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Original article

Lack of correlation between (a)virulence and phylogenetic relationships in root-knot nematodes (*Meloidogyne* spp.)

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Abstract – In tomato, the Mi resistance gene controls the major root-knot nematode species *Meloidogyne arenaria, M. incognita* and *M. javanica.* However, resistance-breaking (*i.e.* virulent) biotypes have been reported from most of the tomato-growing areas in the world. In this study, we assembled a collection of 17 isolates belonging to these three species, either avirulent or virulent against the *Mi* gene, in order to analyze their genetic diversity and phylogenetic relationships. According to parsimony analysis, genomic fingerprints based on RAPD or AFLP markers were independently used to compute trees describing the relationships between the isolates. In both dendrograms, isolates belonging either to the *M. incognita* or *M. javanica* species appeared clustered together, which tends to indicate a monophyletic origin for each of these two species. Conversely, *M. arenaria* isolates were not included in one single clade, which raised questions about the taxonomic status of the currently designated species *M. arenaria.* Moreover, our data showed that clustering of isolates was not associated with their (a)virulence against the tomato *Mi* resistance gene. This lack of correlation between groups determined by molecular markers and virulence indicates that most of the observed DNA polymorphism is independent of virulence, which is presumably under host selection.

***Meloidogyne* spp. / phylogeny / resistance / tomato / virulence**

Résumé – Abscence de corrélation entre (a)virulence et relations phylogénétiques chez les nématodes à galles (*Meloidogyne* spp.) démontrée par analyse RAPD et AFLP. Chez la tomate, le gène Mi de résistance contrôle les principales espèces de nématodes à galles, à savoir *Meloidogyne arenaria, M. incognita* et *M. javanica.* Cependant, des biotypes virulents, c'est-à-dire capables de contourner la résistance, ont été mis en évidence dans la plupart des zones de culture de la tomate à l'échelle mondiale. Au cours de ce travail, nous avons mis en place une collection

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de 17 isolats appartenant à ces trois espèces, caractérisés pour leur avirulence ou leur virulence vis-à-vis du gène *Mi*. L'objectif de notre étude a été d'analyser la diversité génétique et les relations phylogénétiques de ces nématodes, en relation avec leur capacité à se multiplier ou non sur tomate résistante. Les profils moléculaires de chaque isolat, obtenus à partir de marqueurs de type RAPD ou AFLP, ont servi indépendamment de matrice pour construire les arbres phylogénétiques décrivant les relations entre les 17 isolats, par le biais d'une analyse par parsimonie. Une très bonne corrélation a été observée entre les deux types de marqueurs moléculaires. Dans les deux arbres, les isolats appartenant soit à l'espèce *M. incognita*, soit à l'espèce *M. javanica* sont apparus regroupés, ce qui indique très vraisemblablement l'origine monophylétique de ces deux taxons. En revanche, les isolats appartenant à l'espèce *M. arenaria* n'ont pas été distribués au sein d'un unique groupe, ce qui pose la question de leur statut spécifique. De plus, nos résultats ont montré que la distribution des divers isolats n'est pas corrélée avec leur (a)virulence vis-à-vis du gène de résistance *Mi*. Cette abscence de corrélation entre les groupes définis à l'aide de marqueurs moléculaires et selon la virulence des isolats indique que la quasi-totalité du polymorphisme de l'ADN observé est indépendant de la virulence, laquelle est sans doute soumise à la pression de sélection exercée par la plante-hôte.

***Meloidogyne* spp. / phylogénie / résistance / tomate / virulence**

1. INTRODUCTION

Nematodes of the genus *Meloidogyne*, commonly known as root-knot nematodes (RKN), cause loss of yield and quality of a wide range of crops worldwide. Among the most damaging species, *M. arenaria, M. incognita* and *M. javanica* are ubiquitously distributed in tropical and Mediterranean climates. Use of resistant cultivars is the major method of controlling these pests. However, cultivars with specific resistance often become susceptible because of the emergence of new virulent nematode populations. In tomato, the *Mi* resistance gene controls the three previous RKN species, although virulent biotypes have recently been reported from most growing areas in the world [5,11,21]. Therefore, identification and use of new resistance genes is urgently needed for the release of more durable cultivars.

