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Embryo Culture of *Lycopersicon esculentum* × *L. peruvianum* Hybrid Genotypes Possessing Heat-stable Resistance to *Meloidogyne incognita*

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Abstract. **Genotypes of *Lycopersicon peruvianum* (L.) Mill. and *L. peruvianum* var. *glandulosum* (Rick), selected from accessions that possess resistance to *Meloidogyne incognita* [(Kofoid and White) Chitwood] at high soil temperature (30C), were used as male parents in crosses with *L. esculentum* (Mill.) susceptible cultivars UC82, Lukullus, Tropic, and male-sterile line ms-31, respectively. The incongruity barrier between the two plant species was overcome by embryo callus and embryo cloning techniques. Hybridity of the F, progeny obtained from each cross was confirmed by differences in leaf and flower morphology, plant growth habits, and by acid phosphatase isozyme phenotypes using polyacrylamide gel electrophoresis. In greenhouse inoculation experiments, F_1 plants were highly resistant to *M. incognita* in soil at 25 and 30C. These results confirmed the successful transfer and expression of heat-stable resistance to *M. incognita* from *L. peruvianum* to hybrids with *L. esculentum* as a preliminary step to introgressing additional root-knot nematode resistance into tomato.**

The resistance to root-knot nematodes *(Meloidogyne* spp.), present in all resistant commercial cultivars of tomato *(Lycopersicon esculentum* Mill.), is generally considered to be conferred by a single dominant gene, designated *Mi* (Ammati, 1985; Medina Filho and Stevens, 1980; Roberts and Thomason, 1989; Sidhu and Webster, 1981). This resistance was identified in L. *peruvianum* PI 128657. Using embryo rescue, Smith (1944) obtained one resistant F_1 plant from the cross *L. esculentum* Michigan State Forcing x PI 128657. Watts (1947) cloned this unique F_1 plant and obtained the first two backcrosses to *L. esculentum.* Additional backcrosses to the cultivated tomato were obtained by Frazier and Dennett (1949). This material was distributed to tomato breeders and led to the release of the first tomato cultivars resistant to *Meloidogyne incognita.* All cultivars with gene *Mi* have been derived from this one F_1 resistant plant obtained by Smith (1944).

Plants possessing gene *Mi* are resistant to three of the four economically important species of root-knot nematode (RKN): *M. incognita, M. arenaria* (Neal) Chitwood, and *M. javanica* (Treub) Chitwood (Ammati, 1985; Fatunla and Salu, 1977; Medina Filho and Stevens, 1980; Roberts and Thomason, 1989; Sidhu and Webster, 1981). Gene *Mi* does not confer resistance to *M. hapla* Chitwood. In addition, the resistance conferred by *Mi* is not effective in soils above 28C (Ammati, 1985; Ammati et al., 1986; Dropkin, 1969). Nonselected virulent populations of RKN, as well as virulent populations of RKN selected for several generations on plants bearing the *Mi* gene, can overeome the resistance conferred by gene *Mi* (Roberts and Thomason, 1986, 1989; Roberts et al., 1990; Triantaphyllou, 1987).

In view of the narrow genetic basis of resistance to RKN, Ammati et al. (1985, 1986) found additional sources of resistance within some accessions of *L. peruvianum* and *L. peruvianum* var. *glandulosum,* resistance which was expressed at soil temperatures of 28C or above. Additionally some accessions, such as *L. peruvianum* PI 270435 and *L. peruvianum* var. *glandulosum* PI 126440 and PI 126443, were resistant to *M. hapla.* Tests with a cross between two of these accessions showed that the resistance was effective against *M. incognita* isolates selected for virulence on plants that contain gene *Mi* (Roberts et al., 1990). Resistance to many other pests and diseases have been found in wild tomato genotypes, especially in the peruvianum group (Hogenboom, 1979; Medina Filho and Stevens, 1980; Rick, 1976; Rick and Yoder, 1988), but incompatibility barriers between plants of the *peruvianum* and *esculentum* complexes (Ammati, 1985; Hogenboom, 1979; Medina Filho and Stevens, 1980; Poysa, 1990; Rick, 1963; Rick and Yoder, 1986) have made the transfer of these desirable traits to edible tomato difficult.

