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ORIGINAL PAPER

Phenotypic and genetic characterization of the *pistillate* mutation in tomato

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Abstract Many floral phenotypes have been described in decades of tomato genetics, but for very few of them the underlying genes have been identified so far. Because the increasing availability of genome sequence data will facilitate forward genetics in tomato, novel descriptive and map information will help the attribution of genes to phenotypes. In this contribution, we present our work on *pistillate* (*pi*), a genotype that directly recalls mutations affecting class B MADS-box genes, but that has not been further characterized after the first description. Plants homozygous for the *pi* allele appear with Mendelian proportions and, compared to wild-type, show delayed flowering, a frequently modified sympodial segment, higher occurrence of compound inflorescences, and reversion of the floral meristem to vegetative identity. In *pi* mutant flowers, the most striking aberration is the homeotic transformation of stamens into carpels. Ultrastructural analysis also reveals more or less subtle sepaloid features in the three inner floral whorls, mainly based on the presence, distribution and amount of glandular and non glandular trichomes. In the ovary, a 'flower within flower' phenotype was seldom observed; in one instance such phenotype was coupled with the setting of a parthenocarpic fruit, that reiterated the differentiation of a new flower. Mapping experiments positioned *PI* on the distal end of the long arm of chromosome 3. This position was not compatible with any class B or E MADS box gene; differently, the *PI* genetic window contained the *FALSIFLORA* (*FA*) gene, the tomato orthologue of *LEAFY* (*LFY*). The *pi* defects in flowering time and inflorescence development are in agreement with a direct involvement of the floral meristem identity gene. The class B- and E-like phenotypes shown by *pi* mutant plants are likely an indirect consequence because *FA*, as *LFY*, is reported as a positive regulator of homeotic MADS-box genes. Because *fa* mutant plants do not form complete flowers, the *pi* mutation deserves a particular interest, producing four-whorled, although modified, flowers useful to study the functional linkage between flower induction and flower organ identity specification.

Introduction

In addition to its economic importance as vegetable, the tomato (*Solanum lycopersicum* L.) has been a species of choice for studying the genetic control of fruit development and of the determination of its size, shape, and quality (Grandillo et al. 1999; Causse et al. 2004). Mutants involved in the late steps of fruit development have received great attention and many genes have been identified, especially among those controlling the ripening process (Vrebalov et al. 2002; Giovannoni 2007) and fruit pigmentation (Paran and van der Knaap 2007).

Differently, studies on early tomato reproductive biology have lagged behind those of other taxa which have elucidated the molecular control of flower development. Although several monogenic mutants involving flower development have been reported in tomato (Menda et al. 2004; Chetelat 2005), only a few of them have been thoroughly described and for even less the underlying gene has been identified. Some of the genes controlling the transi-

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tion to flowering have been characterized molecularly, such as *SELF PRUNING* (*SP*, Pnueli et al. 1998), *FALSIFLORA* (*FA*, Molinero-Rosales et al. 1999) and *SINGLE FLOWER TRUSS* (*SFT*, Lifschitz et al. 2006) and others only genetically (Quinet et al. 2006; Samach and Lotan 2007).

A key achievement in the understanding of the molecular control of flower development is the ABC(DE) combinatorial model for the specification of floral organ identity. According to the model, the differentiation of the four floral organ types is controlled by the expression of class A genes alone in the first floral whorl, class A and B in the second, class B and C in the third, and C class alone in the fourth (Weigel and Meyerowitz 1994). Additional functions have been discovered as class D genes that are essential for ovule identity (Colombo et al. 1995) and class E genes (*SEPALLATA*, *SEP*) that concur in the specification of all organs except sepals (Honma and Goto 2001; Ditta et al. 2004). Most of these functions are covered by proteins belonging to the MADS box family of transcription factors (TFs, Vandenbussche et al. 2003). In tomato, the characterization of MADS box genes controlling floral organ identity has been mainly addressed through reverse genetics (Pnueli et al. 1991, 1994a, b; Ampomah-Dwamena et al. 2002; Busi et al. 2003; de Martino et al. 2006) and no known mutant has been definitely associated to one of these genes to date.

