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Unilateral incongruity in crosses involving *Lycopersicon pennellii* and *L. esculentum* is distinct from self-incompatibility in expression, timing and location

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Abstract Both interspecific and intraspecific mechanisms restrict the exchange of genes between plants. Much research has focused on self incompatibility (SI), an intraspecific barrier, but research on interspecific barriers lags behind. We are using crosses between *Lycopersicon esculentum* and *L. pennellii* as a model with which to study interspecific crossing barriers. The cross *L. esculentum*×*L. pennellii* is successful, but the reciprocal cross fails. Since the cross can be successfully made in one direction but not the other, gross genomic imbalance or chromosomal abnormality are precluded as causes. We showed that the lack of seed set observed in the cross *L. pennellii*×*L. esculentum* is due to the inability of pollen tubes to grow more than 2–3 mm into the style, whereas SI crosses show continued slow pollen tube growth but, also, fail to set seed. These results indicate that the unilateral response is a barrier distinct from SI, differing from SI in the timing and location of expression in the style. We therefore suggest that this unilateral response in the *L. pennellii*×*L. esculentum* cross is more accurately referred to as "unilateral incongruity" (UI) rather than interspecific incompatibility. Periclinal chimeras were used to determine the tissues involved in UI. The results of crosses with the available chimeras indicate that the female parent must be *L. pennellii* at either LI (layer 1) or both LI and LII (layer 2) and the male parent must be *L. esculentum* at either LII or both LI and LII to observe UI similar to that seen in the *L. pennellii*×*L. esculentum* cross. Pollinations with a mixture of pollen from *L. pennellii* and from transgenic *L. esculentum* plants harboring a pollen-specific GUS reporter gene marker were used to ascertain whether the growth of the pollen tubes of either species was modified as a possible means of overcoming UI. We found no evidence of communication between the two types of pollen tubes to either enhance or restrict all pollen tube growth.

Key words Tomato · Interspecific hybridization · Incongruity · Self incompatibility · Reproductive barriers

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Introduction

Beginning with the initiation of pollen germination, several mechanisms limit successful fertilization within and between species (de Nettancourt 1977; Ascher 1986; Knox et al. 1986; Liedl and Anderson 1993). Intraspecific mechanisms encourage outcrossing within species, while interspecific mechanisms affect speciation and limit the exchange of genes between species. The germplasm of many crops is narrow, due to constraints imposed during their domestication and spread, thus increasing the importance of wild relatives as a rich source of genetic variation. However, transfer of desirable traits to the cultivated species is impeded by interspecific barriers to crossing. This is particularly apparent when attempting to transfer multigenic traits such as yield, quality factors and resistance to insects or diseases from wild to domestic species (Hogenboom 1972; Stalker 1980; Pattee et al. 1991; Mutschler et al. 1993; Sharma 1995). It is therefore imperative to understand the nature and genetic control of the interspecific barriers.

The intraspecific barrier, self-incompatibility (SI), has been the focus of much of the reproductive barrier research in the past 10 years (reviewed in Newbigin et al. 1993). Nearly half of the major crops and ornamental species of the world occur in genera representative of the 71 families known to possess SI (de Nettancourt 1977). SI is a genetically based physiological mechanism promoting outcrossing within a species. As a result of SI, pollen germination or pollen tube growth is slowed or inhibited such that fertilization does not occur when there

is a recognition of like specificities between pollen and pistil (Ascher 1976). Classically, a single locus, *S*, with multiple alleles encodes these specificities. In the gametophytic SI system of the Solanaceae, pollen specificity is generated by the *S* allele of the pollen genome. The system is characterized by slowed growth of incompatible pollen tubes (East and Mangelsdorf 1925).

Research on interspecific barriers is less advanced, despite the existence of these barriers in many genera (de Nettancourt 1977; Liedl and Anderson 1993; Grant 1994; Mutschler and Liedl 1994; Preuss 1994). Intraspecific barriers, such as SI, may also be present in one or both of the interspecific parents, potentially confounding the study of interspecific barriers. Therefore, care must be taken in the design of experiments and interpretation of the results to allow separation of the effects of inter- and intraspecific barriers.

Crossing relationships within the *Lycopersicon* genus demonstrate the existence of several mechanisms to prevent interspecific hybridization (Hardon 1967; Hogenboom 1973, 1979; Rick 1979) and the presence of SI in most of the species (Lamm 1950; Taylor 1986). A strikingly regular pattern of crossability is found in the *Lycopersicon* species (summarized in Mutschler and Liedl 1994). *L. esculentum* acts as the universal female within the genus, accepting pollen from all the other *Lycopersicon* species, regardless of SI or self-compatibility (SC). A universal male in this genus cannot be identified; however, crosses between species are congruous only when the species more closely related to *L. esculentum* is used as the female and the more distantly related species is used as the male parent (i.e. *L. hirsutum*×*L. pennellii* is congruous and *L. pennellii*×*L. hirsutum* is incongruous). Thus, as proposed by Hogenboom (1984), a species barrier may exist which controls this regular pattern of crossing in the genus. However, information on the nature and genetic control of the interspecific barriers and possible interactions with intraspecific barriers such as SI is limited.

The purpose of this study was to determine the timing, location, expression and tissues responsible for the interspecific barrier between *L. esculentum* and *L. pennellii* and whether or not this barrier is related to the intraspecific barrier, SI.