Several tissue culture techniques have been useful in overcoming the unilateral incompatibility. Examples include somatic hybridization (Adams and Quiros, 1985; Glimelius, 1987; Handley et al., 1986; Jain et al., 1987; O'Connell and Hanson, 1986; Wijbrandi et al., 1987; Zelcer et al., 1987), leaf disk transformation mediated by *Agrobacterium tumefasciens* (McCormick et al., 1986), ovule culture (Imanishi, 1988), and embryo culture (Ammati, 1985; Leshem et al., 1989; Neal and Topoleski, 1985; Rick, 1963; Smith, 1944; Thomas and Pratt, 1981; Young et al., 1987). However, none of these techniques has provided an efficient method to transfer genes routinely from the peruvianum to the esculentum complex. The development of bridging lines may prove useful in the future for introgression of desirable traits into *L. esculentum (Poysa, 1990).*

The objective of this work was to obtain hybrids with *L. esculentum* that expressed the heat-stable nematode resistance

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Abbreviations: IR, index of resistance; RKN, root-knot nematode.

gene(s) from *L. peruvianum* as a preliminary step toward its introgression to *L. esculentum* germplasm.

Materials and Methods

Genotypes used in these experiments were *L. peruvianum* (L.) Mill, PI 270435, clones 2R2, 2R3, and 3 MH; *L. peruvianum* var. *glandulosum* (Rick, 1963; Warnock, 1988), PI 126443 clones 1MH, 2MH, and 2R2 and PI 126440 clones 9MH and 4MH. The genotypes were obtained from the wild *Lycopersicou* spp. clone collection maintained by the Dept. of Nematology, Univ. of California, Riverside. Nematode-susceptible *L. esculentum* lines 'UC82', 'Lukullus', 'Tropic', and ms-31 were used as female plants in crosses with the resistant *L. peruvianum* clones. Lines VFN-8 and 'UC82' were used as resistant and susceptible controls, respectively, in the nematode inoculation experiments.

Hybrid embryo callus. Crosses between various genotypes of *L. esculentum* and *L. peruvianum* were made in a greenhouse during Summer 1987 (Table 1). Plants were grown in UC Mix II in 2.5-liter nursery pots (Western Pulp Products Co., Corvallis, Ore.) with a day/night cycle of 27 ± 5C and were fertilized with Osmocote (Sierra Chemical Co., Corvallis). *Lycopersicon peruvianum* genotypes were used as male parents in all crosses. One day before anthesis (when the petals changed from green-yellow to yellow), flowers of *L. esculentum* plants were emasculated, hand-pollinated, and covered with small gelatin capsules.

Fruits from each of the crosses were harvested 25 days after pollination and surface-sterilized (Ammati, 1985). Fruits were dissected under sterile conditions and the immature seeds excised. Seedcoats were removed by abrasion using sterile filter paper. Immature seeds from each fruit were plated in 90 × 15-mm petri dishes containing 10 ml of the culture medium described by Thomas and Pratt (1981). Plates containing the immature seeds were maintained in the dark at 27C for 30 days.

Calli were placed on a shoot regeneration medium containing salts, i-inositol, and vitamins (Murashige and Skoog, 1962) plus 20 g of sucrose/liter and 2 mg of 6-benzylaminopurine (BA)/liter for 30 days. Shoots were transferred to a rooting medium containing MS salts (Murashige and Skoog, 1962) plus (all per liter) 1 mg thiamine·HCl, 100 mg i-inositol, 30 g sucrose, and 1 mg l-naphthaleneacetic acid (NAA). Regeneration of shoots from embryo callus and rooting of the shoots were carried out at 27C. Each plate was exposed for 30 days to a 16-h photoperiod (45 $\mu mol \cdot m^{-2} \cdot s^{-1}$, provided by GroLux fluorescent lamps.

Hybrid embryo cloning. Crosses between ms-31 and PI 126443 clone lMH were made during Summer 1988. The fruit were harvested 25 days after pollination and surface-sterilized (Ammati, 1985). Seeds were excised under a dissecting microscope. Embryos at the heart stage were transferred to petri dishes containing HLH medium (Young et al., 1987). Embryos were incubated at 25C under constant 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescerit light. Adventitious shoots were then rooted in 25-mm test tubes that contained MS basal medium (Murashige and Skoog, 1962) supplemented with 1 mg NAA/liter.

