trees slightly smaller than the largest trees on progeny with curved thorns, and one produced a very small tree. Trees on straight-thorned rootstocks were significantly larger than those on curved-thorned rootstocks, and the latter were similar in size to trees on nucellar 'Flying Dragon' rootstocks (Table 1).

RAPD markers. The two bulked DNA samples from dwarf and nondwarf progeny trees were screened with 280 primers. Several primers amplified DNA segments in one bulk sample but not the other. After testing these primers against all trees in the population, we found that DNA segments amplified by primers H 12,103, and J03 were linked to the dwarfing gene (Table 2). A 1210-bp RAPD amplified with J03 was most closely linked to the dwarfing gene (Fig. 3). The amplification product was observed in all progeny with curved thorns and one of the 11 progeny with straight thorns (Table 2). Primer J03 was also tested with DNA from 'Rubidoux' and 'Pomeroy' seed source trees and zygotic progeny. An amplification product apparently identical to J03-1210 was observed in 'Rubidoux' and some of its self-pollinated progeny, but not in 'Pomeroy' or its progeny.

RFLP and isozyme genotypes of 'Flying Dragon'. The 'Flying Dragon' seed source tree at Riverside had the same genotypes as 'Pomeroy' and 'Rubidoux', 2 nondwarfing cultivars of trifoliolate orange, at 39 of 40 homozygous and heterozygous isozyme and RFLP loci. Four isozyme loci (*Got1*, *Pgm*, *Pgi1*, and *Mdh2*) are known to be heterozygous in these cultivars because of observed segregation in crosses (Torres et al., 1985; Khan and Roose 1988). The RFLP analysis included 29 probes and a total of 54 storable probe-enzyme combinations. Of 36 RFLP loci, 9 are inferred to be heterozygous (when DNA was digested with I or more restriction enzymes) based on phenotypes, segregation in a few *Citrus x Poncirus* hybrids, and a detailed inheritance study in a cross between 2 *Citrus x Poncirus* hybrids (Jarrell et al., 1992). One RFLP probe (pRLC094) differentiated 'Flying Dragon' from 'Pomeroy' but not from 'Rubidoux'.

Discussion

The first question we have addressed is the inheritance of dwarfing by 'Flying Dragon' rootstock. Although the progeny population size is rather small, our results are consistent with inheritance of dwarfing as a single dominant gene. This interpretation assumes that the progeny studied result from self-pollination

of 'Flying Dragon' and not from outcrossing. We can exclude outcrossing to *Citrus*, *Citrus x Poncirus* hybrids, and other genera because progeny had only isozyme and RAPD alleles characteristic of *Poncirus* and *Poncirus* differs from *Citrus* at a high proportion of the markers studied. Outcrossing to other trifoliolate orange genotypes is more difficult to exclude because nearly all trifoliolate orange cultivars have isozyme and RFLP genotypes identical to those of 'Flying Dragon'. Of the zygotic seedlings evaluated, 24 originated from a source tree in Riverside, Calif., that was surrounded by other trifoliolate orange cultivars, 10 originated from a source tree at Exeter, Calif., that was an unknown distance from the nearest other trifoliolate orange tree, and 7 were from a tree in Irvine, Calif., that was at least 1 km from the nearest trifoliolate orange tree. All three of these source trees produced progeny with curved and straight thorns in approximately similar (18:6, 7:3, and 5:2 respectively) ratios, and all produced dwarfing and nondwarfing progeny. It is particularly significant that the isolated tree at Irvine produced nondwarfing zygotic progeny with straight thorns, the phenotype that might otherwise be attributed to outcrossing with normal trifoliolate orange genotypes.

The evaluated trees were planted in 1988 or 1989 and originated from seed planted between 1983 and 1985. Thus, effects of rootstock genotype on tree size might be confounded with tree age. This appears unlikely because, although trees planted in 1989 were 15% to 25% smaller than those planted in 1988, approximately equal proportions of each genotype were planted each year, and neither year of planting nor year of seed collection was significantly related to tree size in two-factor (genotype x year) analyses of variance (data not shown).

Identification of molecular markers linked to the dwarfing gene strengthens the evidence that we have identified a major gene for dwarfing. If dwarfing were determined by a set of minor genes, it is unlikely that we would have been able to identify a set of markers that show strong linkage to dwarfing and to each other. Inheritance of dwarfing from 'Flying Dragon' has not been reported previously. However, our hypothesis of a dominant dwarfing gene is consistent with the observation of Grosser et al. (1988) that somatic hybrids between 'Hamlin' sweet orange and 'Flying Dragon' expressed the curved-thorn trait and dwarfing ability (Gmitter, personal communication) when used as rootstocks.