Materials and methods

Plant material and growing conditions

The plant materials used included *L. esculentum* cv. New Yorker (Le) which, like all accessions of *L. esculentum*, is self-compatible. Two self-compatible *L. pennellii* accessions, LA716 and LA2963, and three self-incompatible *L. pennellii* accessions, LA1340, LA1376 and LA2560, were obtained from Dr. C.M. Rick, University of California, Davis. Plants were grown in a greenhouse in soilless medium (Boodley and Sheldrake 1982). The medium for the *L. pennellii* accessions was mixed with an equal volume of sterilized sand to increase drainage. The media for all plants were supplemented with Osmocote and fertilized weekly with Peters 9–14–15. Natural day length conditions were supplemented to 12 h (0600–1800 h) by high-intensity discharge lights. Standard fungicide and insecticide practices were followed.

A series of periclinal chimeras were also used. Classification of periclinal chimeras is based on the division of the apical zone into one or more tunicas and a corpus (Tilney-Bassett 1986). The duplex apex, common to most angiosperms (Gifford and Corson 1971), is composed of two tunicas, the outer tunica (LI) and the inner tunica (LII), and a corpus (LIII) (Satina and Blakeslee 1941). With few exceptions, these three layers in dicots are generally responsible for generating the following tissues (Marcotrigiano and Bernatzky 1995). The LI gives rise to the epidermis of the plant and can be responsible for the stigmatic surface of the pistil, integuments of the ovary and the transmitting tract of the style. The LII generates the subepidermal tissue, including most of the leaf blade tissue, and the micro- and macrospore mother cells, which produce the gametes. The LIII generates the central tissue which gives rise to much of the vascular tissue (Satina and Blakeslee 1941; Tilney-Bassett 1986; Marcotrigiano and Bernatzky 1995). The names used to identify the different chimeras are derived by using a code for each species or hybrid at the appropriate position of the layer (Kirk and Tilney-Bassett 1978).

The four interspecific periclinal chimeras (PPE, PEE, F_1F_1E and F_1EE) available were derived from a stock of *L. esculentum* carrying the visible leaf color marker *Xa*, and the SC accession *L. pennellii* LA716 or the interspecific F_1 created from these two parents (Szymkowiak and Sussex 1992). PPE has LI and II of *L. pennellii* LA716 over LIII of *L. esculentum* and PEE has LI of *L. pennellii* LA716 over LII and III of *L. esculentum*. The chimeras F_1EE and F_1F_1E, have LI of the interspecific F_1 over the *L. esculentum* LII and III and the LI and II of the same F_1 over the *L. esculentum* LIII, respectively.

L. esculentum lines homozygous for the LAT59-GUS chimeric gene construct (*L. esculentum*^{T} or Le^T) were previously described by Twell et al. (1990). LAT59 directs strong GUS expression in post-meiotic microspores, mature pollen and pollen germinating in vitro or in vivo (Twell et al. 1990).

In vivo pollen tube analysis

Pollen tube growth in vivo was assayed using a method based upon fluorescence of callose in pollen tubes after staining with aniline blue (Linskens and Esser 1957; Kho and Baer 1968) or GUS staining and clearing of the style using the procedure described in Liedl et al. (1993). Manual self pollinations were made on buds emasculated when the anthers were turning yellow, but prior to dehiscence of the pollen. Pollinations were made at 70°C in growth chambers having the same light regime as the greenhouse. For the GUS studies, *L. esculentum*^{T} pollen was mixed with pollen from *L. pennellii* LA2963 (Lp) to an approximate ratio of 1:1. This pollen mixture was then applied to the stigma. Pollen in this and all other manual pollinations was applied with forceps rinsed in 95% ethanol between pollen sources. To observe pollen tube growth with aniline blue fluorescence, pollinated flowers were harvested at different times post pollination and fixed in FPA (5% formalin, 5% propionic acid and 50% ethanol). The petals and anthers were removed from the pistils, which were subsequently softened overnight in 8 N NaOH. After being washed three times with double-distilled H_20, the pistils were stained with 0.1% aniline blue in 0.1 M K_3PO_4 for 4 h in the dark. The aniline blue-stained samples were then placed in a drop of glycerin on a microscope slide, covered with a cover slip, squashed and examined by fluorescent light microscopy (Zeiss standard microscope no. 2 filter). Samples stained for GUS activity were analyzed using a dissecting scope.

The number of styles analyzed for each time point depended on the number of flowers available to pollinate and whether the flowers abscised from the plant. On average, five flowers were analyzed for each time point, with a range from 2 to 20. Due to variation in the length of the styles within and between the species used, pollen tube growth is presented as the distance pollen tubes travelled divided by the length of the style. This gives a value of 0.0 for a style without pollen tubes and 1.0 for pollen tubes tra-