So far, little is known about the genetic relationships that may occur among avirulent and virulent RKN species and populations. However, such information would be of significant importance for the breeding of new resistant cultivars and for the development of new strategies for monitoring virulent populations in the field. In a previous study, we analyzed the molecular diversity of RKN, without being able to identify markers of (a)virulence [15]. The objectives of the research reported here were to study the genetic relationships among a collection of *Mi*-avirulent and *Mi*-virulent isolates belonging to *M. arenaria, M. incognita* and *M. javanica* species, using both RAPD and AFLP markers, in order to determine whether (a)virulence could be related to the deduced phylogenetic structure of this genus.

Table I. Origin of the *Meloidogyne* spp. isolates used in this study and their (a)virulence against the tomato *Mi* resistance gene.

Species	Isolate code	Geographic origin	Virulence against *Mi*
M. arenaria	AN13	Grau-du-Roi, France	yes
	AN14	Château Belair, St Vincent Island	yes
	AN7	Monteux, France	no
	AN8	Saint Benoît, France	no
	AN22	Espiguette, France	no
M. incognita	AN1	Valbonne, France	yes
	AN15	N'Gorom, Senegal	yes
	B6612	The Netherlands	yes
	AN9	Antibes, France	no
	AN31	Taiwan	no
	INCR2	California, USA	no
M. javanica	AN16	The Canary Islands	yes
	AN17	Niaga, Senegal	yes
	AN18	Turkey	yes
	AN10	Avignon, France	no
	AN40	Oualidia, Morocco	no
	AN41	La Réunion Island	no

2. MATERIAL AND METHODS

2.1. Nematodes

Five isolates of *M. arenaria*, six isolates of *M. incognita* and six isolates of *M. javanica* were used in this study (Tab. I). Each isolate consisted of the progeny of a single female selected from the originally sampled natural population and was considered as a clonal line, due to the mitotic parthenogenetic mode of reproduction of these nematodes.

2.2. DNA extraction

Total genomic DNA was extracted from 200 to 300 μl of nematode eggs of each isolate that had been stored at -80 °C before use. Eggs were frozen in liquid nitrogen and ground with a mortar and pestle. The DNA was purified from the resulting powder by a phenol-chloroform extraction [14]. Following ethanol precipitation, DNA was resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA).

Table II. Oligonucleotide primers used for RAPD analysis.

Code	Sequence (5′ to 3′)	% G+C
J10	AAGCCCGAGG	70
J20	AAGCGGCCTC	70
K07	CCCTACCGAC	70
K14	AGCGAGCAAG	60
K09	CCCTACCGAC	70
K14	CCCGCTACAC	70
K16	GAGCGTCGAA	60
K19	CACAGGCGGA	70
M10	TCTGGCGCAC	70
N10	ACAACTGGGG	60
P01	GTAGCACTCC	60

2.3. RAPD procedure

Genomic DNA was diluted to a concentration of 10 ng·μl^{-1}, and 10-mer oligonucleotide primers were tested in RAPD experiments (Tab. II). RAPD-PCR was performed in a final volume of 25 μl containing 10 ng of total genomic DNA, 80 pM of primer, dATP, dCTP, dGTP and dTTP each at 200 μM final concentration, 1× Taq incubation buffer and 0.25 U Taq polymerase (Appligene). Each reaction mixture was overlaid with mineral oil. Amplification was performed on a Biometra TRIO Thermoblock thermal cycler. The cycling program was 1 min at 94 °C; 40 cycles of 20 s at 94 °C; 30 s at 36 °C; 2 min at 70 °C and a final incubation of 10 min at 70 °C. Amplification products were separated by electrophoresis in 1.4% agarose gels in TBE buffer at a constant current of 150 mA for approximately 3 h, and visualized with ethidium bromide (0.5 μg·ml^{-1}) under UV light.