Plant conditioning. After 30 days on rooting medium, plantlets were transferred from test tubes to 250-cm^3 paper cups filled with sterilized vermiculite and sealed with plastic bags. The plants were placed in a growth chamber held at 25C and with a 16-h photoperiod (45 $\mu mol \cdot m^{-2} \cdot s^{-1}$, provided by fluorescent light. The plastic bags were perforated progressively to allow acclimatization to ambient humidity. When the plants showed shoot elongation and leaf expansion, they were transferred to a greenhouse and maintained at 27C and 30% to 40% relative humidity.

Electrophoresis. Protein extraction from young, fully expanded true leaves was performed according to Suurs et al.

Table 1. Comparisons between *L. esculentum* × *L. peruvianum* hybrids for number of seeds produced, number of seeds that produced calli, and number of plants regenerated.

Crosses	No. seeds	No. calli[z]	No. plants regenerated
L. esculentum x *L. peruvianum*			
UC82 270435	135	82	None
UC82 270435-2R2	67	11[y]	One
Lukullus 270435-2R2	36	13	None
Lukullus 270435-2R3	15	15	None
UC82 270435-2R3	40	15	None
ms-31 270435-2R3	0	0	---
Tropic 270435-2R3	13	13	None
Lukullus 270435-3MH	16	9	None
UC82 270435-3MH	0	0	---
L. esculentum x *L. p. glandulosum*			
ms-31 126443-1MH	116	0	---
Tropic 126443-1MH	68	48	None
Tropic 126443-2MH	0	0	

(1989). Tissue (100 mg) was ground in 50 µl of extractant buffer that contained 10 ml of 0.05 M tris, 2 ml of glycerol, and 100 µl of 2-mercaptoethanol. The extracts were centrifuged at 11,500 × g for 3 min and then 10 µl of sample was loaded directly on the gel. Polyacrylamide gel electrophoresis was carried out on 7% gel slabs (0.75 × 75 × 80 mm) at a constant 100 V until the front line had migrated 65 mm (≈ 1 h). The electrode buffer was 0.025 M tris and 0.076 M glycine solution, pH 8.3, and the gel buffer was 0.378 M tris·HCl, pH 8.9. Staining procedures for acid phosphatase were as described by Vallejos (1983). Composition of the stain solution was 20 ml of 0.5 M Na acetate, pH 4.5, 3 ml of 0.05 M $MgCl_2 \cdot 6H_2O$, 250 mg of Fast Black K Salt, 5 ml of 1% β-naphthyl acid phosphate (in 50% acetone), and 172 ml of H_2O.

Nematode cultures. The culture of *M. incognita* was started from a single egg mass on greenhouse-grown 'Tropic' tomato plants. The identity of the nematode isolate was confirmed morphologically by microscopic examination of perineal patterns of adult females (Eisenback, 1985) and by isozyme (esterase and malate dehydrogenase) phenotypes (Esbenshade and Triantaphyllou, 1987, 1990; Fox and Atkinson, 1989).

Resistance test. One-month-old seedlings or rooted cuttings were used for tests of host reaction to nematodes. Single plants were grown in l-liter polyethylene plastic cups (Louisiana Plastic, St. Louis) filled with steam-sterilized loamy sand and fertilized with Osmocote. Because the experiments were carried out in water-bath temperature tanks, each cup was nested in another empty cup without drainage holes in the bottom. The thin walls of the cups assured a uniform' distribution of the temperature in the root ball. Soil temperature in the cups was maintained at either 25 or 30C for each experiment.

Inoculum was prepared by the method of Hussey and Barker (1973). A water suspension of 5000 eggs and infective second-stage juveniles was pipetted into holes in the soil around the plant roots. Plants were arranged in each temperature tank in a completely randomized design. Nematode egg production on roots was evaluated after the accumulation of ≈ 500 degree days (12,000 heat units above the nematode developmental threshold temperature of 10C). This procedure allowed completion of one nematode generation (Tyler, 1933). Root systems were washed free of soil, damp-dried with paper towels, and weighed. Eggs were extracted by crushing the roots in 1% NaOCl solution, and eggs in a known volume of suspension were counted under a dissecting microscope (Hussey and Barker, 1973).

The number of eggs per gram of root was calculated by dividing the total number of eggs per root system by the total root weight to develop an index of resistance (IR). A plant was considered resistant when the number of eggs per gram of root was < 10% of the eggs per gram of root on the susceptible control 'UC82'. Data on the number of eggs per gram of root were transformed by obtaining the square root and subjected to analysis of variance tests.