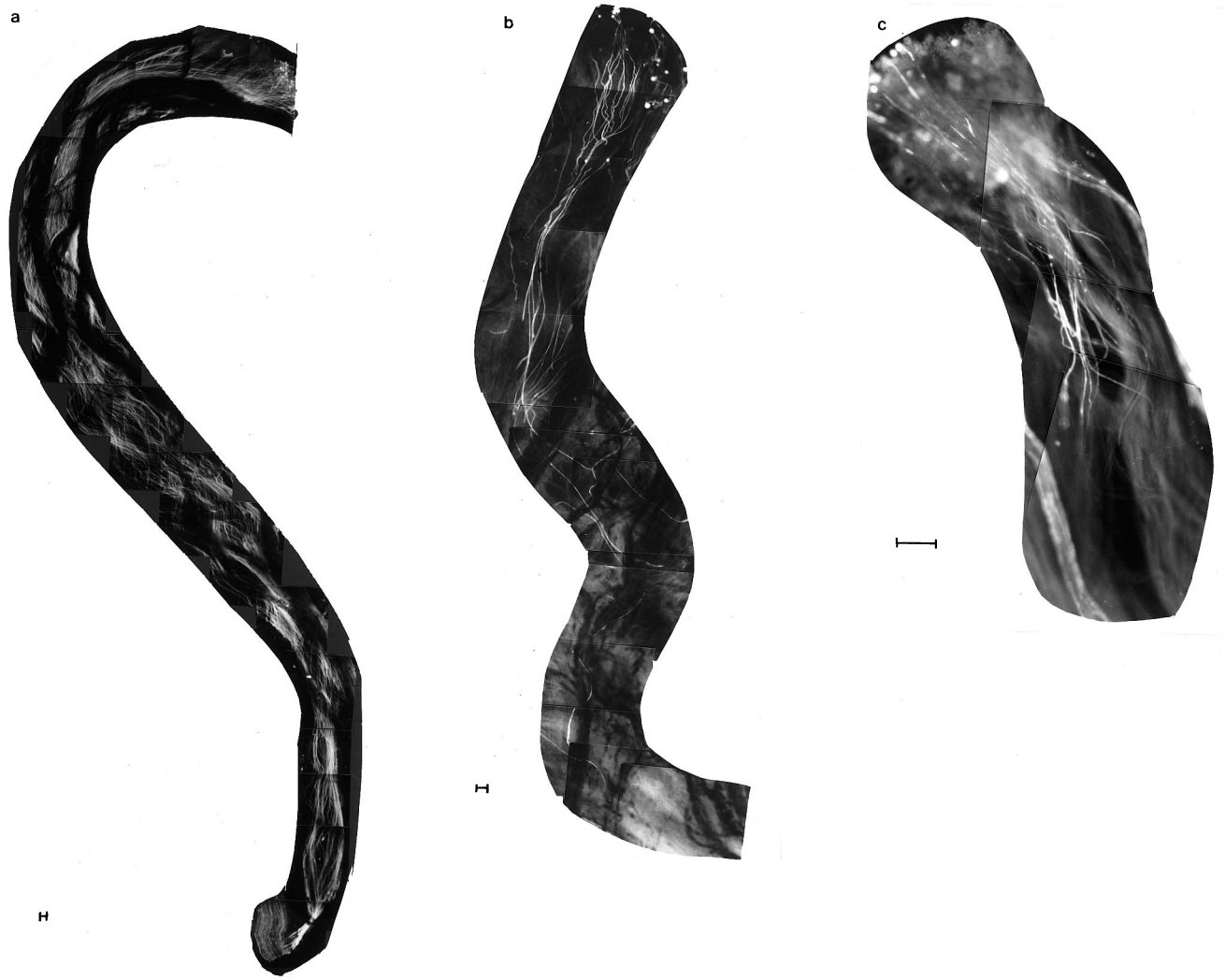

Fig. 1a–e Pollen tube growth at the stigmatic surface, in the style and in the ovary for **a** *L. esculentum* selfed (similar response seen for self pollinations of the SC accessions *L. pennellii* LA716 and LA2963 and the interspecific F_1 hybrid (*L. esculentum*×*L. pennellii* LA716)), **b** the SI accession *L. pennellii* LA1376 selfed (similar response seen for self pollinations of all SI *L. pennellii*) and **c** SC *L. pennellii* LA2963×*L. esculentum* (similar response seen for either SI or SC *L. pennellii*×*L. esculentum*). *Bars each* 100 μm

versing the entire length of the style. Standard deviations for average pollen tube growth were based on a minimum of four pollinations.

Results and discussion

Pollen tube growth in self and cross pollinations of *L. esculentum* and *L. pennellii*

Pollen tube growth was examined as a first step in testing whether or not the expression of SI and the interspecific barrier were similar. Pollen tubes traverse the length of the style by 24 h post pollination (hpp) in self pollinations of *L. esculentum* and the SC accessions of *L. pennellii* (Fig. 1a). The tubes elongated straightforwardly to the ovary and callose plugs were uniformly deposited at regular intervals.

L. esculentum and *L. pennellii* LA2963 have very similar pollen tube growth curves (Fig. 2). These sigmoid curves suggest a biphasic growth with a transition around 8 hpp, which was also seen by other researchers (Cresti et al. 1980; Mulcahy and Mulcahy 1983, 1988). Self pollinations of *L. pennellii* LA716 also show a sigmoid pollen tube growth curve. Selfed styles of LA716 exhibit more variability in the number of pollen tubes growing in and down the style than pollen tubes in self pollinations of LA2963, resulting in a lower average pollen tube growth rate with a higher standard deviation (data not presented). The transition between the two growth phases for LA716 is also later (14 hpp). We believe the poorer performance of LA716 is due in part to the unusually fragile junction between the style and ovary in this accession, generally poorer quality pollen (reduced percent pollen stainability and in vitro germination) and the need for manual self pollination to obtain seed set. Pollen tube growth curves in reciprocal crosses between the two SC *L. pennellii* accessions are similar to that in self pollinations of LA716, but exhibit less variability in pollen tube length at each time point (Fig. 3).

The growth curve of the interspecific F_1 was intermediate between those of the two parents, *L. esculentum* and LA716, but exhibited the familiar sigmoid shape.