2.4. AFLP procedure

The AFLP procedure was performed essentially as previously described [16] with minor modifications. Nematode DNAs (1 μg) were digested using *Hin*dIII and *Mse*I restriction endonucleases. Ligation of the specific adapters was conducted in RL buffer (10 mM Tris pH 7.5, 10 mM MgAc, 50 mM Kac, 5 mM DTT, 50 ng·μl^{-1} BSA) for 3 h at 37 °C. Primers used in the first pre-selective amplification step were: 5′-GACTGCGTACCAGCTTA-3′ (H+A) and 5′-GATGAGTCCTGAGTAAA-3′ (M+A), which are complementary to

the core sequence of the HindIII and MseI adapters, respectively. Amplification was performed for 28 cycles according to the following cycle program: 1 min at 94 °C, 1 min 20 s at 60 °C, 1 min at 72 °C. Preamplification products were diluted five-fold in TE buffer. Primers used in the second (selective) amplification step were H + AAA and H + AAC in combination with M + AAA, M + AAC, M + AAG, M + AAT, M + ACA, M + ACC, M + ACG, M + ACT, M + AGA, M + AGC, M + AGG, M + AGT, M + ATA, M + ATC, M + ATG, M + ATT. Thus, a total of 32 primer combinations was used. The *Hin*dIII primers were labeled using 1 μCi of ^{33}P-dATP and 0.2 U of T4 polynucleotide kinase for 45 min at 37 °C and 15 min at 70 °C. Amplification of 1 μL of diluted products from the selective pre-amplification was performed according to the following temperature profile: 1 min at 94 °C, 1 min 20 s at 65 °C and 1 min at 72 °C. The annealing temperature was decreased each cycle by 0.7 °C for 12 cycles and was continued at 56 °C for the remaining 24 cycles. The amplification products were mixed with an equal volume of formamide dye (98% formamide, 10 mM EDTA, 0.025% each of xylene cyanol and bromophenol blue) and electrophoresed in 4.75% denaturing polyacrylamide gels for 2 h at 50 W. The gels were dried on Whatmann 3MM paper under vacuum and exposed to X-ray film to produce autoradiographs.

2.5. Data analysis

In both RAPD and AFLP experiments, bands were scored as present or absent directly from the gels or the autoradiographs. Experiments were repeated at least once, and only DNA fragments consistently present or absent between repeats were recorded and considered as binary characters. DNA fingerprints from each isolate were converted to a 0-1 matrix, and phylogenetic analyses were conducted using the computer program PAUP 3.1 [18], according to the following options. Characters were run unordered with no weighting, and the heuristic search algorithm was used to find the most parsimonious tree. Consistency indices were calculated automatically during analyses, and characters that were uninformative were deleted, *i.e.* bands invariant between all isolates or present in one single isolate only [13]. Five hundred bootstrap replicates were performed to test the support of branches for the most parsimonious tree [6], and a 50 per cent majority-rule consensus tree was computed for each analysis.

3. RESULTS

3.1. DNA variation revealed by RAPD

The 17 Meloidogyne isolates were separated based on amplification product patterns from 170 combinations of primer-DNA templates. Results of this analysis are summarized in Table III. Under the reaction conditions described,

the number of amplified fragments per primer varied from 8 to 19, and their size ranged from 500 to 3 000 bp. Of the 125 reproducible RAPD bands obtained with the ten random primers, 10 were monomorphic (*i.e.* present in all the isolates), and 20 were species-specific (4 for *M. arenaria*, 9 for *M. incognita* and 7 for *M. javanica*).

Within *M. incognita* and *M. javanica*, the total number of polymorphic bands was rather low (29 and 18, respectively). Moreover, when the phylogenetically uninformative characters were removed from the whole data set (*i.e.* any amplified fragment present, within one species, in a single isolate only), this number significantly decreased (7 and 8 uninformative characters were amplified from *M. incognita* and *M. javanica* genomic DNA, respectively). For *M. arenaria*, the total number of polymorphic fragments was much higher (52), even though half of them (25) were considered as uninformative characters. Depending on the random primer used, differences were noted in the information provided. For example, most primers differentiated the three RKN species tested, but two did not (primers K09 and N10). In some other cases, no infraspecific polymorphism could be revealed (with primers K14, K16 and K19 for *M. incognita*, and with primers J10, K14, K16 and P01 for *M. javanica*). Moreover, within each species, the number of polymorphic amplified fragments was primer-dependent (for example, within *M. incognita*, from no polymorphic fragments with primers K14, K16 and K19 to 11 polymorphic fragments with primer K07). Nevertheless, from the total data set, it clearly appeared that *M. arenaria* was much more variable than the two other species.

