Theor Appl Genet (1997) 95:992–996

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Breakdown of self-incompatibility in tetraploid *Lycopersicon peruvianum*: inheritance and expression of *S*-related proteins

Received: 11 September 1996 / Accepted: 21 March 1997

Abstract *Lycopersicon peruvianum* displays gametophytic self-incompatibility (GSI). We have isolated self-compatible (SC) tetraploids of *L. peruvianum* from tissue-cultured leaves and have explored the expression and inheritance of their *S*-related proteins. The *S*-related protein profiles of styles of SC tetraploids were indistinguishable from the diploid self-incompatible (SI) explant source based on SDS-PAGE. All progeny obtained from self-fertilization of two tetraploids were SC. Cloned cDNA sequences of the *S*-related proteins were used to determine the inheritance of this locus in these progeny through Southern hybridization. The allelic ratio, as determined from the intensity of DNA restriction fragments, was consistent with the predicted ratio if only pollen bearing two different alleles was successful in achieving fertilization. All progeny obtained had at least one copy of each allele, and individuals fully homozygous for either allele were not found, indicating that pollen grains bearing two identical alleles were inhibited. In addition, the level of expression of the *S*-related proteins in the progeny correlated with the allelic dosage at the DNA level. We demonstrate that the observed self-compatibility in the tetraploids was not caused by an alteration in the expression of *S*-related proteins.

Key words Self-incompatibility · Tetraploids · Heterogenic pollen · Plant breeding · Tomato · *Lycopersicon peruvianum*

Publication No. 3212 of the Massachusetts Agricultural Experiment Station

Communicated by B. S. Gill

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Introduction

Members of the family Solanaceae exhibit gametophytic self-incompatibility (GSI) that is controlled by a single major locus, the *S* locus, and the incompatibility phenotype is controlled by the genotype of the pollen and the style (de Nettancourt 1977). The *S*-related proteins of the styles are basic, glycosylated proteins of low molecular weight (Mau et al. 1986; Anderson et al. 1986; Ai et al. 1990; Kaufmann et al. 1991). These proteins exhibit ribonuclease activity and are referred to as *S*-RNases (McClure et al. 1989). They have been shown to be essential for the GSI response in transgenic plants (Lee et al. 1994; Murfett et al. 1994). During an incompatible response the *S*-RNases are involved in the degradation of pollen RNA, which results in the disruption of protein synthesis and eventually the arrest of pollen-tube growth (McClure et al. 1990).

Polyploidy is known to result in the breakdown of GSI in the Solanaceous species *Nicotiana* (Pandy 1968), *Petunia* (Stout and Chandler 1942), *Solanum* (Livermore and Johnstone 1940), and *Lycopersicon* (de Nettancourt et al. 1974), as well as in the non-Solanaceous, *Trifolium* (Brewbaker 1954), *Pyrus* (Crane and Lewis 1942), and *Oenothera* (Lewis 1947).

In a previous study Frary (1990) recovered rare self-compatible SC *Lycopersicon peruvianum* plants from tissue-cultured leaves of SI plants. These plants had increased leaf and guard cell size, which suggested that they might be autopolyploids. Herein we describe the breakdown of the GSI response in tetraploid *L. peruvianum* heterozygous for the *S* alleles for which cDNA clones of both the alleles were available (Rivers et al. 1993; Liang et al. 1994).

Materials and methods

Plant material

A single plant of *Lycopersicon peruvianum* (from accession LA 2163, kindly provided by C. M. Rick, University of California, Davis) from which the S_{m1} and S_{m2} alleles were cloned served as the source of diploid tissue from leaf explants (Chawla PhD thesis). We refer to the diploid genotype (having one copy each of S_{m1} and S_{m2} as "S_{m12}", the tetraploid genotype bearing two copies of each allele as "S_{m1122}", etc. A "related *L. peruvianum*" refers to a plant belonging to the same accession used in the study but differing in genotype at the *S* locus. The ploidy status of two SC regenerants used in further genetic studies was determined by pollen size and leaf guard cell measurements (Wilson et al. 1981). Their pollen diameter was 20–30% larger and guard cells 40% larger than those of the diploid GSI plants, indicating that they were tetraploid (Dowley et al. 1975). Chromosome counts in pollen mother cells (Belling 1926) and from roots originating on leaf cuttings further confirmed that they were tetraploid ($2n = 48$).