Pollen tubes traverse only half of the length of the style (approximately 3.5–4.5 mm) by 24 hpp in self pollinations of each of the SI accessions of *L. pennellii* (Fig. 1b). Later, the pollen tubes slowly traverse the remainder of the style and enter the ovary by 48 hpp (Table 1). Thus, pollen tubes were in the ovary of compatible pollinations (such as self pollinations of *L. esculentum* or SC *L. pennellii* accessions) 24 h prior to self pollinations of SI *L. pennellii* accessions. Pollen tube growth rate also appears to vary between the three SI *L. pennellii* accessions used, since at 24 hpp the distance the pollen tubes grew ranged from approximately 1/4 to 1/2 the length of the style. Thus, the expression of SI occurred as pollen tubes grew down the style and resulted in a reduction in the pollen tube growth rate in self pollinations of SI relative to the pollen tube growth rate in self pollinations of SC *L. pennellii* accessions or *L. esculentum*. This reduction in growth rate is in agreement with many reports of SI in the Solanaceae (Yasuda 1934; Straub 1946; McGuire and Rick 1954; Schlösser 1961; Hardon 1967; Ascher 1976; Herrero and Dickinson 1980, 1981). However, other researchers have reported that SI causes pollen tubes to stop and/or burst in the Solanaceae (de Nettancourt et al. 1973; Williams and Knox 1982; Rivers and Bernatzky 1994). This apparent contradiction might result from artifacts caused by histological fixation, the limited number of times samples were taken post pollination, or environmental differences affecting pollen tube development (Ascher 1984; Webb and Williams 1988). McGuire and Rick (1954) found swollen pollen tubes in all their samples and, thus, found no correlation between swollen pollen tubes and type of compatibility. Therefore, it is important to sample at least twice post pollination (at 24 and 48 h) to differentiate pollen tubes which are exhibiting an incompatible reaction from those which are slow growing for a reason unrelated to SI, yet congruous.

All SI and SC accessions of *L. pennellii* produced similar results in reciprocal crosses with *L. esculentum* (Fig. 1c, Table 1). The results of the interspecific cross *L. esculentum*×*L. pennellii* are similar to the self pollination of *L. esculentum* or of SC *L. pennellii* in that the pollen tubes traveled the length of the style by 24 hpp and successfully set seed. In contrast, the pollen tubes in the reciprocal cross (*L. pennellii*×*L. esculentum*) penetrated the stigmatic surface, entered the transmitting tissue of the style, and stopped growth approximately 2–3 mm into the style by 24 hpp (Figs. 1c, 4). No additional pollen tube elongation was observed in these crosses at later times, explaining the lack of seed set in this type of

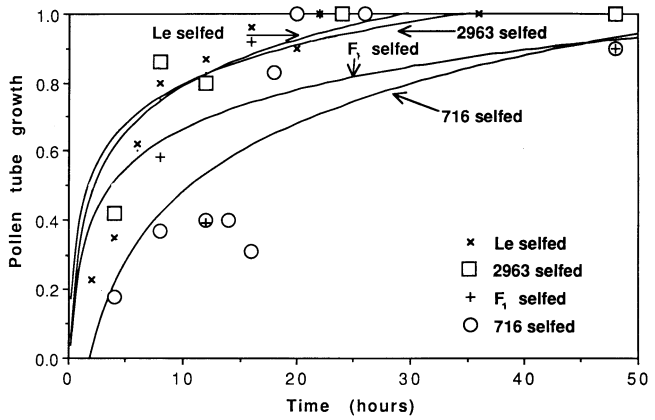

Fig. 2 Pollen tube growth over time in self pollinations of *L. esculentum* cv. New Yorker (*Le*), SC *L. pennellii* LA716 (*716*), SC *L. pennellii* LA2963 (*2963*) and the interspecific F_1 hybrid (*L. esculentum*×*L. pennellii*)

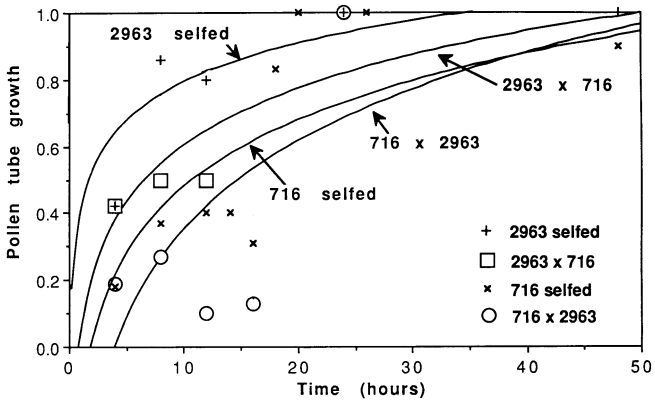

Fig. 3 Pollen tube growth over time in intraspecific pollinations between SC *L. pennellii* LA716 (*716*) and SC *L. pennellii* LA2963 (*2963*)

Table 1 Pollen tube growth at 24 and 48 h post pollination (*hpp*) in pollinations with *L. pennellii* (*Lp*) self-incompatible accessions (LA1340, LA1376 and LA2560), self-compatible accessions (LA716 and LA2963) and *L. esculentum* (*Le*). Pollen tube growth is expressed as the distance pollen tubes travelled divided by the length of the style. Values in parentheses are standard deviations for average pollen tube growth (based on a minimum of four pollinations)

Cross	SI *L. pennelli* accessions						SC *L. pennellii* accessions			
	LA1340		LA1376		LA2560		LA716		LA2963	
	24 hpp	48 hpp	24 hpp	48 hpp	24 hpp	48 hpp	24 hpp	48 hpp	24 hpp	48 hpp
Lp selfed	0.23 (0.21)	1.00 (0.00)	0.50 (0.00)	1.00 (0.00)	0.57 (0.17)	1.00 (0.00)	0.83 (0.25)	0.88 (0.19)	1.00 (0.00)	1.00 (0.00)
Le x Lp	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	0.80 (0.34)	0.90 (0.29)	1.00 (0.00)	1.00 (0.00)
Lp x Le	0.11 (0.00)	0.00	0.12 (0.03)	0.14 (0.00)	0.23 (0.16)	0.00	0.25 (0.13)	0.31 (0.13)	0.23 (0.14)	0.25 (0.00)