Results

Hybrid embryo callus. The number of seeds in each cross was highly variable and some of the crosses did not produce seeds (Table 1). A total of 766 seeds were plated. After 1 month of culture, most of the immature seeds showed some callus development but became discolored and no further callus growth was observed. Only one seed, from a cross between 'UC82' and PI 270435 clone 2R2, developed a normal white callus that grew vigorously. This callus. was transferred to a shoot regeneration medium. Shoots formed 4 weeks later. Ten shoots were excised and rooted as described above and four vigorous plants were obtained. Their morphological characters of leaf, hirsuteness, growth habit, and length of style resembled those of the *L. peruvianum* clone used as the male parent or were intermediate between the two parents (Table 2, Fig. 1).

Zymograms for acid phosphatase 1 *(Aps 1)* (Fig. 2) showed the slow band at the variant allele *Aps* $1^{1/1}$ in a homozygous condition for PI 270435 clone 2R2 *(Aps* $1^{1/1}$*);* the mother plant 'UC82' was homozygous for the fast allele *(Aps* $1^{+/+}$*)*. Each of the four hybrids showed the heterozygous phenotype *Aps* $1^{1/+}$ with the slow band, the fast band, and a third intermediate band. Each of the four F_1 plants were backcrossed to 'UC82' and five well-developed embryos were isolated and rescued from seven seeds. Three BC_1 plants were regenerated using the procedure described below, but they did not survive the transfer from tissue. culture media to soil.

Hybrid embryo cloning. Three large embryos at the heart stage were rescued from several hundred degenerated seeds. Most of the immature seeds produced small, brownish, globular-stage embryos. No somatic embryogenesis was observed, but adventitious shoots were obtained. Subsequently, the shoots were rooted, and several plantlets were transferred to pots after conditioning. The morphological characters of the leaf, growth habit, hirsuteness, and length of style more closely resembled the male parent or were intermediate between the two parents (Table 2, Fig. 3). "

Zymograms for *Aps 1* (Fig. *2)* showed the heterodimer phenotype for *L. peruvianum* var. *glandulosum* PI 126443 clone 1MH, with a slow band corresponding to the variant allele *Aps* $1^{1/1}$, a fast band corresponding to a new allele *Aps* $1^{2/2}$, and an intermediate band belonging to the heterodimer *Aps* $2^{1/2}$. The allele *Aps* $1^{2/2}$ was first reported by Fobes and Rick (1976) in all tested *L. chmielewskii* Rick, Kes., Fob., and Hone and *L. parviflorum* Rick, Kes., Fob., and Hone genotypes. This allele gives a slower band than *Aps* $1^{+/+}$ and has also been observed in this particular clone (126443 1MH) (V.M. Williamson, Univ. of California, Davis, personal communication).

The male-sterile line ms-31 is homozygous for the fast allele *Aps* $1^{+/+}$. Hybrids between line ms-31 × PI 126443 clone 1MH showed the heterozygous phenotype *Aps* $1^{2/+}$, but only the slow band *(Aps* $1^{2/2}$ allele) and the intermediate band *(Aps* $1^{2/+}$ allele) were observed. The fast band belonging to the *Aps* $1^{+/+}$ allele was not expressed.

Resistance tests. Resistance to *M. incognita* was tested at 25 and 30C (Table 3). The number of eggs per gram of root on VFN-8 was 0 at 25C, indicating resistance, but 2300 (IR = 87) at 30C, confirming its susceptibility at high temperature. The numbers of eggs per gram of root on the two hybrids and their

Table 2. Morphological characteristics of *Lycopersicon esculentum* and *L. peruvianum* parents and their F_1 progeny.