Pollination studies

Reciprocal crosses were performed between self-fruitful regenerants and the original diploid plants. The aniline-blue fluorescence technique (Martin 1959) was used to observe pollen-tube growth within the style. For tetraploid × diploid, and diploid × diploid crosses, the styles were removed from the flowers 72 h after pollination (to allow for completion of the GSI response). For the diploid × tetraploid crosses the styles were removed 48 h after pollination as flowers usually abscised by 72 h post-pollination. Fixation and staining was done as previously described (Williams and Webb 1987).

Gel electrophoresis of style proteins

Proteins from three styles and stigmas of mature flowers of individual plants were extracted and analyzed on 20% SDS-polyacrylamide gels after staining with Coomassie blue (Bernatzky 1993).

Southern analysis

DNA was extracted from the young leaves by a modified miniprep method (Bernatzky and Tanksley 1986). Approximately 2 ug DNA was digested overnight with *Eco*RI as per the manufacturer's instructions (Promega). Electrophoresis, Southern blotting onto Hybond N^+ membranes (Amersham), and hybridization were done as described before (Bernatzky and Schilling 1992). Probes were made by random priming (Feinberg and Vogelstein 1984) of inserts of S_{m1} and S_{m2} cDNA clones. Equal counts (approximately 5×10^7 Bq/μg template) of both probes were added to the hybridization solution. The filters were washed at a final stringency of $0.5 \times$ SSC, 0.1% SDS at 68°C and exposed to X-ray film at −70°C with an intensifier screen (Dupont).

Results

Breeding behavior

Two self-fertile fruitful plants were selected from tissue-culture regenerated shoots. Germination studies (not presented) showed that seeds of these plants had a germination percentage comparable to that for control diploid crosses ($S_{m12} \times$ a related *L. peruvianum*). In reciprocal crosses, when the diploid was used as a female and the tetraploid as the male, fruit set was always observed. Fruits remained small and contained many undeveloped seeds. This was expected as $2n♀ \times 4n♂$ cross leads to collapse of the seeds due to abnormal endosperm development (Cooper and Brink 1945). Since we did observe undeveloped seeds, we classified this cross as compatible.

In the reciprocal cross, tetraploid ♀ × diploid ♂, only 3 of 32 pollinations resulted in fruit set. These fruits remained small and had on average five collapsed seeds per fruit. This suggests that the style tissue of the tetraploid exhibited an incompatible but perhaps weakened response. All progeny obtained from the self-pollination of tetraploid regenerants were SC based on fruit set and seed development. Two progeny from each genotypic class (based on DNA and protein profiles, see below), i.e. S_{m1122}, S_{m1112}, S_{m1222}, were self-pollinated and seed viability determined. These representative progeny, irrespective of their genotype, set viable seeds, and the seed number per fruit was comparable to that of a diploid compatible cross. The diploid ♀ × tetraploid ♂ resulted in the presence of many pollen tubes at the base of the style 48 hr after pollination. At the same point in time the pollen tubes showed very little growth in the reciprocal crosses. However, at 72 h post-pollination there was an average of four to five tubes at the basal end of the style for the tetraploid ♀ × diploid ♂ cross (two observations). The self-pollinated diploid did not have any pollen tubes at the basal end of the style after 72 h.

Genetic analysis

SDS-PAGE of the stylar extracts revealed no obvious differences between the diploid GSI source of explant and the regenerants (Fig. 1). The two *S*-locus-associated proteins of MW 24–26 kDa corresponding to the products of the S_{m1} and S_{m2} alleles were seen in both the original GSI explants and the two SC regenerants, indicating that the expression of *S*-related proteins was similar. Southern blotting of the diploid parent DNA digested with *Eco*RI and probed with their respective cDNA clones showed two bands of 5 kb and 1.3 kb for the S_{m1} allele and two of 4.2 kb and 0.6 kb for the S_{m2} allele. Since the DNA blots were probed with equal amounts of S_{m1} and S_{m2} probes, we expected to see bands of equal intensity in the diploid (S_{m12}) and the tetraploid (S_{m1122}) plants and shifts in intensity proportional to allelic dosage in the progeny. For example, a S_{m1112} plant would show an approximately threefold higher signal for the S_{m1} restriction fragments as compared to that for the S_{m2} allele (Fig. 2). On the basis of random chromosome segregation, five different

