A New Graft Transmissible Disease Found in Nagami Kumquat

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ABSTRACT. An undescribed graft transmissible disease has been found on Nagami kumquat. Three types of symptoms have been observed: 1) vein clearing on Pineapple sweet orange, Troyer citrange, sour orange, Marsh grapefruit, Orlando tangelo, Dweet tangor and Alemow, but not on Mexican lime, Etrog citron, Cleopatra mandarin, rough lemon, Eureka lemon, Volkamer lemon, trifoliate orange, Nules clementine and Parson's special mandarin; 2) stem pitting on Etrog citron, but not on the other species and hybrids; and 3) graft incompatibility of Nagami kumquat on Troyer citrange, but not on rough lemon. Vein clearing symptoms were more severe in seedlings grown at 18-25°C than at 27-32°C. Stem pitting was induced only at 18-25°C. Some kumquat plants obtained by shoot-tip grafting *in vitro* were compatible with Troyer citrange, and did not induce vein clearing, but still produced stem pitting. These data suggest the presence of more than one pathogen on the original plants. Preliminary electron microscopy studies have shown the presence of some virus-like particles about 800 nm long in extracts of infected Troyer citrange and sweet orange plants. Diseased kumquats gave negative reactions by ELISA using four different tristeza antisera.

A Citrus Variety Improvement Program (CVIPS) was started in Spain in 1975 to recover virus-free plants from all commercial varieties and other citrus species, valuable for research work or for a germplasm bank (1, 2, 3). In this program, Nagami kumquat from two origins was selected and indexed for virus content. One of the kumquat sources was selected from a private variety collection at Alhama, Murcia, Spain, (Alhama kumquat), and the other was introduced from the Station des Recherches Agrumicoles at Saint Giuliano, Corsica, France, (SRA-153, kumquat), kindly provided by Dr. Vogel). Both kumquats were propagated on rough lemon and Troyer citrange in the greenhouse and graft-inoculated to Mexican lime, Pineapple sweet orange, Etrog citron and Parsons Special mandarin to test for tristeza, psorosis and/or concave gum, exocortis and xyloporosis, respectively. Alhama kumquat indexed negative for xyloporosis, positive for tristeza and exocortis and gave conspicuous vein clearing on Pineapple. SRA-153 kumquat indexed negative for xyloporosis, tristeza and exocortis, but it also induced vein clearing on Pineapple sweet orange.

This type of vein clearing symptoms on sweet orange are usually not induced by tristeza in Spain. Furthermore, SRA-153 kumquat indexed negative for tristeza on Mexican lime and by ELISA. Thus, the possibility existed that vein clearing induced on Pineapple by the two kumquat sources was due to a new pathogen. In addition, both kumquats grew well on rough lemon but were apparently incompatible with Troyer citrange.

In this paper we describe experiments directed to determine the characteristics of the agent or agents inducing vein clearing on Pineapple and to establish a possible relationship between those agents and the incompatibility between Nagami kumquat and Troyer citrange. In most experiments described below we used SRA-153 kumquat to avoid possible interference from tristeza and exocortis which were present in Alhama kumquat.

EXPERIMENTS AND RESULTS

Citrus species and varieties susceptible to Nagami kumquat in-

oculum. Four seedlings of each of the citrus species and varieties listed in table 1 were graft-inoculated with two bark patches from SRA-153 kumquat and incubated in a greenhouse at 18-25°C. New leaves were observed in each flush for symptoms, and eight months after inoculation plants were peeled to look for stem pitting.

Vein clearing symptoms, similar to those induced by tristeza on Mexican lime, were induced not only on Pineapple (fig. 1), but also on Comuna sweet orange, Troyer citrange (fig. 2), Alemow, *Marsh* grapefruit, Orlando tangelo, Dweet tangor, sour orange, and Cleopatra mandarin.

Stem pitting symptoms, similar to those caused by tristeza, were induced only in Etrog citron (fig. 3).

Vein clearing usually appeared in half to fully expanded leaves in the first flush (one month or less after grafting) and sometimes later disappeared. In subsequent flushes,

TABLE 1
SYMPTOMS OBSERVED ON DIFFERENT CITRUS SPECIES AND VARIETIES GRAFT-INOCULATED WITH NAGAMI KUMQUAT SRA-153.