The relationship between rootstock dwarfing and the curved-thorn morphological marker trait is likely to be simpler than it appears in the analysis presented here. The rootstock dwarfing effect and the curved-thorn trait are probably pleiotropic effects of the same gene. Although four trees with straight thorns produced trees scored as dwarfed, all 30 progeny with curved thorns produced trees scored as dwarfed. Selfing of trifoliolate orange leads to some inbreeding depression, as evidenced in the smaller mean size of trees on 'Flying Dragon' progeny with curved thorns compared

Table 2. Joint segregations of dwarfing and thorn morphology with RAPD markers.

Trait-pair	Phenotype	N	Phenotype	N	Phenotype	N	Phenotype	N	G test
Dwarfing-Thorn	D_1_	30	D_cc	4	ddC_	0	ddcc	7	23.1***
Dwarfing-J03	D_1_	30	D_00	4	dd1_	1	dd00	6	15.2***
Dwarfing-103	D_1_	29	D_00	5	dd1_	3	dd00	4	5.2*
Dwarfing-H 12	D_1_	27	D_00	7	dd1_	1	dd00	6	10.9***
Thorn-J03	C_1_	30	C_00	0	cc1_	1	cc00	10	38.9***
Thorn-103	C_1_	28	C_00	2	cc1_	4	cc00	7	14.0**
Thorn-H12	C_1_	26	C_00	4	cc1_	2	cc00	9	17.2***

D = dwarfing allele, C = allele for curved thorns, 1 = dominant RAPD allele (product), 0 = recessive RAPD allele (no product).

, *Significant at $P < 0.01$ or 0.001 .

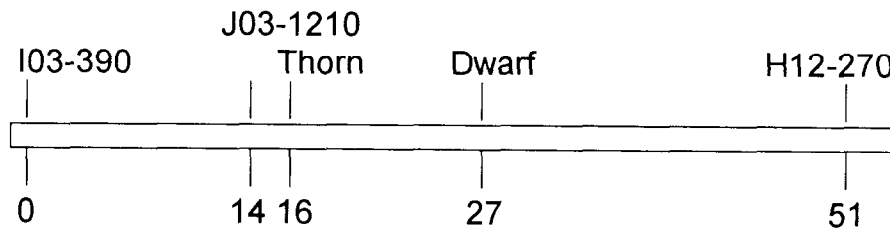


Fig. 3. Linkage map of the dwarfing gene region of 'Flying Dragon' trifoliolate orange. Map distances are shown in centimorgans computed using the Kosambi mapping function. Thorn and dwarf may be pleiotropic effects of the same gene (see text).

with trees on nucellar 'Flying Dragon' (Table 1). Because of inbreeding depression, it is expected that some selfed progeny will perform poorly as rootstock, producing abnormally small trees. This effect is expected to result in misclassification of some straight-thorned progeny without the dwarfing gene as dwarfing, but not the reverse. Second, the effect of each progeny genotype on tree size when used as a rootstock was evaluated from size of a single tree and is therefore also likely to be influenced by environmental variation. Thus, genetic and environmental factors are expected to reduce the accuracy of the phenotypic evaluation of this trait. Finally, the near identity of alleles in the three trifoliolate orange cultivars at many molecular marker loci clearly indicate that 'Flying Dragon' is differentiated from nondwarfing cultivars of trifoliolate orange by the accumulation of one or more mutations without any sexual recombination. If the dwarfing effect were caused by a separate mutation from the curved thorn trait, it is unlikely that these mutations would be closely linked. We therefore conclude that dwarfing effect as a rootstock and curved thorn and stem growth are probably pleiotropic effects of a single dominant mutation.

Since 'Flying Dragon' and 'Rubidoux' are both heterozygous for RAPD marker J03-1210, while 'Pomeroy' is homozygous recessive for this marker, it appears that 'Flying Dragon' derives from a genotype more closely related to 'Rubidoux' than to 'Pomeroy'. The banding patterns detected with RFLP probe pRLc094 are also consistent with this relationship. 'Flying Dragon' is used as a bonsai cultivar in Japan (Bitters et al., 1979) and possibly it was originally selected for this use. Inheritance of its unusual morphological traits as a heterozygous dominant gene is consistent with this hypothesis.