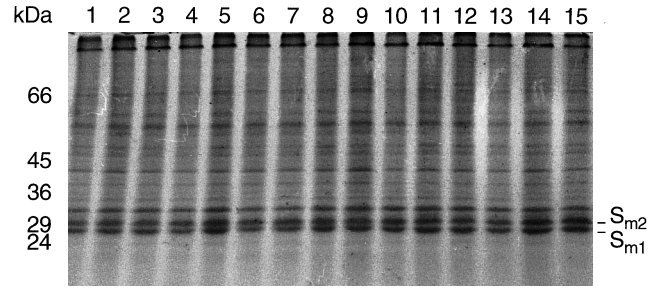

Fig. 1 SDS-PAGE analysis of stylar extracts of the diploid and its tissue-cultured regenerants. The *S*-associated proteins are indicated at right. Lane 1 is the original diploid; *lanes 2–15* are the tissue-cultured regenerants. *Lanes 2* and *3* were subsequently found to be tetraploids. Molecular-weight estimates are indicated on the *left* in kilodaltons (kDa)

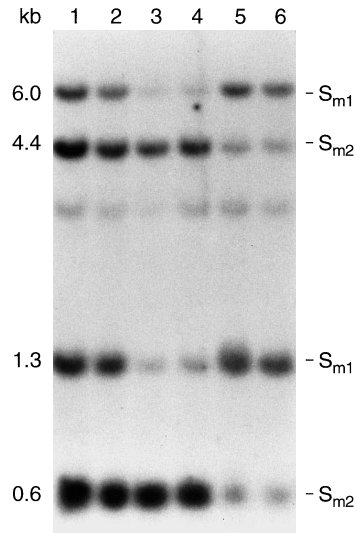

Fig. 2 DNA analysis of progeny obtained from self-pollination of tetraploids. DNA was digested with *Eco*RI, the fragments separated by gel electrophoresis, blotted, and hybridized to cloned S_{m1} and S_{m2} cDNA fragments. The approximate sizes of the fragments are indicated on the left in kilobases (kb). Fragments of approximate sizes 6.0 kb and 1.3 kb corresponded to the S_{m1} allele, those of 4.4 kb and 0.6 kb to the S_{m2} allele. The genotypes represented in the lanes are as follows: lanes 1 and 2 S_{m1122}, lanes 3 and 4 S_{m1222}, lanes 5 and 6 S_{m1112}

genotypes (i.e., S_{m1111}, S_{m1122}, S_{m1112}, S_{m1222}, and S_{m2222}) should be observed amongst the progeny from the selfing of S_{m1122}, if all pollen genotypes (S_{m11}, S_{m12}, S_{m22}) were equally likely to achieve fertilization. We analyzed 87 and 91 progeny – obtained from the selfing of two independent tetraploid regenerants – for DNA signal intensity for both alleles. Theoretically, if all the pollen was able to achieve fertilization, we would expect one S_{m1111} and one S_{m2222} for every 36 offspring (i.e., be tetraplex for that allele). We observed 14 S_{m1112}, 54 S_{m1122}, and 19 S_{m1222} in one population and 18 S_{m1112}, 62 S_{m1122}, and 11 S_{m1222} in the second population. However, we did not recover a single tetraplex individual in either population (i.e., S_{m1111} or S_{m2222}). In addition, both of these sets of progeny conformed to a ratio of 1 (S_{m1112}) : 4(S_{m1122}) : 1(S_{m1222}), which fits the expected ratio if only the heterogenic pollen (S_{m12}) achieved fertilization (Table 1). The stylar extracts of individual offspring from the self-pollinated tetraploids were subjected to SDS-PAGE analysis in order to see whether the *S*-protein dosage correlated with the allelic dosage at the *S* locus. A random sample of these protein profiles is show in Fig. 3. The two *S*-locus-associated proteins showed dosage differences that correlated with allelic dosage based on DNA hybridization. Although this correlation was based on visual inspection, it suggests that the level of protein expression matches the allelic dosage based on DNA. Therefore, the expression of *S*-related proteins of the style is not disturbed in the SC tetraploids or their progeny.