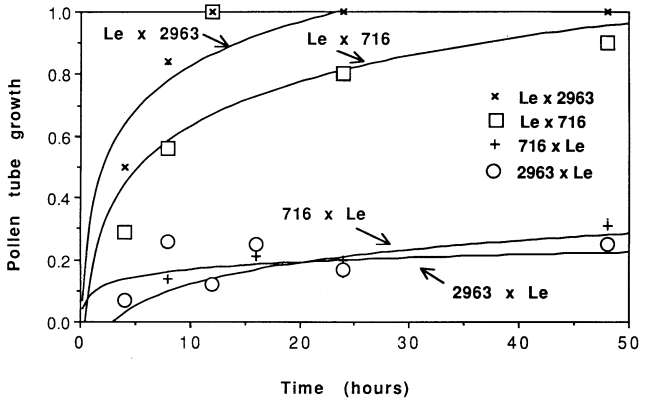

Fig. 4 Pollen tube growth over time in pollinations among *L. esculentum* cv. New Yorker (*Le*), SC *L. pennellii* LA716 (716) and SC *L. pennellii* LA2963 (*2963*)

cross. The termination of pollen tube growth in *L. pennellii*×*L. esculentum* crosses is quite distinctive and visually distinguishable from the reduction in pollen tube growth seen in self pollinations of SI *L. pennellii*.

In summary, pollen tube growth in successful interspecific and compatible intraspecific crosses is indistinguishable and fits prior descriptions of normal pollen tube growth (Cresti et al. 1980; Mulcahy and Mulcahy 1983, 1988). Thus, no a priori reason exists to prevent *L. esculentum* pollen from growing the length of the *L. pennellii* style. The unilateral interspecific barrier appears to function on a species-specific basis. The characteristic effects of UI on pollen tube growth also occurs regardless of whether a SC or SI accession of *L. pennellii* is used.

Pollen tube growth in backcross pollinations of the interspecific F_1 and the two species

The interspecific F_1 between *L. esculentum* and either of the SC accessions of *L. pennellii* used is also SC. Pollen tube growth in self pollinations of these interspecific F_1 and in the backcrosses *L. esculentum*×F_1 and F_1×SC *L. pennellii* is similar to pollen tube growth of the parental species and both set seed readily (data not presented). Pollen tube growth in the reciprocal backcrosses, F_1×*L. esculentum* and *L. pennellii*×F_1, is slower and more variable. We found that the pollen tubes were half way down the style at 16 hpp and reached the ovary by 24 hpp. Hardon (1967) investigated pollen tube growth in the same type of cross, but used a different *L. pennellii* accession; he found that most of the pollen tubes were inhibited after growing a shorter distance than in an incompatible pollination. Overall, pollen tube growth in crosses with the F_1 is more variable. The two backcrosses, F_1×*L. esculentum* and *L. pennellii*×F_1, achieve seed set rarely (Mutschler and Liedl 1994) or very rarely (Mutschler and Cobb 1985). This raises the question of whether the cause of failure of either or both of the backcrosses is due to a weaker form of UI or to a different interspecific barrier.

Relationship between the unilateral interspecific crossing barrier and self incompatibility

Several researchers have suggested that unilateral crossing barriers and SI are related phenomena (Lewis and Crowe 1958; Abdalla and Hermsen 1972; de Nettancourt 1977; Chetelat and DeVerna 1991). However, the unilateral crossing barrier is still observed when SC accessions of an SI species are used in interspecific crosses (Martin 1964; Rick 1969) and conditions that overcome SI do not generally overcome the unilateral response (Van Tuyl et al. 1982; Ascher 1986). Chetelat and DeVerna (1991) suggested that *L. pennellii* alleles at one or more loci in regions on chromosomes 1, 6 and 10 control what is assumed to be the expression of an unspecified unilateral interspecific barrier. The region on chromosome 1 includes the *S* locus, which encodes the specificities for the self-incompatible reaction in *Lycopersicon* (Tanksley and Loaiza-Figueroa 1985). However, we believe the data of Chetelat and DeVerna (1991) are insufficient to support a relationship between their barrier and SI due to the single time point used to observe pollen tube growth, the existence of aberrant segregation of markers in the genomic regions analyzed and the complex tri-species population used. An alternative explanation for their observations can be given based on the functioning of SI and their interspecific barrier in their populations, which are controlled by separate and unlinked loci (Mutschler and Liedl 1994).

Kuboyama et al. (1994) also found temporal and morphological differences in pollen tube growth in unilateral crosses within *Nicotiana*. In addition, they also suggest that the incongruity they observed must be controlled by mechanisms other than SI, since the S-glycoprotein identified as important in the SI response for SI *N. alata* is not found in styles of SC *N. tabacum.*

Thus, the data available to date do not support a model involving the *S* locus in the operation of the unilateral response seen in the cross *L. pennellii*×*L. esculentum*. This does not imply that SI has no effect in populations derived from interspecific crosses; however, it indicates that there may be other barriers. If SI and the unilateral response are indeed separate barriers, then incongruous crosses involving an SI species or its progeny as the male and *L. esculentum* as the female are essentially doubly blocked, and any attempt to make this cross would have to overcome both barriers to succeed.