Citrus species and varieties	Symptoms*	
	Vein clearing	Stem pitting
Pineapple sweet orange	+	−
Comuna sweet orange	+	−
Troyer citrange	+	−
Alemow	+	−
Marsh grapefruit	+	−
Orlando tangelo	+	−
Dweet tangor	+	−
Sour orange	+	−
Cleopatra mandarin	+	−
Etrog citron	−	+
Volkamer lemon	−	−
Mexican lime	−	−
Rough lemon	−	−
Eureka lemon	−	−
Parson Special mandarin	−	−
Nules clementine	−	−
Trifoliate orange	−	−

*+, positive; −, negative.

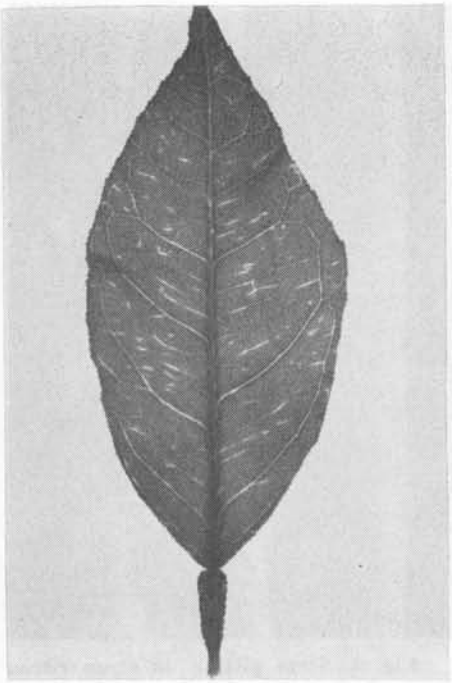

Fig. 1. Pineapple sweet orange leaf showing vein clearing induced by SRA-153 kumquat inoculum.

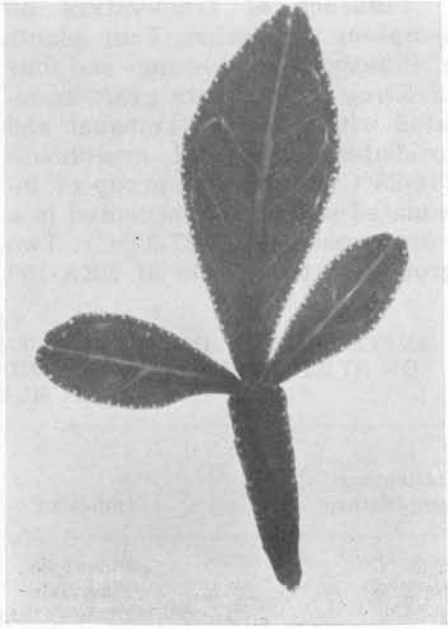

Fig. 2. Troyer citrange leaf showing vein clearing induced by SRA-153 kumquat inoculum.

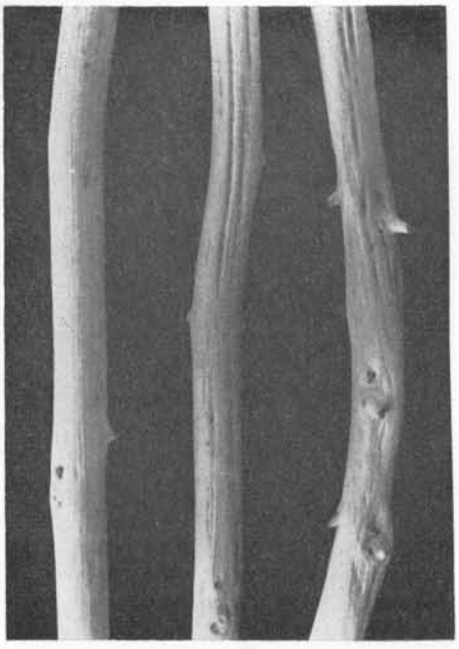

Fig. 3. Stem pitting on etrog citron stems. Left, uninoculated control; center, inoculated with SRA-153 kumquat; right, inoculated with tristeza.

vein clearing was rarely observed. Stem pitting on Etrog citron sometimes appeared as early as two months after grafting.