3.2. DNA variation revealed by AFLP

Results of the AFLP analysis are summarized in Table III. Fingerprinting of the 17 *Meloidogyne* isolates with 32 primer combinations revealed a total of 2,104 reproducible amplified DNA fragments, of which 1,550 were polymorphic among all RKN isolates (on the basis of their presence/absence). Depending on the primer combination and DNA template used, 33 to 114 bands were observed, with a number of polymorphic fragments ranging from 19 to 53. The size of the amplified DNA fragments ranged from 50 to 600 bp.

Each primer combination used in this study unambiguously allowed the differentiation of the three species tested. Among the polymorphic fragments, 197 appeared as species-specific characters: 20, 122 and 55 for *M. arenaria*, *M. incognita* and *M. javanica*, respectively. Considering the whole data set, notable differences in polymorphism were observed within each of the three species. In *M. arenaria*, 798 amplified fragments were polymorphic, and 444 of them appeared as phylogenetically informative characters (as defined previously for the RAPD analysis). Significantly fewer polymorphic bands were amplified for both *M. incognita* and *M. javanica*, with 128 and 111 polymorphic fragments, respectively. But unlike with *M. arenaria*, most of these bands (112 and 99, respectively) were shared by at least two isolates within one species

Table III. Comparative parameters of the RAPD and AFLP analysis conducted on 17 root-knot nematode isolates.

	RAPD analysis	AFLP analysis
Primers (or primer combinations) tested	10	32
Reproducible amplified bands	125	2,104
Amplified bands per primer or primer combination (average value)	8 to 19 (12.5)	33 to 114 (65.8)
Size of the amplified bands	500 to 3,000 bp	50 to 600 bp
Monomorphic bands (%)	10 (8.0%)	554 (26.3%)
Species-specific bands (%)	20 (16.0%)	197 (9.3%)
Amplified / polymorphic (%) / phylogenetically informative bands within:		
– *M. arenaria*	86 / 52 (60.5%) / 27	1,856 / 798 (43.0%) / 444
– *M. incognita*	66 / 29 (43.9%) / 22	1,185 / 128 (10.8%) / 112
– *M. javanica*	67 / 18 (26.9%) / 10	1,351 / 111 (8.2%) / 99

(*i.e.* phylogenetically informative). Using appropriate primer combination(s), each of the 17 RKN isolates could be easily differentiated from all the others.

3.3. Phylogenetic analysis

Parsimony analysis of the 0-1 data matrix using the heuristic search algorithm resulted in 6 and 8 shortest trees with 185 and 1,884 steps for the RAPD data and AFLP data, respectively. The consistency index (CI), including or excluding uninformative characters, was 0.622 or 0.583 for the RAPD data, and 0.765 or 0.731 for the AFLP data (data not shown).

For each of the two data sets, bootstrap analysis with 500 replicates resulted in a majority rule consensus tree (Fig. 1) with almost exactly the same topological configuration as the shortest consensus trees previously obtained. Although not identical, the RAPD-based tree and the AFLP-based tree appeared largely similar, and inferred a number of relationships between the RKN isolates tested. In both analyses, our results indicated that the species *M. incognita* and *M. javanica* form each a monophyletic group, with bootstrap values of 100 per cent for the two clusters. Conversely, the 5 *M. arenaria* isolates were not clustered together, suggesting a polyphyletic origin for these isolates. Of particular concern, isolates AN22 and AN13 appeared very distinct from all other isolates. At the infraspecific level, no evidence was provided for any significant clustering, except for two subsets of isolates that were found to be associated in the AFLP tree only: isolates INCR2 and AN15 in *M. incognita* clade, and isolates AN18, AN40 and AN41 in the *M. javanica* clade, with bootstrap values of 0.97 and 0.89, respectively. Within each of the three RKN species, virulent isolates were not clearly differentiated from the avirulent ones, based either on RAPD or AFLP data, but appeared randomly distributed in the trees. Overall, no correlation could be achieved between (a)virulence of the isolates and the topology of the phylogenetic trees computed.

RAPD and AFLP data were further combined in a single matrix, which was analyzed using parsimony under the same options chosen for the two individual analysis. The resulting majority rule consensus tree showed the same general topology (and the same bootstrap support at interspecific nodes) as observed previously (Fig. 2), *i.e.* no grouping of the 5 *M. arenaria* isolates tested, and lack of correlation between (a)virulence and phylogenetic clustering of the isolates.