Among the tomato mutations that show homeotic transformation of floral organs, the allelic series of *stamenless* (*sl*) has received much attention in the past (Sawhney 1992; Gómez et al. 1999). Recently, the *Sl* locus has been suggested as the tomato orthologue of the B-function *DEFICIENS* (*DEF*) gene of *Antirrhinum majus* (Gómez et al. 1999; Mazzucato et al. 2008), although definite evidence for that has not been produced. Other tomato mutations recalling a disruption of the B function are *pistillate* (*pi*, Rick and Robinson 1951) and *pi-2* (synonymous *green pistillate*, Rasmussen and Green 1993; Rick 1993). Both these mutants show strong carpellization of stamens, while the modification of the corolla is subtle for *pi* (Rick and Robinson 1951) but very severe for *pi-2* (Rasmussen and Green 1993). It is not known whether these mutants are allelic and no more details have come to light since they were first described.

In this research, we set up to characterize the *pi* mutation of tomato. The complete description of this phenotype and its integration into the tomato genetic map indicated that *pi* likely represents a leaky mutant allele of the *FA* transcription factor. Differently from *fa*, *pi* produce four-whorled, although modified, flowers; thus, it offers novel opportunities to address the study of the functional linkage between flower induction and flower organ identity specification.

Materials and methods

Plant material and morphological analysis

An F_2 seed sample segregating the *pi* mutation in the background of the cv San Marzano (SM) was obtained from the Tomato Genetics Stock Center (TGRC), University of California, Davis, USA (acc. No. 2-137). Thirty-six F_2 plants were raised in a heated tunnel during the autumn–winter season at Viterbo, Italy (42°26′N, 12°04′E) and seed was harvested from all plants with wild-type (WT) phenotype. An F_3 seed stock that was shown to segregate *pi* by progeny testing was sown in spring and a population of totally 118 plants was grown to maturity in unheated tunnel with standard agronomic practices.

At flowering, each plant was classified as WT or *pi* based on floral morphology. Time to flowering was recorded as the number of leaves to the first inflorescence and the regularity of the sympodial segment as the number of leaves between the first and the second inflorescence. Moreover on the second inflorescence, the inflorescence structure (number of ramifications) and determinacy (number of vegetative elements on the inflorescence, including leafy sepals, leaves or complete shoots), and the number of flowers (only on simple inflorescences) were recorded.

Flower morphology was examined in more detail on 25 WT and *pi* flowers harvested before anthesis, corresponding to the stage 3 of Mazzucato et al. (1998). Using a stereomicroscope, on a single flower basis, the number of sepals and petals was counted and the length and width of the longer organ in each whorl was measured. After removing the perianth, anther length (in *pi* flower only anthers retaining staminoid features were measured) and ovary length and width were measured. After dissecting a portion of the pericarp, ovule morphology was observed.

Seed set was recorded on 25 WT and 4 *pi* plants by harvesting four to eight fruits per plant derived by open-pollination (OP). Seed set after crossing was evaluated after 25 flowers per genotype were emasculated and subjected to hand-pollination (HP) with abundant, 90% stainable WT pollen. From the ripe fruits collected, seed was extracted, counted and referred to as number of seeds per unit fruit.

Histology and scanning electron microscope (SEM) analysis

Flowers of WT F_3 plants were sampled at the stages 2 and 3 of Mazzucato et al. (1998) that correspond to flower bud 9–12 mm-long and opening flower, respectively. Flowers of the *pi* mutant were collected at comparable stages taking into account the different sepal length between the two genotypes. For light microscopy, specimens were fixed, embedded and sectioned essentially as described (Mazzu-

cato et al. 1998) with the difference that Paraplast (Sigma) was used for embedding instead of Technovit.