Discussion

Tissue culture was used to regenerate tetraploid SC plants from diploid GSI explants. Although cytochimerism has been observed in in vitro-regenerated *L. peruvianum* (Sree Ramulu et al. 1976), we

Table 1 Observed and expected genotypic ratios obtained from self-pollination of tetraploids

Genotype of self-pollinated progeny	Expected ratio without pollen selection[a]	Observed ratio in tetraploid I[b]	Observed ratio in tetraploid II[b]	Expected ratio with selection against homogenic pollen
S_{m1111}	0.03	0.00	0.00	0.00
S_{m1112}	0.22	0.16	0.20	0.17
S_{m1122}	0.50	0.62	0.68	0.67
S_{m1222}	0.22	0.22	0.12	0.17
S_{m2222}	0.03	0.00	0.00	0.00

[a] Based on random chromosome assortment

[b] Observed ratio was significantly different from expected ratio without pollen selection at $P > 0.05$ for tetraploid I and tetraploid II ($\chi^2 = 17.1$ and 15.7, respectively) but not significantly different from the expected ratio with pollen selection ($\chi^2 = 2.62$ and 1.72, respectively)

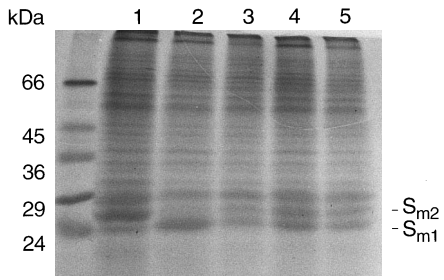

Fig. 3 SDS-PAGE of the stylar proteins of selected offspring of one of the SC tetraploids whose genotype was determined by the DNA dosage analysis described in Fig. 2. The *S*-associated proteins are indicated. The genotypes of the samples as determined by DNA dosage are as follows: *lane 1* S_{m1222}, *lane 2* S_{m1112}, *lanes 3* and *4* S_{m1122}. Lane 5 is the diploid explant source, S_{m12}. Molecular-weight estimates listed on the *left* in kiloDaltons

eliminated the possibility that our selected regenerants were cytochimeras by making chromosome counts or using phenotypic analysis for the derivatives of each shoot apical cell layer. In most flowering plants, stomates are L1 derivatives, pollen L2 derivatives and adventitious roots are L3 derivatives (Tilney-Bassett 1986). All three cell layers of the SC regenerants displayed increased ploidy: larger stomates, larger pollen grains, increased number of chromosomes in root tips. In addition, our plants were regenerated from leaf tissue, and it has been shown that most shoots regenerated in this fashion are non-chimeric even if the source tissue is chimeric (Marcotrigiano 1986).

Tetraploidy has been known to breakdown GSI in many species. Lewis (1947) proposed that heterogenic pollen loses its GSI phenotype leading to SC. Our study on the progeny obtained from the selfing of *L. peruvianum* tetraploids showed that the genotypic ratio of the progeny, based on direct analysis of *S*-related proteins and their corresponding DNA, was consistent with the expected ratio when only the heterogenic pollen was able to achieve fertilization. The expected genotypic ratios obtained from selfing the tetraploids showed a close fit to ratios based on random chromosome assortment (Table 1).

All the progeny obtained from selfing the tetraploids, irrespective of their genotype, were SC. This was expected as each would produce some amount of heterogenic pollen, which has been proposed to be responsible for the SC behavior. The cross diploid ♀ × tetraploid ♂ was also compatible, which was expected as the tetraploid-derived SC heterogenic pollen would presumably be able to grow down the GSI diploid style and effect fertilization. These results are in contrast with the observations of de Nettancourt et al. (1974). In their study of a self-pollinated tetraploid of *L. peruvianum* they found the SC character was transmitted to approximately half the progeny whereas the remaining plants were self-sterile. The self-sterile condition could be attributed to poor pollen fertility. However, in a number of their self-sterile plants pollen abortion was low, and in some cases, pollen-tube inhibition was typical for an GSI reaction. Since the SC tetraploids of de Nettancourt et al. (1974) were derived after four generations of forced inbreeding, the effects of inbreeding on sterility could not be separated from responses due to GSI.

In our experiment, the reciprocal cross (tetraploid × diploid) was mainly incompatible but some fruit set was observed albeit at a low frequency (< 10%). These fruits remained small and contained collapsed seeds, indicating that fertilization had taken place. Since pollen from the GSI diploid parent would carry a single *S* allele, the GSI response of the tetraploid style to the haploid pollen may differ from that of a diploid style. This was confirmed by style squashes which showed that the pollen tubes grew much longer in a 4n ♀ × 2n ♂ cross than in an incompatible cross, though not more than four to five tubes on average passed through the base of the style.