We have observed that the curved-thorn trait is expressed in some, but not all hybrids between 'C-35' citrange (*C. sinensis* x *P. trifoliata*) and 'Flying Dragon', but these trees are still too young to allow us to evaluate their dwarfing ability. The J03-1210 RAPD marker was inherited with the curved thorn trait in these populations. This observation and the dwarfing property of sweet orange + 'Flying Dragon' somatic hybrids suggest that the dwarfing gene will be expressed in *Citrus* x *Poncirus* hybrids.

The presence of additional dwarfing factors in 'Flying Dragon' is suggested by the observation that, when used as rootstock for Valencia orange, zygotic progeny of 'Flying Dragon' with straight thorns produce significantly smaller trees than zygotic genotypes of other trifoliolate orange cultivars. This partial dwarfing effect of straight-thorned progeny of 'Flying Dragon' cannot be attributed to inbreeding depression because trees on straight-thorned 'Flying Dragon' rootstock are smaller than trees budded on equally inbred (selfed one generation) progeny of 'Pomeroy' and 'Rubidoux', genotypes nearly isogenic to 'Flying Dragon'. We cannot formally exclude environmental effects as the cause of the reduced size of trees on straight-thorned progeny of 'Flying Dragon' because most trees on 'Flying Dragon' progeny were planted in different (but adjacent) rows than those on 'Pomeroy' and 'Rubidoux' progeny.

Thus, an environmental gradient perpendicular to row orientation could explain the difference in tree sizes between these genotypes. However, we have no evidence for the existence of such a gradient. Some studies have noted that trees on 'Flying Dragon' rootstock are too small for commercial acceptance (Wheaton et al., 1991). If it proves to be inherited, the partial dwarfing effect of these straight-thorned 'Flying Dragon' progeny may be valuable to rootstock breeders and horticulturists because it may result in more nearly optimal tree sizes than are achieved with the stronger dwarfing gene we have identified. Thus, both classes of zygotic progeny from 'Flying Dragon' appear worthy of evaluation as citrus rootstock. Initial observations suggest that most progeny of 'Flying Dragon' produce predominantly nucellar seedlings, and we are evaluating these progeny for iron-chlorosis resistance, citrus tristeza virus resistance, effects on yield, and other horticultural characteristics when they are used as rootstock for citrus.

The contorted trunk growth characteristic of 'Flying Dragon' has been observed in other plants. Apparent mutants of 'Troyer' and 'Carrizo' citranges (*C. sinensis* x *P. trifoliata*) with zig-zag stem growth but straight thorns have been reported (Russo and Starrantino, 1989), but the BA-300 mutant derived from 'Troyer' does not have a dwarfing effect when used as a rootstock (Reforgiato Recupero et al., 1992). The effect of the 'Carrizo Contorto' mutant on tree size has not been reported, but it produced a revertant branch with more normal morphology. *Morus bombycis* var. *unryu* and *Salix matsudana* var. *tortuosa* are morphologically similar to 'Flying Dragon'. It is not known whether these represent mutations at homologous genes, or simply superficially similar morphologic.

The mechanism of dwarfing by 'Flying Dragon' rootstock is not known. When used as a rootstock for 'Valencia' orange and other citrus, the trunk circumference of 'Flying Dragon' rootstock is much larger than that of the scion, whereas the scion and stock circumferences of similar-age trees on nondwarfing trifoliolate genotypes are more similar. In addition to its dwarfing action when used as a rootstock, when 'Flying Dragon' is used as an interstock with nondwarfing rootstock, tree size is intermediate to that of trees on 'Flying Dragon' rootstock and standard trifoliolate orange cultivars such as 'Rubidoux' (Roose, 1990). On these trees, the 'Flying Dragon' interstock is larger in circumference than either rootstock or scion. One mechanism consistent with these observations and the observed dominance of the dwarfing gene is that 'Flying Dragon' tissue irreversibly binds or inactivates a translocated growth regulator produced by roots.

We present evidence that a single major gene is responsible for dwarfing by a citrus rootstock, the first such example in any tree crop. Several DNA and morphological markers are closely linked to the dwarfing gene. It may be possible to apply these markers in map-based selection to select Flying Dragon-type dwarfing rootstock. Development of a high-density map of the dwarfing region appears possible and may facilitate map-based cloning of the dwarfing gene.

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