4. DISCUSSION

Based on their ability to reproduce or not on resistant tomato cultivars bearing the Mi gene, RKN isolates belonging to the three species *M. arenaria*, *M. incognita* and *M. javanica* can be classified as virulent or avirulent, respectively. The main question related to our study was whether any association occurs between the (a)virulence of isolates and their (phylo)genetic background. In spite

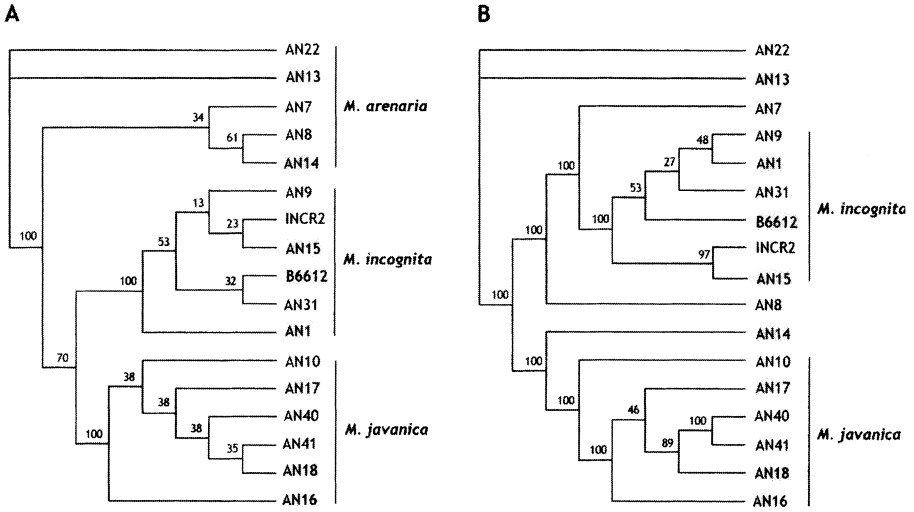

Figure 1. Majority-rule consensus trees describing the phylogenetic relationships of *Meloidogyne* spp. isolates. (A) Tree based on RAPD data. (B) Tree based on AFLP data (B). Numbers at nodes represent the percentage of bootstrap values supporting each internal branch. Grey boxes correspond to isolates virulent on *Mi*-resistant tomato. Population codes are given in Table I.

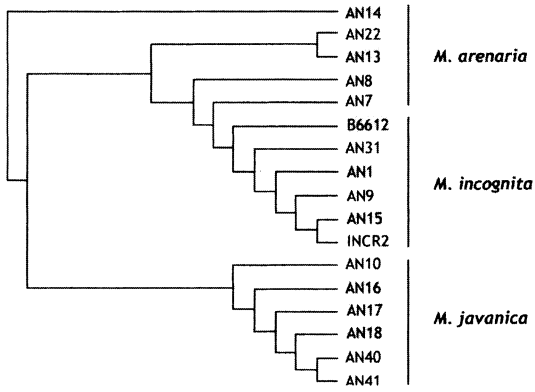

Figure 2. Majority-rule consensus trees describing the phylogenetic relationships of *Meloidogyne* spp. isolates based on the combination of RAPD and AFLP data. Numbers at nodes represent the percentage of bootstrap values supporting each internal branch. Grey boxes correspond to isolates virulent on *Mi*-resistant tomato. Population codes are given in Table I.

of the variability detected, our results indicated that virulent isolates were not clearly distinct from the avirulent ones, either within RKN as a whole or within any of the three species tested. Indeed, the fact that neutral markers differentiate avirulent and virulent RKN would have been quite unexpected, although