L. esculentum UC82 and ms-31 female parents	Hybrids	*L. peruvianum* male parents
Determinate	Indeterminate	Indeterminate
Self-compatible	Self-incompatible	Self-incompatible
Large sepals, hairy	Short sepals, not hairy	Short sepals, not hairy
Style inserted in anther cone	Style slightly exserted from anther cone	Style exserted from anther cone
	Leaf morphology intermediate between both parents	

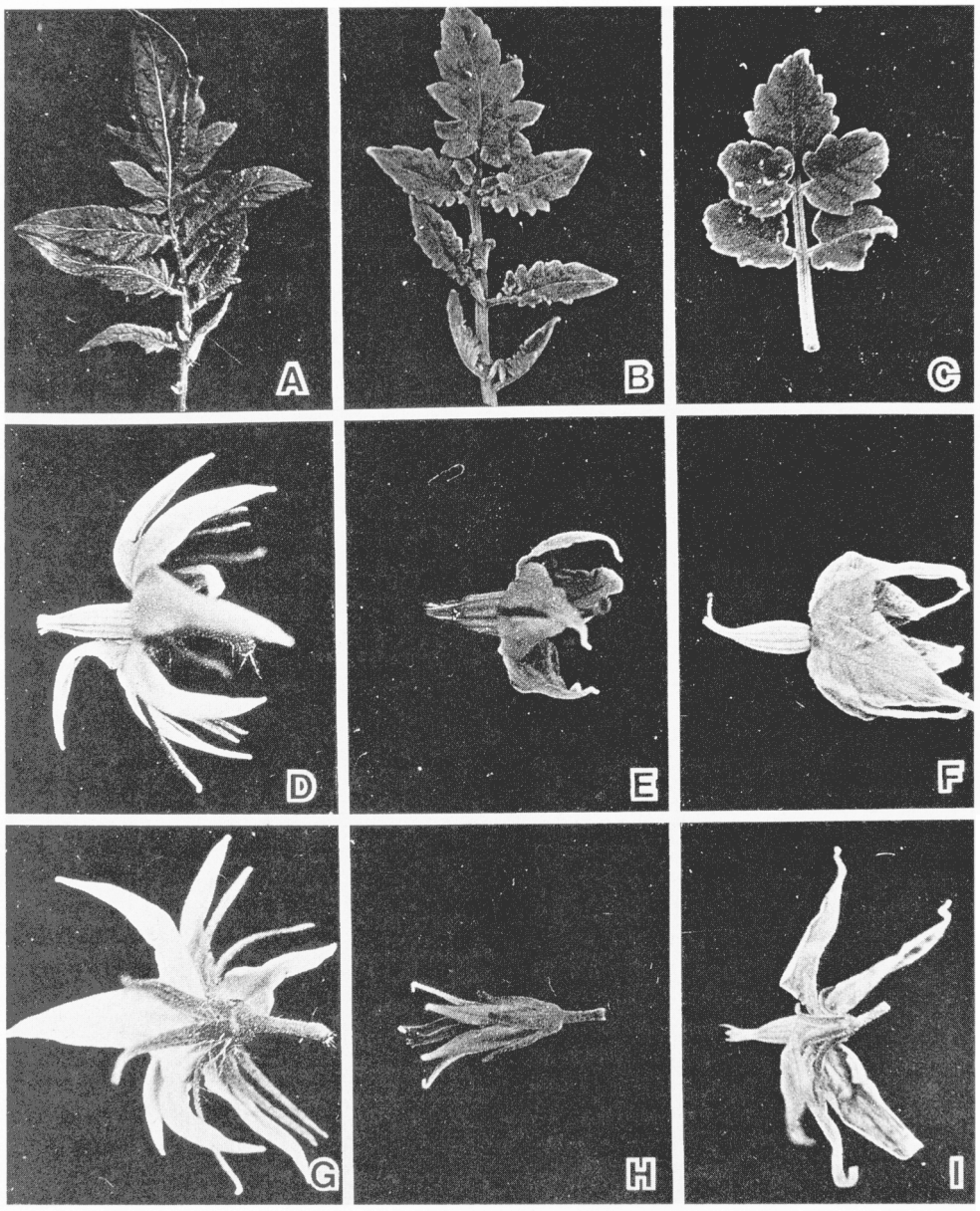
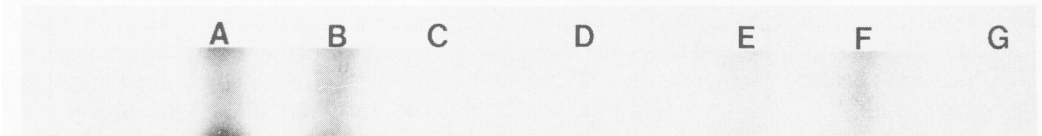


Fig. 1. Morphological characteristics of the hybrid embryo callus-derived plant and its parents. **(A, D, G)** *L. esculentum* cv. UC82-leaf. style under anther cone, and large hairy sepals, respectively, **(B, E, H)** hybrid—leaf, partially exserted style, and small glabrous sepals, respectively **(C, F, I)** *L. peruvianum* 270435 clone 2R2-leaf, exserted style, and small glabrous sepals, respectively.