To determine the percentage of stainable pollen, five flowers per genotype were used. One anther per WT flower was dissected, squashed on a microscope slide and the released pollen grains stained in a drop of 1% acetic orcein and 50% glacial acetic acid solution. Stainable pollen was calculated as the percentage of plump, purple pollen grains appearing in a sample of at least 200 grains. From *pi* flowers, anthers retaining staminoid features were selected, dissected with a razor blade and processed as WT anthers. A sample size of 200 pollen grains could not always be reached in these slides.

For SEM analysis, stage 2 flowers were fixed, processed and observed as reported earlier (Mazzucato et al. 1998).

Mapping of the *Pi* locus

Molecular mapping of the *Pi* locus took advantage from the indication of linkage with the *Parthenocarpic fruit* (*Pat*) locus. F_1 hybrids from the cross between a *pat* and a *pi* mutant line showed that the two mutations were not allelic, but the absence of double mutants in the F_2 generation suggested tight linkage (Mazzucato et al. 2008). Therefore, because the *Pat* locus was previously located on the distal end of the long arm of tomato chromosome 3 (Beraldi et al. 2004), three *pi* plants segregating from the original stock were used as female parents in crosses with a suitable introgression line (IL3-5; Eshed and Zamir 1994), where an introgression from *S. pennellii* spanned the target region. Out of several crosses attempted, a few flowers set the fruit and produced F_1 seed. F_1 hybrids were grown to maturity and left to OP in order to harvest F_2 seed. Ninety-one F_2 plants were raised to maturity in unheated tunnel in Viterbo in spring and classified according to the phenotype at the *Pi* locus. All the F_2 WT plants ($n = 70$) were progeny tested by growing to flowering 10–20 F_3 progeny plants.

Total DNA was extracted from F_2 plants according to Doyle and Doyle (1990). Each plant was analysed for the genotype at marker loci located on the distal end of the long arm of chromosome 3 (TomatoEXPEN 2000 map, http://www.sgn.cornell.edu) with the primers and PCR conditions given in Table 1. PCR amplifications were performed in a 20 µl total volume, containing 20 ng of genomic DNA template, 50 pmoles of each of the two primers, 200 µM dNTPs, 2 mM $MgCl_2$, 1× Taq polymerase buffer, and 1 U Taq DNA polymerase (New England Biolabs, Beverly, MA, USA). The amplifications were conducted with an ini-

Table 1 Primer sequences and PCR reaction parameters for the markers used

Marker name	Position[a]	Primer sequence (5′–3′)	Annealing temperature (°C)[b]	No. of cycles[c]	PCR product length (bp)[d]
T1235	160.00	F CGTCTCCCATTTCAATTGG R AAAGCAACTGTTCCCTTCAA	55	32	1700_l, 1600_p
T1143	159.00	F GGAGAATGGGCATCTACAA R CCTTTAGGATGGATTCCG	55	32	1100_l, 900_p
T0796	157.00	F TCGGAGTATTGATGGCCTGTC R TCAACATGAGGACCACGGC	60	32	1950_{EcoRI}
T0668	137.50	F CGGTGATAAGGAGGTGGT R CAAATTTCGGCATTACTGG	65–56	12 + 20	1100_p, 1000_l
T0581	137.00	F CGACTGATGTTCGTAAGGCCC R AGCGTAGAGCTTGCGTTCACA	60	32	500
T0482	137.00	F TTGCTCGCTGCAGAGAAGAAA R CCCAGCTATAGGTGCATCACG	nd	nd	565
KFG-FA	135.00	F AGGGGAAGAGGATGAGGAAA R AGCCACCAATGGCTTGTAAC	60	32	850_{SstI}
T0761	133.00	F GGACTTTGTGCACAGTTGG R TCTTCCTCCTCATCAACCTT	65–56	12 + 20	2600_{HinfI}

[a] Marker position on the long arm of tomato chromosome 3 according to the Tomato-EXPEN 2000 map, (http://www.sgn.cornell.edu) ordered from the most distal to the most proximal

[b] A range in annealing temperature indicates a touch-down cycle as detailed in Materials and methods. *ND* not determined

[c] A double value indicates a touch-down cycle as detailed in "Materials and methods"; *ND* not determined