Influence of temperature on symptom expression. Four plants of Pineapple sweet orange and four of Etrog citron, were graft-inoculated with SRA-153 kumquat and incubated in a cool greenhouse (18-25°C). A similar group of inoculated plants was incubated in a warm greenhouse (27-32°C). Two groups of ten plants of SRA-153 kumquat on Troyer citrange were incubated in the cool and warm greenhouses respectively.

Vein clearing symptoms on Pineapple were more intense at cool than at warm temperature (table 2). Stem pitting on Etrog citron only appeared in plants incubated at the cool greenhouse. Incompatibility symptoms of kumquat on Troyer citrange were similar in plants incubated at both temperatures.

Transmission experiments. a. **Graft transmission.** Vein clearing of sweet orange and stem pitting of citron were readily transmitted by grafting. All inoculated citron plants showed pitting, and about 75 percent of sweet orange plants, inoculated in different experiments, showed vein clearing.

b. **Dodder transmission.** A citron plant inoculated with SRA-153 kumquat and showing stem pitting, was connected by *Cuscuta subinclusa* Dur. and Hilg. to eight healthy citron plants for two months. Five months later none of the eight citron plants showed stem pitting.

c. **Mechanical transmission from citrus to citrus.** Mechanical transmission from citron to citron was assayed by cutting citron stems 100 times with a knife contaminated each time by cutting infected plants. To inoculate the negative control a healthy instead of an infected citron plant was cut with

TABLE 2
INFLUENCE OF INCUBATION TEMPERATURE OF INDICATOR PLANTS ON SYMPTOM EXPRESSION INDUCED BY INOCULUM FROM NAGAMI KUMQUAT SRA-153

Incubation temperature	Indicator	Symptoms* Vein clearing	Stem pitting
18-25°C	Pineapple	++	—
27-32°C	Pineapple	+	—
18-25°C	Etrog Citron	—	+++
27-32°C	Etrog Citron	—	—

*—, negative; +, mild; ++, moderate; +++, severe.

the knife. Positive control was a citron plant graft-inoculated from the same source of inoculum.

None of the four citron plants mechanically inoculated showed symptoms, whereas the positive controls showed stem pitting.

d. **Mechanical transmission to herbaceous hosts.** Plants of Rutgers tomato, Blackeye Cowpea, Long vert maraichere cucumber, *Physalis floridana* Rydb. and Xanthi tobacco were dusted with carborundum and then mechanically inoculated with leaf extracts of Troyer citrange, Pineapple sweet orange or Dweet tangor with vein clearing symptoms. Extracts were prepared by mortar and pestle using three different buffers (0.1 M citrate, pH 5.8; 0.1 M phosphate, pH 6.5; and 0.1 M Tris-HCl, pH 7.9) to each of which was added 2 per cent mercaptoethanol. Nine to twenty plants were inoculated with the extract from diseased plants in each case, and five to ten more were inoculated with similar extracts from healthy citrus plants.

No difference was found between plants inoculated with extracts from healthy or diseased citrus plants.

Movement of the infectious agents within citrus plants. Twelve Pineapple seedlings were graft-inoculated with SRA-153 kumquat bark patches and immediately girdled above or below the inoculum graft.

None of six plants girdled above the inoculum showed vein clearing in leaves from flushes produced above the girdle, whereas three of them did show vein clearing in new leaves produced below the girdle. Two of the six Pineapple seedlings girdled below the inoculum showed vein clearing in upper leaves but none of them showed symptoms in leaves produced below the girdle.

Incompatibility studies. Several trials were done to propagate Alhama and SRA-153 kumquats on Troyer citrange. In all cases grafted plants showed incompatibility symptoms. Growth of kumquat buds was very poor, with small sized and yellow leaves (fig. 4) that usually dropped. Budunions were abnormally weak.