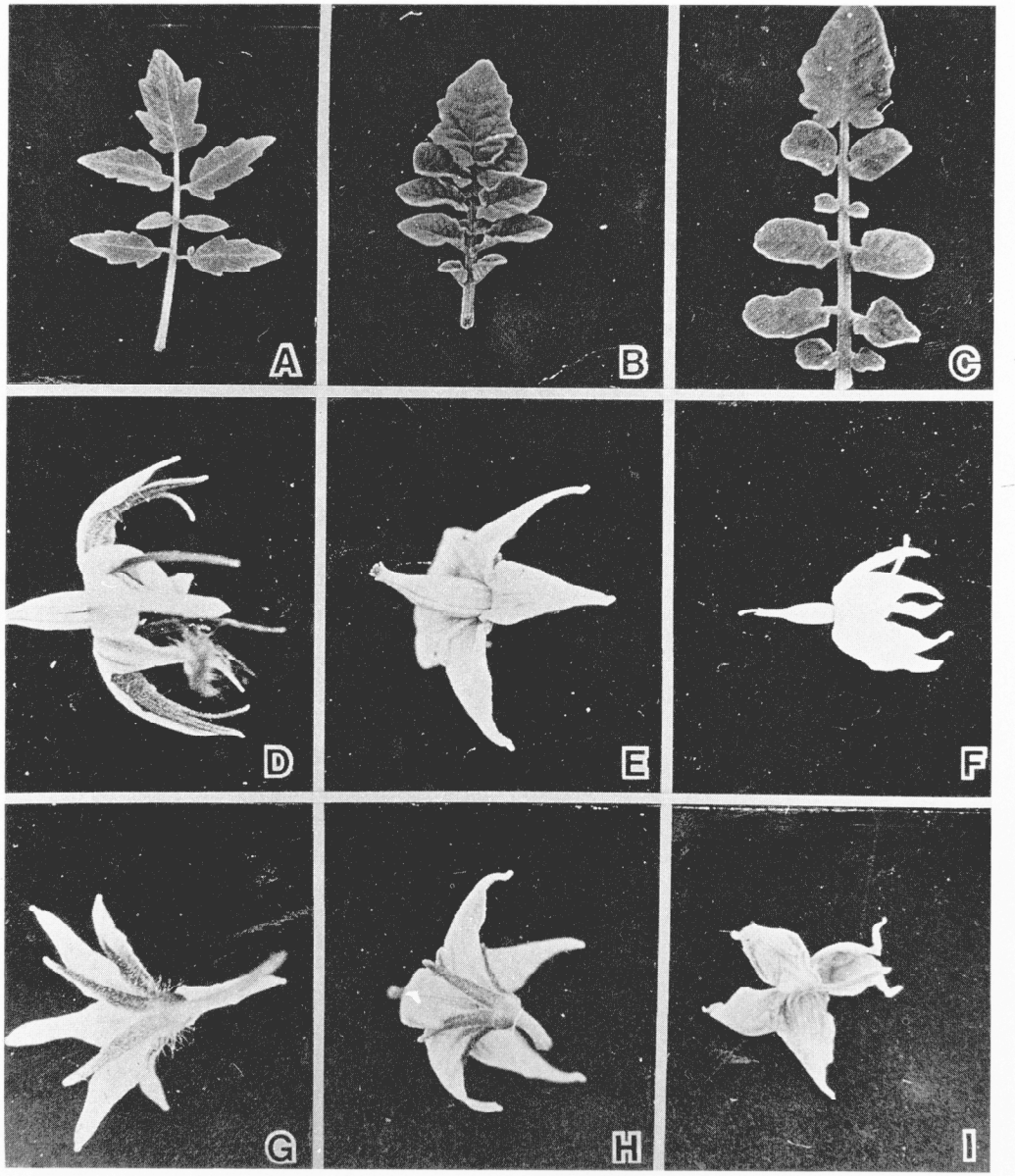


Fig. 3. Morphological characteristics of plants derived from embryo cloning. **(A, D, G)** *L. esculentum* line ms-31–leaf, style deep inside anther cone, and large hairy sepals, respectively, **(D, E, H)** hybrid-leaf, style just inside anther cone, and small glabrous sepals, respectively, **(C, F, I)** *L. peruvianum* var. *glandulosum* 126443 clone 1MH-leaf, exserted style, and small glabrous sepals, respectively.