We still do not know why heterogenic pollen is able to achieve fertilization. Lewis (1947) proposed a competition theory which postulated that the products of the two alleles competed in a diploid pollen grain for a limited amount of substrate, so that neither of the alleles produced its normal effect of GSI. A major drawback to this theory is that it does not explain the GSI behavior of homogenic pollen (i.e., pollen having the same *S* alleles) as presumably competitive interactions would also occur in this pollen (de Nettancourt 1977). In triploids of *L. peruvianum* (de Nettancourt 1977; Sree Ramulu et al. 1977), SC does not occur even though gamete viability is high and a portion of the pollen is expected to be heterogenic. Instead, the reaction is typical of GSI. The model of competitive interaction is further compromised in that different tetraploid lines bearing the same *S* alleles in *L. peruvianum* (de Nettancourt et al. 1974) and in *N. alata* (Pandy 1968) display large variations in SC. The self-sterile features of triploids and the variability among tetraploids has led to the conclusion that the interaction between *S* alleles in pollen depends on the genetic background and possibly on genetic dosages of other factors in the pollen grain (de Nettancourt 1977).

The nature of the pollen component and the interactions between pollen and style are not yet known. Two models have been advanced to explain the specific interactions between the pollen and the style. One (Haring et al. 1990) assumes that the specificity of the GSI response lies in the specific uptake of the *S*-RNases, whereas the other (Thompson and Kirch 1992) proposes that the specificity lies in the inhibition of RNases once they enter the pollen by *S*-specific inhibitors. In such a specific *milieu*, it is likely that the presence of two different factors in a single pollen could lead to the failure of its recognition by the stylar component bearing the same alleles. These two

receptors/factors would probably occupy similar domains that would lead to their abnormal conformation, or if dimerization of the pollen component is involved, that would lead to the formation of unproductive heterodimers. Both of these scenarios would, in effect, block the interaction between the pollen and the stylar protein. Since only one type of "factor" is present in homogenic pollen, there is no failure of recognition in this case, and the reaction is GSI. It is evident that further research needs to be performed to elucidate the recognition factors controlling pollen-style interactions in plants possessing an SI system.

Acknowledgments We thank Drs. D. Stein and T. H. Boyle for their critical reviews of the earlier drafts of this manuscript. This paper was submitted in partial fulfillment of the PhD of Bindu Chawla.

References

Ai Y, Singh A, Coleman CE, Ioerger TR, Kheyr-Pour A, Kao T-H (1990) Self-incompatibility in *Petunia inflata*: isolation and characterization of cDNAs encoding three *S* allele associated proteins. Sex Plant Reprod 3:130–138

Anderson MA, Cornish EC, Mau SL, Williams EG, Hoggart R, Atkinson A, Bonig I, Grego B, Simpson R, Roche PJ, Haley JD, Penschow JD, Niall HD, Tregear GW, Coghlan JP, Crawford RJ, Clarke AE (1986) Cloning of cDNA for a stylar glycoprotein associated with the expression of self-incompatibility in *Nicotiana alata*. Nature 321:38–44

Belling J (1926) The iron-acetocarmine method of fixing and staining chromosomes. Biol Bull 50:160–162

Bernatzky R (1993) Genetic mapping and protein product diversity of the self-incompatibility locus in wild tomato (*Lycopersicon peruvianum*). Biochem Genet 31:173–183

Bernatzky R, Tanksley SD (1986) Genetics of actin-related sequences in tomato. Theor Appl Genet 72:314–321

Bernatzky R, Schilling A (1992) Methods for Southern blotting and hybridization. In: Beckmann JS, Osborn TC (eds) Plant genomes: methods for genetic and physical mapping. Kluwer Academic Publ, Dordrecht, pp 15–31

Brewbaker JL (1954) Incompatibility in autotetraploid *Trifolium repens* 1. Competition and self-compatibility. Genetics 39:307–316

Chawla B (1996) Genetic analysis of the breakdown of self-incompatibility in *Lycopersicon peruvianum*. PhD thesis, University of Massachusetts, Amherst

Cooper DC, Brink RA (1945) Seed collapse following matings between diploid and tetraploid races of *Lycopersicon pimpinellifolium*. Genetics 30:376–401

Crane MB, Lewis D (1942) Genetical studies in pears. III. Incompatibility and sterility. J Genet 43:31–42

de Nettancourt D (1977) Incompatibility in Angiosperms. Monographs on theoretical and applied genetics. Springer, Berlin Heidelberg New York

de Nettancourt D, Saccardo F, Laneri U, Capaccio E (1974) Self-compatibility in a spontaneous tetraploid of *Lycopersicon peruvianum* Mill. In: Polyploidy and induced mutations in plant breeding. IAEA, Vienna, pp 77–84

Dowley LJ, Routley DG, Pierce LC (1975) Effect of ploidy level on stomate number and guard cell length in tomato. Irish J Agric Res 14:220–221

Feinberg AP, Vogelstein B (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 134:266

Frary A (1990) The stability of self-incompatibility in *L. peruvianum* plants regenerated from in vitro cultures of leaf and cotyledon tissue. Honor's thesis, Biology Dept. Mount Holyoke College, South Hadley, Mass.