[d] Values are PCR product lengths in base pairs (bp) approximated by comparison with molecular weight ladders. Pedices indicate product length in *Solanum lycopersicum* (l) or *S. pennellii* (p) for in/del markers or the restriction enzyme used to detect polymorphism for CAPS markers. PCR product length for marker T0482 is that expected from the mRNA sequence, because amplification from genomic DNA was not obtained

tial 2 min at 94°C that was followed by 32 cycles of 1 min at 94°C, 1 min at the annealing temperature reported in Table 1, and 1 min at 72°C, plus 5 min final extension at 72°C. For markers T0668 and T0761, a touch down cycle was adopted in order to avoid aspecific amplification products, where the annealing temperature was lowered from 65 to 56°C in the first 12 cycles and left at 56°C for the remaining 20 cycles. For marker T0482 no amplification product could be obtained with the designed primers, either modifying the annealing temperature, the number of cycles or the type of PCR program. This marker was therefore left out from the analysis. The amplification products were resolved by electrophoresis on 2% (w/v) agarose gels and stained with ethidium bromide.

Whereas amplicons obtained with T1235, T1143, and T0668 turned out to be useful as co-dominant in/del markers, polymorphisms between *S. pennellii* or IL3-5 and *S. lycopersicum* for the other marker loci were evidenced as cleaved amplified polymorphic sequences (CAPS) after attempts with several 4- and 6-cutter restriction enzymes. CAPS polymorphisms were found for T0796, KFG-FA (the *Eco*RI digestion suggested at the http://www.sgn.cornell.edu website did not cut the PCR product obtained from our F_2 plants), and T0761 (Table 1), whereas no enzyme cutting the T0581 amplicon in a polymorphic way was found. CAPS polymorphisms were obtained by digesting 10 µl of the PCR reaction with 5 U of the appropriate enzyme (Invitrogen, Carlsbad, CA, USA) and resolving the restriction fragments on 2.5% (w/v) agarose gels.

Once a polymorphism was detected, its map position was controlled by screening a set of five introgression lines carrying different introgressions of *S. pennellii* chromosome 3 (IL 3-1 to 3-5; Eshed and Zamir 1994).

The PCR product obtained with the KFG-FA marker primers from the parents (SM-*pi* plant and IL3-5), a homozygous *Pi* and a homozygous *pi* F_2 plant and from a WT SM plant was sequenced on both strands using an ABI PRISM 310 automatic sequencer (PerkinElmer, Boston, MA, USA).

Genetic relationship between *Pi* and *Pi-2*

To explore the genetic relationship between *Pi* and *Pi-2*, a number of WT plants of a population segregating *pi-2* (TGRC acc. No. 3-802) were pollinated with pollen from plants of the IL3-5 line. F_1 hybrids were all normal confirming the recessivity of *pi-2*. Twenty-nine plants of an F_2 population which was assessed to segregate both *pi-2* and the IL3-5 markers were grown to maturity, classified for the phenotype at the *Pi-2* locus and used to extract the DNA. Plants were then analyzed with the chromosome 3 markers T1143, T0668, and T0761 with the same procedures described above.

Data analysis

Chi-squared (χ^2) tests were performed to examine the goodness-of-fit between the expected Mendelian ratio and the segregation data for the mutations and markers for all the populations described. Differences between WT and *pi* plants for morphological traits were tested by Student's *t* test.

The software package JoinMap® 3.0 (Van Ooijen and Voorrips 2001) was used to perform the linkage analysis between the molecular markers and the *Pi* and *Pi-2* loci: a LOD score of 3.0 or above was specified. The Kosambi mapping function was used to convert recombination frequencies into map distances.

Results

Vegetative development, floral transition and inflorescence development

The original seed stock segregated 25 and 11 plants bearing WT (Fig. 1a) and *pi* mutant (Fig. 1b, c) flowers, according to the Mendelian ratio for a single recessive allele (χ^2 0.59, $P > 0.05$). The studied F_3 population also segregated the *pi* mutation in a Mendelian fashion (84:34, χ^2 0.92, $P > 0.05$).