To elucidate if graft incompatibility of Nagami kumquat on Troyer citrange was due to the presence of pathogens or was of genetic origin, a few plants were obtained from SRA-153 by shoot-tip grafting (4). Graft success, growth *in vitro* of shoot-tip grafted plants and growth of those plants after transplanting to soil were normal and no symptoms of incompatibility were observed. Three of the micrografted plants were indexed by graft inoculation to 4 Troyer citrange, 4 Pineapple sweet orange, 4 Dweet tangor and 4 Etrog citron plants. All inoculated citron plants showed stem pitting

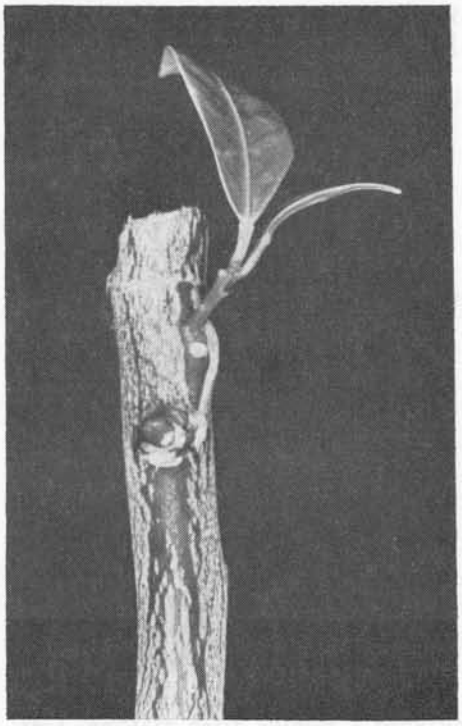

Fig. 4. Growth of SRA-153 kumquat on Troyer citrange 7 months after grafting.

whereas none of the Troyer citrange, Pineapple sweet orange or Dweet tangor plants showed vein clearing.

To confirm the compatibility of kumquat plants obtained by shoot-tip grafting on Troyer citrange, ten buds from a micrografted plant and ten more from the original SRA-153 were propagated on Troyer citrange. All plants from the micrografted source grew vigorously whereas plants from the original source showed severe incompatibility symptoms, (fig. 5). Total growth of micrografted plants 7 months after propagation averaged 200 cm per plant whereas growth of plants from the original source averaged only 0.5 cm per plant.

Another Nagami kumquat found later in Sevilla, that had been introduced from California, was compatible on Troyer citrange. Indexing of this kumquat disclosed that it was free of tristeza, it did not induce vein clearing on Pineapple sweet orange but it induced stem pitting on Etrog citron.

Fig. 5. Nagami kumquat on Troyer citrange 7 months after grafting. Left, original SRA-153 kumquat; right, SRA-153 kumquat obtained by shoot-tip grafting *in vitro*.

Since incompatibility between kumquat and Troyer citrange was associated with the presence of the agent inducing vein clearing, an experiment was done to find out whether this agent was also able to induce incompatibility between Pineapple sweet orange and Troyer citrange.

Healthy Pineapple sweet orange buds were propagated on ten seedlings of Troyer citrange previously graft-inoculated with bark patches of SRA-153 kumquat. Ten healthy seedlings of Troyer citrange were grafted with Pineapple buds infected with the vein clearing agent and ten more were budded with healthy Pineapple.

Growth of healthy and inoculated plants was similar and no incompatibility symptoms were observed.

Electron microscopy studies. Formvar-coated grids were floated for 10 minutes on a drop of leaf extract (200 mg of leaf homogenized in 1 ml of 0.1 M Tris-HCl buffer pH 7.6) or, alternatively, leaf pieces were dipped for 1 min in one drop of 0.1 M phosphate buffer pH 7.8. Fully expanded leaves with vein clearing symptoms and equivalent leaves from healthy control plants were used. Grids were washed by floating them for 1 min on a drop of distilled water, stained for 4 min with 2% phosphotungstic acid adjusted to pH 7, washed for 1 min with distilled water and blotted with a filter paper.

Grids were observed in a JEOL JEM 100S electron microscope at 80 KV. Four to six openings of each grid were thoroughly scanned at a magnification of 21,000X and at least the same number of observations were made on negative controls.

A few virus-like particles about 800 nm long were found in some

preparations from Troyer citrange and sweet orange with vein clearing and from kumquat obtained by shoot-tip grafting from SRA-153 kumquat. No such particles were observed in negative controls.