Failure of the cross *L. pennellii*×*L. esculentum* is due to unilateral incongruity

Several authors have reported interspecific crosses in which only one direction resulted in successful seed production (Kostoff 1930; Stout 1952; McGuire and Rick 1954; Martin 1961; Hogenboom 1984; Kuboyama et al. 1994), similar to what we observed in the cross *L. pennellii*×*L. esculentum*. Several names have been suggested for this type of general interspecific barrier: unilateral in-

congruity (Hogenboom 1984), unilateral stylar incompatibility (de Nettancourt et al. 1973, 1974), unilateral incompatibility (Lewis and Crowe 1958) and interspecific incompatibility (de Nettancourt 1977). "Incongruity" as defined by Hogenboom (1973, 1975) encompasses passive reproductive barriers which evolve due to isolation of taxa and better describes the nonfunctioning of interspecific relationships than does the term "incompatibility". For this reason, and because the information available to date does not indicate whether SI actually plays a role in limiting gene exchange in this interspecific cross, we propose that the unilateral interspecific barrier between *L. pennellii* and *L. esculentum* be called "unilateral incongruity" (UI).

Tissue and genome specificity of unilateral incongruity

Crosses were made with two of the interspecific periclinal chimeras PPE, and F_1F_1E (Szymkowiak and Sussex 1992) and the lines used to create them in order to determine the tissues involved in UI in the cross *L. pennellii*×*L. esculentum.* PPE and F_1F_1E both self to set viable seed. Thus, since seed set was possible using both the male and female gametes of these chimeras, failure of any crosses using these chimeras should be due to the operation of a reproductive barrier, rather than to a simple lack of functional pistils, ovaries or pollen from the chimeric parent.

Pollen tube growth and the ability to set seed in crosses involving PPE and F_1F_1E chimeras were examined to determine whether LIII created or released any interspecific barriers to fertilization and seed set. PPE successfully crosses in either direction with *L. pennellii*. The progeny resulting from these crosses and the self progeny of PPE are indistinguishable from *L. pennellii* plants. PPE is also similar to *L. pennellii* in that PPE will not set seed if pollinated with *L. esculentum*. The progeny derived from self seeds of the F_1F_1E chimera were similar to an F_2 population and pollen from *L. esculentum, L. pennellii* and the F_1 grew into the ovary within 24 hpp. Both chimeras, PPE and F_1F_1E, function similarly to *L. pennellii* and the interspecific F_1, respectively, and thus there is no indication that LIII is involved in UI. As a result, further studies focused on the use of the chimeras differing in the first two layers (PEE and F_1EE) to study the action of LI and/or LII in UI.

We formulated several models concerning the tissues and genomes involved in the early arrest of pollen tube growth seen in *L. pennellii*×*L. esculentum* crosses. The models are based upon observations of male and female interactions in self and cross pollinations among *L. esculentum,* SC *L. pennellii* and their interspecific F_1 as detailed in the prior section. The models hypothesized the involvement of either individual layers, or combinations of two or more layers in control of pollen tube growth or seed set. In some instances, the model cannot be limited to the prediction of a successful or unsuccessful pollination, and an alternative based on a proportion of the tissues involved may be possible. We included the chimeras with the F_1 to compare the two types of pollen tube growth found in the crosses *L. pennelli*×*L. esculentum, L. pennellii*×F_1 and F_1×*L. esculentum*. Pollen tube with that growth and seed set of crosses using PEE and F_1EE as male and female parents were compared to the predictions of the models (Tables 1, 2).

Crosses using the PEE and F_1EE chimeras as the male parent indicated that LII is involved in control of the male component of UI, but the data were insufficient to determine whether LII is solely responsible or interacts with LI. The pollen tube growth and seed set in the cross *L. pennellii*×PEE shows early arrest of pollen tube growth and subsequent failure to set seed, similar to the *L. pennellii*×*L. esculentum* cross (Tables 2 and 3). The cross *L. esculentum*×PEE shows that PEE pollen is capable of normal growth to the ovules. If LI were the only layer of the male involved in UI, then the cross *L. pennellii*×PEE should have normal pollen tube growth, since the cross would be equivalent to *L. pennellii*×*L. pennellii*. However, this cross shows the abnormal pollen tube growth pattern of the *L. pennellii*×*L. esculentum* cross, indicating that possession of only LI of *L. pennellii* in the male does not cause or release the barrier that results in early arrest of the growth of pollen tubes and failure to set seed.

Other crosses using the chimeras as males and the F_1 or *L. pennellii* as female result in variable pollen tube growth, reminiscent of the variable pollen tube growth in backcrosses of the interspecific F_1 with the species. Crosses between the *L. pennellii*×F_1EE and the interspecific F_1 with both of the chimeras resulted in a variable response with some pollen tubes growing to the end of the style and others only half way down the style by 24 hpp. The results of crosses between the chimeras eliminate many of the models of possible tissue interactions as causes of the early arrest of pollen tube growth similar to that in the cross *L. pennellii*×*L. esculentum,* but we could not distinguish among the four remaining models with the crossing data available (Tables 2, 3). The one exception to the predictions is the variable pollen tube growth seen in the *L. pennellii*×F_1EE cross. We are unable to determine whether the variable pollen tube growth is the result of UI observed between the parent species or of another barrier, since the possibility exists for cell-cell interactions between adjoining layers. Therefore, a final determination of the correct model is not possible without crossing data involving a more complete set of chimeras.