Early vegetative development was similar in all F_3 plants. Later, it was observed that the *pi* mutants showed delayed transition to flowering, because they produced on average two leaves more than the WT before differentiating the first inflorescence (Table 2). In addition, *pi* plants presented a sympodial segment modified with respect to the WT: instead of the normal sequence of three leaves and one inflorescence (Pnueli et al. 1998), they developed on average more than four leaves between the first two inflorescences (Table 2).

Whereas the WT presented almost always simple inflorescences, as expected for SM plants (G.P. Soressi, personal communication), the *pi* mutant inflorescences showed on average more than two ramifications (Fig. 1d; Table 2). As reported earlier (Rick and Robinson 1951), in the inflorescence of the *pi* mutant the floral meristem often lost its identity and reverted to a vegetative developmental program; this happened with a penetrance of 84% and a mean of more than four vegetative elements were found per inflorescence (Table 2). Leafyness of the inflorescence occurred with different severity degrees, ranging from a single leaf, to the development of a new vegetative shoot (Fig. 1d). Sometimes loss of floral identity occurred in the form of a leafy sepal (Fig. 1e). Sometimes, the primary *pi* meristems reverted to vegetative growth, so that inflorescence reversion is also possible in *pi* in addition to floral reversion.

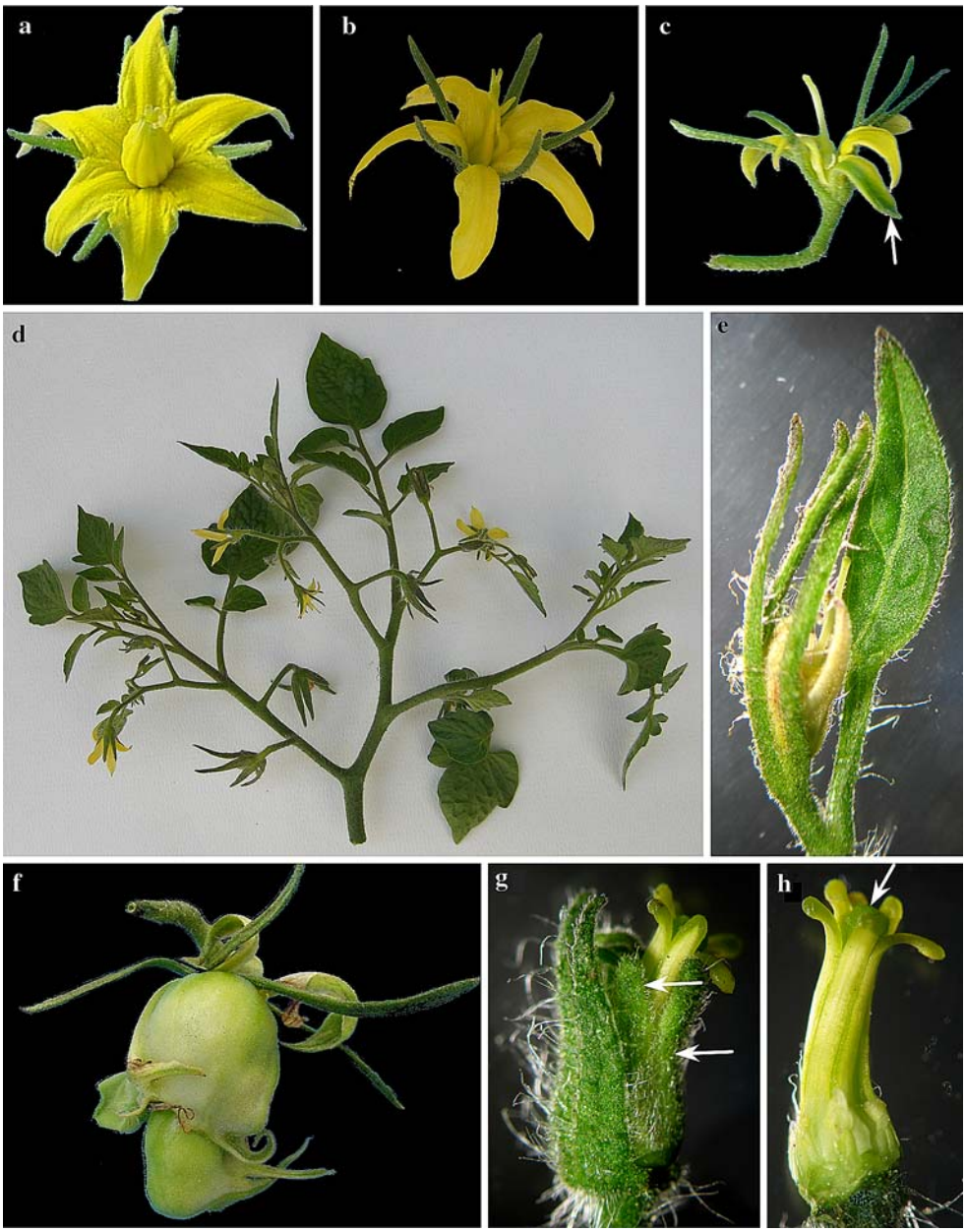

Fig. 1 Flowers at anthesis of **a** wild-type (*WT*) and **b, c** *pi* mutant plants (the *arrow* points to a petal with greenish vein). **d** Compound inflorescence of a *pi* plant, and **e** leafy phenotype of a putative *pi* sepal. **f** Ectopic fruit and sepal- or shoot-like outgrowths in a parthenocarpic fruit set on a *pi* plant. **g** Sepals and sepalized petals (*arrows*) and **h** carpellized stamens and true stigma (*arrow*) of a *pi-2* flower

Finally, when only simple inflorescences were compared, the mutant presented a number of flowers significantly lower than the WT (Table 2).

Flower development