Haring V, Gray JE, McClure BA, Anderson MA, Clarke AE (1990) Self-incompatibility: a self-recognition system in plants. Science 250:937–941

Kaufmann H, Salamini F, Thompson RD (1991) Sequence variability and gene structure at the self-incompatible locus of *Solanum tuberosum*. Mol Gen Genet 226:457–466

Lee HS, Huang S, Kao T-H (1994) *S*-proteins control rejection of incompatible pollen in *Petunia inflata*. Nature 367:560–563

Lewis D (1947) Competition and dominance of incompatibility alleles in diploid pollen. Heredity 1:85–108

Liang W, Rivers BA, Bernatzky R (1994) Molecular cloning of a second *S*-allele from *L. peruvianum* and assessment of allele frequencies in populations based on DNA hybridization. Rep Tomato Genet Coop 44:22–23

Livermore JR, Johnstone FE (1940) The effect of chromosome doubling on the crossability of *Solanum chacoense*, *S. jamesii* and *S. bulbocastanum* with *S. tuberosum*. Am Potato J 17:170–173

Marcotrigiano M (1986) Origin of adventitious shoots regenerated from cultured tobacco leaf tissue. Am J Bot 73:1541–1547

Martin FW (1959) Staining and observing pollen tubes in the style by means of fluorescence. Stain Technol 34:125–128

Mau S-L, Williams EG, Atkinson A, Anderson MA, Grego B, Simpson RJ, Clarke AE (1986) Style proteins of wild tomato associated with the expression of self-incompatibility. Planta 169:184–191

McClure BA, Haring V, Ebert Pr, Anderson MA, Simpson RJ, Sakiyama F, Clarke AE (1989) Style self-incompatibility gene producs of *N. alata* are ribonucleases. Nature 342:955–957

McClure BA, Gray JE, Anderson MA, Clarke AE (1990) Self-incompatibility in *Nicotiana alata* involves degradation of pollen rRNA. Nature 347:757–760

Murfett J, Atherton TL, Mou B, Gasser CS, McClure BA (1994) *S*-RNase expressed in transgenic *Nicotiana* causes *S*-allele specific pollen rejection. Nature 367:563–566

Pandy KK (1968) Colchicine induced changes in the self-incompatibility behavior of *Nicotiana*. Genetica 39:257–271

Rivers BA, Bernatzky R, Robinson SJ, Jahnen-Dechent W (1993) Molecular diversity at the self-incompatibility locus is a salient feature in natural populations of wild tomato (*Lycopersicon peruvianum*). Mol Gen Genet 238:419–427

Sree Ramulu K, Carluccio F, de Nettancourt D, Devreux M (1977) Trisomics from triploid-diploid crosses in self-incompatible *Lycopersicon peruvianum*. I. Essential features of aneuploids and self-compatible trisomics. Theor Appl Genet 50:105–119

Sree Ramulu K, Devreux M, Ancora G, Laneri U (1976) Chimerism in *L. peruvianum* plants regenerated from *in vitro* cultures of anthers and stem internodes. Z Pflanzenzuecht 76:299–316

Stout AB, Chandler C (1942) Hereditary transmission of induced tetraploidy and compatibility in fertilization. Science 96:257

Thompson RD, Kirch HH (1992) The *S*-locus of flowering plants: When self-rejection is self interest. Trends Genet 8:383–387

Tilney-Bassett RAE (1986) Plant chimeras. Edward Arnold, London

Williams EG, Webb MC (1987) Enclosure of pollinated flowers weakens the gametophytic self-incompatibility response in *Lycopersicon peruvianum*. J Exp Bot 38:1756–1764

Wilson CL, Pusey Pl, Otto BE (1981) Plant epidermal sections and imprinting using cyanoacrylate adhesives. Can J Bot 61:781–782