Crosses using PEE and F_1EE as the female indicate that the female must have the *L. pennellii* genotype at either LI or both LI and LII to show the early arrest of pollen tube growth. Crosses of PEE and F_1EE with either parental species produce normal pollen tube growth with the exception of the cross PEE×*L. esculentum* (Table 2). Pollen tube growth in the cross PEE×*L. esculentum* is normal in appearance but more variable, with pollen tubes ranging from half way down the style to all the way down the style in 24 h. This cross fails to set seed, even though some of the pollen tubes reach the ovary (Tables 2, 3). Non-functional ovules are not responsible for the failure to set seeds in the cross PEE×*L. esculentum,* since seeds are obtained from

Table 2 Summary of hypothetical and observed pollen tube growth in crosses with periclinal chimeras. *LI, LII* Layers of periclinal chimers (see Materials and methods for description), *both LI and LII* both layers must support pollen tube growth of other parent to extent predicted, *either LI or LII* either layer can support pollen tube growth of other parent to extent predicted. Le *L. esculentum*, Lp *L. pennellii*, F_1 interspecific F_1 (*L. esculentum*×*L. pennellii*), *PEE*, F_1*EE* periclinal chimeras (see Materials and methods for description). *S* Pollen tube growth stopped in style, *O* pollen tube growth to ovary in 24 h post pollination, *V* variable pollen tube growth (roughly equivalent of F_1×*L. esculentum* cross), *S/O* either outcome (S vs O) or an intermediate may be possible, *V/O* either outcome (V vs O) or an intermediate may be possible

Cross	Layers of the chimeral parent hypothesized to be involved in pollen tube growth and predicted responses				Results Observed
	Male LI	Male both LI & II	Male either LI or II	Male LII	
Le × PEE	O	O	O	O	O
Lp × PEE	O	S/O	O	S	S
Le × F_1EE	O	O	O	O	O
Lp × F_1EE	O	S/O	O	S	V
F_1 × PEE	O	V/O	O	V	V
F_1 × F_1EE	O	V/O	O	V	V
	Female LI	Female both LI & II	Female either LI or II	Female LII	
PEE × Le	S	S/O	O	O	V
PEE × Lp	O	O	O	O	O
F_1EE × Le	V	V/O	O	O	O
F_1EE × Lp	O	O	O	O	O
PEE × F_1	O	O	O	O	O
F_1EE × F_1	O	O	O	O	O
	Female LI Male both LI & II	Female LI Male LII	Female both LI & II Male both LI & II	Female both LI & II Male LII	
PEE × PEE	S/O	S	S/O	S/O	S
PEE × F_1EE	S/O	S	S/O	S/O	S
F_1EE × PEE	V/O	V	V/O	V/O	V
F_1EE × F_1EE	V/O	V	V/O	V/O	O

Table 3 Summary of hypothetical and observed seed set in crosses with periclinal chimeras. *LI, LII* Layers of periclinal chimers (see Materials and methods for description), *both LI and LII* both layers must support successful seed set, *either LI or LII* either layer can support successful seed set. *Le L. esculentum*, *Lp L. pennellii*, F_1 interspecific F_1 (*L. esculentum*×*L. pennellii*), *PEE*, F_1*EE* periclinal chimeras (see Materials and methods for description). *Yes* seed set, *No* seed did not set, *n.a.* data not available

Cross	Layers of the chimeral parent hypothesized to be involved in seed set and predicted responses				Results Observed
	Male LI	Male both LI & II	Male either LI or II	Male LII	
Le × PEE	Yes	Yes	Yes	Yes	n.a.
Lp × PEE	Yes	No	Yes	No	No
	Female LI	Female both LI & II	Female either LI or II	Female LII	
PEE × Le	No	No	Yes	Yes	No
PEE × Lp	Yes	Yes	Yes	Yes	Yes
	Female LI Male both LI & II	Female LI Male LII	Female both LI & II Male both LI & II	Female both LI & II Male LII	
PEE × PEE	No	No	No	No	No
PEE × F_1EE	No	No	No	No	No
F_1EE × F_1EE	No	No	No	No	No

PEE×*L. pennellii*. The pollen tube reaction in the cross PEE×*L. esculentum* does not fully fit either model. However, it resembles the cross between F_1×*L. esculentum*, in which pollen tube growth is slowed, but eventually reaches the ovary and usually fails to set seed. If the *L. pennellii*×*L. esculentum* barrier is dominant, then we would expect to find the backcrosses to be dominant. Since this is not the case, the barrier in the backcrosses (i.e. *L. pennellii*×F_1 and F_1×*L. esculentum*) may be either a different barrier or the same barrier, but not fully dominant in its expression. This

Table 4 Results of crossing within and between *L. esculentum* with a GUS construct driven by a pollen specific promoter and *L. pennellii* (LA2963). Le *L. esculentum*, *Le*^{T} *L. esculentum*, carrying GUS construct (see Materials and methods for description), *Lp L. pennellii*, *mix* mixture of *L. esculentum*^{T} and *L. pennellii* pollen (see Materials and methods for description).

Cross			Seed set	Pollen tubes reach ovary	GUS expression in pollen tubes	Location of GUS along length of pollen tube
Le	×	Le^T	Yes	Yes	Yes	Entire length
Le	×	Lp	Yes	Yes	No	–
Le	×	mix	Yes	Yes	Yes	Entire length
Lp	×	Le^T	No	No	Yes	Top 1/4
Lp	×	Lp	Yes	Yes	No	–
Lp	×	mix	Yes	Yes	Yes	Top 1/4

suggests that there is a weakening of the barrier in the F_1, or an interaction between LI and LII which results in a weakened response of the barrier between the species, or that another reproductive barrier(s) exists.