an AFLP marker linked to avirulence was recently identified in the Asian rice gall midge [2]. In fact, our result indicates that most of the DNA polymorphism revealed by RAPD or AFLP markers in these nematodes is largely independent of (a)virulence. The three RKN species studied here reproduce exclusively by apomictic (*i.e.* mitotic) parthenogenesis [19]. A direct relationship between phylogenetic and virulence groupings would have indicated that virulent biotypes share a common ancestor, and would suggest that the current worldwide distribution of virulent isolates is the result of genetic variation restricted to this particular ancestor (from one or a few centres of origin?) and subsequent random dissemination of its progeny through agronomical practices [20]. Therefore, because of the inability to identify markers correlated with virulence, it seems unlikely that virulent biotypes are of monophyletic origin within RKN. In this regard, it can be hypothesized that the virulent lineages appeared after the establishment (and dissemination?) of the clonal isolates within each species, probably as the result of independent mutational events. Similar experiments conducted on the closely related potato cyst nematodes from the genus *Globodera* revealed contrasted features. In *G. rostochiensis*, a number of AFLP markers were identified which were specific to different avirulence phenotypes (= pathotypes). On the contrary, the clustering of *G. pallida* isolates revealed limited resemblance with their pathotype classification [8]. In the case of these amphimictic species, it has been suggested that the difference in distribution of polymorphisms among *G. rostochiensis* and *G. pallida* isolates might indicate a higher level of gene flow among the *G. pallida* isolates compared to the *G. rostochiensis* isolates [8].

Comparison of RAPD and AFLP analysis demonstrated the high potential of AFLP to generate infraspecific polymorphism, with 5- to 10-fold more bands amplified with the latter procedure compared to the RAPD methodology (on average, 12.5 bands per primer to 65.8 bands per primer combination amplified in the RAPD and AFLP analysis, respectively). Although some authors questioned the validity of phylogenetic inference based on RAPD markers [1,3], a number of studies demonstrated that evolutionary trees built upon such markers are consistent with trees inferred from other characters in systems with well-known phylogenies [4]. Moreover, similar conclusions were provided by RAPD and AFLP analysis as far as phylogenetic relationships were concerned [7,8,17]. The general agreement found here between the dendrograms based on AFLPs and RAPDs, and also the high internal consistency of the data at the specific level, as inferred from bootstrap support of the nodes for *M. incognita* and *M. javanica*, supported the reliability of our analysis. Moreover, as the RAPD and AFLP markers generated here could reasonably be considered as independant characters, and although no statistical test to support the homogeneity between the two data sets had been performed, they were combined to run a global parsimony analysis, according to the "total evidence" approach [12]. Since combining data led to the same conclusions than the individual analysis did, we decided to consider that the resulting tree provided an accurate

estimate of the phylogenetic relationships of the isolates studied [9]. Overall, isolates belonging either to the *M. incognita* or *M. javanica* species were clustered together, which suggested a monophyletic origin for each of these two species. Conversely, *M. arenaria* isolates were not included in one single clade, even though the tree topologies deduced from RAPDs and AFLPs were not identical. This result is consistent with previous data showing a high heterogeneity within *M. arenaria* compared with both *M. incognita* and *M. javanica*. Cytological studies reported the occurrence of at least three forms (a major triploid form with a somatic chromosome number ranging from 50 to 56, and also diploid and hypotriploid forms) and raised questions about the taxonomic status of the diploid and hypotriploid isolates [19]. In comparison to the other species tested, mtDNA RFLP analysis showed a significant dichotomy within *M. arenaria* [10]. Recently, AFLP fingerprinting also revealed such intraspecific diversity [16]. Undoubtedly, an expanded molecular phylogenetic study of a large collection of isolates currently designated as belonging to *M. arenaria*, including the different cytogenetic forms, would clarify patterns of diversification and relationships within this group. If such an analysis confirms that the species *M. arenaria*, as presently considered, is not of monophyletic origin, then revision of this taxon might be examined.

The recent identification of new resistance genes in the wild species *Lycopersicon peruvianum* [22–24] offered new perspectives in terms of tomato breeding. However, it is not yet known whether these genes will be overcome by virulent isolates in the near future, although such information would be of outstanding importance in evaluating the durability of the resistance they might confer to new commercial tomato cultivars. In this study, isolates virulent against the *Mi* resistance gene only were tested, because such material alone was available at that time. Assuming that isolates virulent against other tomato resistance genes are identified soon, their molecular fingerprinting would be of interest, in order to test whether the conclusion of the present work (*i.e.* lack of correlation between (a)virulence against a resistance gene and phylogenetic relationships) can be generalized to a wider range of RKN-tomato genotypic interactions.

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