resistant parents were significantly less than on the susceptible parents at both temperatures. The IR was very low for the two hybrids and the resistant parents. These results provided additional confirmation that transfer of gene(s) promoting heat-stable resistance to RKN has been accomplished.

known. Embryos are heterotrophic during the globular stage and maintain this condition until late heart stage. During the globular stage, embryos obtain their nutrient and growth-regulating substances mainly from the cells of the endosperm, rather than from their own cells. After the late heart stage, they become autotrophic and are culturable in vitro in any regular medium con-

Table 3. Reproduction of *Meloidogyne incognita (eggs* per gram of root) at 25 and 30C soil temperature on *Lycopersicon esculentum* and *L. peruvianum* genotypes and their F_1 progeny.

Genotype	25C eggs/g root	25C IR[z]	30C eggs/g root	30C IR[z]
L. peruvianum glandulosum 126443-1MH	85(3)[y] b[x]	3	1(12) c	0
L. peruvianum 270435-2R2	45(3) b	1	24(12) c	1
L. esculentum ms31	2641(3) a	84	1542(3) b	59
L. esculentum UC82	3153(3) a	100	2625(9) a	100
L. esculentum VFN8	0(3) b	0	2300(10) a	87
L. esculentum UC82 x *L. peruvianum* 270435-2R2	64(3) b	2	50(24) c	2
L. esculentum ms31 x *L. peruvianum glandulosum* 126443-1MH	92(3) b	3	0(3) c	0

[z]Low value = high resistance.
[y]Values in parentheses indicate number of replications.
[x]Mean separation within columns by Duncan's multiple range test *(P* ≤ 0.05).

nature of the hybrid with *L. peruvianum* PI 270435 clone 2R2. Although the hybrid with *L. peruvianum* var. *glandulosum* PI 126443 clone 1MH did not express the *Aps* $1^{+/+}$ allele, the hybrid nature of the plants was determined by expression of the *Aps* $1^{2/2}$ allele from the father plant and, the intermediate band *Aps* $1^{2/+}$. The *Aps 1* phenotype observed for *L. peruvianum* var. *glandulosum* PI 126443 clone 1MH was not the same as described previously by Ammati et al. (1985). The *Aps* $1^{2/+}$ phenotype for *L. peruvianum* var. *glandulosum* PI 126443 also has been observed (V.M. Williamson, personal communication).

The expression of resistance to *M. incognita* in both hybrids at moderate (25C) and high (30C) soil temperatures provides strong evidence for the presence of new alleles at the *Mi* locus, or gene(s) that are different than the heat-sensitive *Mi* gene. Our data suggest that the expression of the resistance is not affected by *L. esculentum* background, since levels of reproduction in both male parents (PI 270435 clone 2R2 and PI 126443 clone 1MH) were similar at both temperatures with those of their respective F_1 hybrids. Roberts et al. (1990) pointed out that virulent populations of RKN selected on *Mi* gene-bearing plants for several generations and used to inoculate a hybrid with combined resistance to high soil temperatures and to *M. hapla* (PI 270435 clone 3MH × PI 126443) were unable to reproduce on this hybrid, suggesting that the resistance factor present in the plants was different from the *Mi* gene.

The expression of the resistance in these hybrids could have important implications for the future of tomato production. Since most of the important nematicides are no longer legal in the United States, the availability of diverse sources of resistance to RKN in *L. esculentum* genotypes is an imperative goal. In production regions where cultivars with the *Mi* gene are used extensively, additional resistance could be useful in reducing the occurrence of virulent nematode populations selected on plants having the heat-sensitive *Mi* gene. In tropical and subtropical areas, where resistant tomato cultivars are overcome by RKN because of high soil temperatures, heat-stable resistance could have important utility. Further, the partial introgression of this (these) new gene(s) into *L. esculentum* constitutes an important step for the improvement of its genetic diversity.

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