ELISA tests. To confirm the absence of tristeza virus in SRA-153 kumquat the following groups of plants were tested by ELISA using 4 antisera (873 and 879 provided by S. M. Garnsey, R4 provided by D. J. Gumpf and M1 obtained in our laboratory): a) SRA-153, Alhama, Sevilla, SRA obtained by shoot-tip grafting, and SRA-153 seedling kumquats; b) Mexican lime, Etrog citron Arizona 861-S1, alemow and Pineapple sweet orange plants inoculated with bark patches of SRA-153 kumquat or tristeza T-300 and c) noninoculated controls.

Only Alhama kumquat and plants of group *b* inoculated with tristeza gave positive reactions. No difference was observed between the rest of the plants and the negative controls with any of the four antisera.

DISCUSSION

A new graft transmissible disease inducing incompatibility between Nagami kumquat and Troyer citrange has been found. Inoculum from affected kumquats induced stem pitting on Etrog citron and vein clearing in several citrus species and varieties (table 1). Although symptoms are similar to those induced by tristeza the host range of both diseases are different. Common Spanish tristeza strains and kumquat inoculum induce vein clearing on *C. macrophylla* and stem pitting on Etrog citron, but only tristeza strains induce stem pitting on Mexican lime and vein clearing on Mexican lime and Etrog citron. Only kumquat inoculum induced vein clearing on sour orange, Orlando tangelo, Dweet tangor, Marsh grapefruit, Troyer citrange, Cleopatra mandarin and Comuna and Pineapple sweet oranges.

These data indicate that the kumquat disease is not caused by common Spanish tristeza strains. Results of ELISA tests confirm that the causal agent of this new disease is not related to tristeza.

Compatibility between kumquat obtained from shoot-tip grafting and Troyer citrange suggests that incompatibility of SRA-153 and Alhama kumquats was not genetic, but was due to the presence of some pathogen. The ability of micrografted kumquat to induce stem pitting on Etrog citron, but not vein clearing on Pineapple and other hosts is an indication that at least two pathogens were present in the original kumquat and that the incompatibility between Nagami kumquat and Troyer is associated with the agent inducing vein clearing. The later discovery of a different kumquat source which was compatible with Troyer citrange and also induced stem pitting on Etrog citron, but not vein clearing, supports the association between the incompatibility and the vein clearing agent.

The pathogens inducing vein clearing and stem pitting were readily transmitted from citrus to citrus by grafting. Dodder and mechanical transmission from citron to citron were unsuccessful; however, these experiments were carried out before there was any evidence of the presence of two pathogens in kumquat. Thus, the absence of symptoms on inoculated citron plants only indicates that the stem pitting agent was not transmitted. No data is available on dodder and mechanical transmissibility of the vein clearing agent. All attempts to mechanically transmit the agents of the kumquat disease to a limited range of herbaceous hosts were also unsuccessful. Failure to transmit those

agents to noncitrus hosts may indicate that, like most citrus virus and virus-like pathogens, they have a narrow host range.

Results of the girdling experiments indicate that the agent inducing vein clearing on Pineapple is restricted to the outer tissues of the stems of infected plants. These experiments were also carried out before evidence of the presence of two pathogens in kumquat was available. Thus, no conclusion can be drawn about the localization of the stem pitting agent within the infected plant.

A few virus-like particles were observed in extracts of infected plants that were not found in healthy plants. The presence of these particles in different hosts may be an indication that they are associated with the disease. In addition, finding of those particles in micrografted plants suggests that they may be related to the agent inducing stem pitting on citron but not with the vein clearing or incompatibility agents(s).

The fact that the kumquat disease is readily graft-transmitted, the type of symptoms produced on different indicators and observation of virus-like particles on diseased but not on healthy plants is an indication that at least one virus may be implicated in the disease complex.

So far, incompatibility induced by this complex has been found affecting Nagami kumquat on Troyer citrange, but experiments underway show that infected kumquat may also be incompatible on other rootstocks. Although no incompatibility was observed on Pineapple sweet orange grafted on Troyer citrange inoculated with SRA-153 kumquat, other commercial varieties could become incompatible. In this case, the disease could become a serious problem for the citrus industry.

The agents inducing vein clearing and stem pitting have been found in Nagami kumquat from different origins. This is an indication that these pathogens may be present in several countries and it could be an explanation why certain lines of kumquat have been considered incompatible on Troyer citrange.

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