The number of perianth organs was lower in the mutant compared to the WT (Table 3). Sepals were significantly longer in *pi* flowers (Fig. 1e–f); conversely, petals were longer in the WT (Table 3). Mutant petals were usually distorted, wilty and sometimes reduced (Fig. 1b). In the petal, greenish veins were often observed on the adaxial surface (Fig. 1c, arrow).

The most striking aberration in *pi* mutant flowers was the strong modification of (Fig. 1b) or the apparent lack (Fig. 1c) of the staminal cone. At a closer inspection it was evident that in both cases stamens were homeotically transformed into carpels and therefore appeared styliform and fused to the true pistil. When the third whorl retained staminoid characters, sub-normal anthers were shorter and less developed than in the WT (Table 3).

Table 2 Transition to flowering and inflorescence traits in wild-type (WT) and *pi* mutant plants

Trait	Phenotype at the *Pi* locus	
	WT	*pi*
No. of leaves to the first inflorescence	8.6 ± 0.2	10.3 ± 0.2
No. of leaves between the first and second inflorescence	3.2 ± 0.05	4.8 ± 0.11
No. of ramifications on the second inflorescence	0.24 ± 0.07	2.14 ± 0.27
No. of vegetative elements occurring in the inflorescence	0.28 ± 0.07	4.18 ± 0.30
No. of flowers per inflorescence[a]	8.8 ± 0.2	7.4 ± 0.2

A total of 118 plants, 84 WTs and 34 *pi* mutants, were screened in the F_3 generation. All differences are significant for $P \leq 0.001$ according to the *t* test

[a] Calculated only considering simple inflorescences

Table 3 Floral traits and seed set in wild-type (WT) and *pi* segregants

Trait	Phenotype at the *Pi* locus		Significance level[b]
	WT	*pi*	
No. of sepals	5.54 ± 0.10	5.00 ± 0.00	***
No. of petals	5.50 ± 0.10	5.08 ± 0.10	**
Sepal length	8.66 ± 0.21	12.00 ± 0.36	***
Sepal width	1.22 ± 0.05	0.97 ± 0.02	***
Petal length	9.92 ± 0.17	8.44 ± 0.44	**
Petal width	2.19 ± 0.12	1.73 ± 0.12	**
Anther length[a]	9.52 ± 0.14	6.97 ± 0.79	***
Ovary length	2.06 ± 0.07	1.84 ± 0.06	*
Ovary width	1.57 ± 0.04	1.75 ± 0.05	**
No. of seeds per fruit under OP	20.0 ± 4.2	0	***
No. of seeds per fruit under HP	16.6 ± 4.1	13.8 ± 5.4	NS