The results of crosses between the chimeras eliminate many of the possible models of tissue interaction that cause the early arrest of pollen tube growth similar to that in the cross *L. pennellii*×*L. esculentum* (Tables 2, 3). The male component responsible for the arrest of pollen tube growth is either LII alone or both LI and II. The alternative models with only LI or either LI or II controlling the male component would predict that all crosses would be successful, which was not observed. The female component responsible for the arrest of pollen tube growth is either LI only or both LI and II. The alternative models with only LII or either LI or II controlling the female component would predict that all crosses would be successful, which was not observed. The four combined models possible using the two models for each component (male component, LII only or both LI and II; female component, LI only or both LI and II) were tested in crosses between the two chimeras (Tables 2, 3). Observed pollen tube growth in crosses between the two chimeras meets the possible expectations in all but one model (male component, LII only and female component, LI only). This exception is observed only in the cross between F_1EE×F_1EE, which results in pollen tubes in the ovary rather than the variable pollen tube growth predicted. Since this cross involves the interspecific hybrid, which doesn't respond in a manner identical to that of the cross between the species, we cannot eliminate either of the following alternative possibilities: (1) expression of UI similar to variable pollen tube growth observed in crosses of the interspecific hybrid with the two species or (2) the function of another interspecific barrier. Thus, it is difficult to eliminate any of the four remaining models. In the case of seed set, the expectations for all four models are identical. Therefore, final identification of the correct model is not possible without a more complete set of chimeras.

Investigating communication between male gametophytes during mixed pollinations

Mentor pollen or mixed pollinations have been suggested as methods to study communication between the pollen tubes and the female sporophyte or gametophyte, or to overcome crossing barriers (Stettler and Ager 1986). The limitations of such experiments were the inability to distinguish among the pollen tubes as they grow down the style and the necessity in many studies of using chemical or irradiation methods to kill pollen from one of the sources prior to mixing with the second pollen source. Pollen from transformed plants carrying a pollen-specific GUS construct provides an innocuous method to identify one of the pollen sources used in a mixed pollination and, thereby, determine if signaling exists between the pollen tubes in a style.

Control pollinations were made to compare the growth of GUS-expressing *L. esculentum* pollen tubes with normal *L. esculentum* pollen tube growth. Pollen tubes were in the ovary within 24 h in the crosses *L. esculentum*×*L. esculentum*^{T} and *L. esculentum* selfed. Growth of the pollen from *L. esculentum*^{T} was limited in the cross *L. pennellii*×*L. esculentum*^{T} (Table 4) in the same manner as described previously for the cross *L. pennellii*×*L. esculentum*. In both cases, pollen tube growth is inhibited and reaches only 2–3 mm down the *L. pennellii* styles. Therefore, possession of the GUS construct does not affect the expression of the unilateral interspecific barrier in crosses involving *L. esculentum*^{T} pollen.

Mixtures of pollen from *L. esculentum* (with or without the GUS construct) and *L. pennellii* were used to pollinate *L. esculentum* or *L. pennellii* pistils. At least some of the pollen resulting from the mixed pollination of both *L. esculentum* and *L. pennellii* pistils was normal, with pollen tubes reaching the ovary as expected (Table 4). In mixed pollinations of *L. esculentum* pistils, the GUS-expressing *L. esculentum*^{T} pollen tubes reached the ovary. However, in mixed pollinations of *L. pennellii* pistils the *L. esculentum*^{T} pollen tubes (GUS-expressing) were all localized to the top 1/4–1/3 of the style, and pollen tubes not expressing GUS (i.e. *L. pennellii*) were found in the ovary (Table 4). From this we conclude that the unilateral barrier operates on pollen tubes individually (i.e. the barrier restricts only the *L. esculentum* pollen in an *L. pennellii* style, but not *L. pennellii* pollen), rather than promoting or inhibiting all pollen tubes in the presence of some arrested ones. These data also demonstrate the absence of pollen mentoring. If mentoring existed between the two types of pollen, the GUS-expressing pol-

len tubes (*L. esculentum*^{T}) in a mixture of *L. pennellii* and *L. esculentum*^{T} pollen should grow further down the *L. pennellii* style than was found in the control cross of *L. pennellii*×*L. esculentum*^{T}. However, there is no evidence of signaling between the two pollen sources to either enhance or restrict pollen tube growth, since the GUS-expressing *L. esculentum*^{T} pollen tubes were only in the first 2–3 mm of the style in *L. pennellii*×(*L. pennellii*+*L. esculentum*^{T}) crosses.

Future work on unilateral incongruity

Our objective in studying the mechanisms underlying UI is to provide information that can be used to reduce or overcome the barrier(s). Several questions remain regarding the mechanisms involved in the UI reaction. Pollen tube growth and seed set data obtained using a partial set of periclinal chimeras showed that any of four models could explain the tissue interactions of UI. Discrimination among these four models will require additional chimeras (i.e. EPP). We demonstrated that the GUS reporter gene can be used to identify pollen tubes in vivo and that pollen tube growth of such pollen is not altered; however, we were unable to obtain evidence of either an enhancement or restriction of pollen tube growth after mixed pollinations with *L. pennellii* and *L. esculentum*^{T} pollen. While mixed pollinations do not overcome UI, this pollen-specific GUS reporter gene system may prove useful for further studies of pollen-pistil interactions in UI. One hint of a method with which to overcome UI comes from the observation of variable pollen tube growth in crosses between the parental species and the interspecific F_1. In fact, preliminary studies indicate that individual F_2 plants show variation in the extent of pollen tube growth in incongruous crosses (Liedl and Mutschler, unpublished results). Our future studies will focus on genetically mapping factors that contribute to this variable pollen tube growth and in determining whether the barrier is developmentally regulated during pollen and/or pistil development.

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