A total of 25 flowers from WT and *pi* F_3 plants were dissected and measured as detailed in Materials and methods. The No. of seeds per fruit was calculated on 25 WT and 4 *pi* plants producing fruits in open-pollination (OP) and after 25 WT and *pi* flowers were subjected to hand-pollination (HP) with fertile pollen

NS not significant

[a] The datum for *pi* flowers refers to anthers retaining at least some male features

[b] Differences significant for $P \leq 0.05$ (*), 0.01 (**), or 0.001 (***) according to the *t* test

The mutant ovary was smaller than the WT in length; however, because of the contribution given by the fused stamens, ovary width in the mutant was higher (Table 3). After dissecting the pericarp, mutant ovules appeared normal; in some cases sepaloid outgrows were found inside the ovary, more or less replacing the placenta and ovules, and recalling a 'flower within flower' phenotype (not shown). In one instance, such phenotype was evidenced by the setting of a parthenocarpic fruit, that contained new sepals and a new, parthenocarpic ovary inside (Fig. 1f). Throughout the analysis, aged *pi* flowers seldom showed enlarged ovaries; in the original F_2 population, that was raised in autumn–winter conditions, the development of small parthenocarpic fruits was observed in *pi* plants.

Under our experimental conditions, seed production per unit fruit of WT F_3 plants was 20.0 ± 4.2 under OP, a yield similar to that of fruits obtained by HP (Table 3). Mutant plants were completely sterile under OP and no seed could be produced. Also obtaining fruit set after HP was extremely difficult; out of 25 *pi* flowers pollinated, only four set fruits and seeds. They gave a seed yield comparable to that in WT plants (Table 3).

Histological and ultrastructural analysis

Compared to the WT (Fig. 2a), histological sections of *pi* flowers confirmed the strong homeotic transformations of the anther cone in the mutant (Fig. 2b). Carpellized anthers were composed of mosaic tissue, with portions eventually featuring a normal techa where meiosis had occurred (Fig. 2c). Developing pollen grains found in these rather normal anther portions were scarce and generally not stainable (Fig. 2e). A portion of the carpelloid anthers could bear external ovules (EOs) (Fig. 2b, c).

Tissue sections showed that often the receptacle of *pi* flowers was abnormally elongated and floral organ insertion was not regular but alternate (Fig. 2b, compare with Fig. 1e). In about 30% of the 15 *pi* flowers observed, it was possible to detect vegetative buds developing at the sepal axil; although leafy sepals were observed (Fig. 1e), evidence for the development of these axillary buds was not reported in mature of aging flowers.

At the SEM analysis, sepal morphology did not reveal any homeotic conversion, but, compared to the WT (Fig. 3a), a higher number of hairs was observed in the mutant (Fig. 3b). The main distinctive feature of the abaxial sepal surface was the presence of long, multicellular non glandular hairs mounted on large foot cells (Fig. 3a, b, arrows; Chandra Sekhar and Sawhney 1984). On the adaxial sepal surface, hairs whit foot cells were absent in both genotypes; however, whereas in the WT hairs were rare at the basis (Fig. 3c), the mutant showed dense hairs also in this region (Fig. 3d). Moreover, in the WT glandular hairs with club-shaped stalks bearing four-lobed glands were present only along sepal margins (Fig. 3e, arrows), whereas in the mutant they were widespread on the whole sepal adaxial surface (Fig. 3f, arrows).

On the abaxial WT petal surface, hairs were mainly present on the distal third (Fig. 3g). In the mutant, the same pattern was observed with the difference that also hairs with

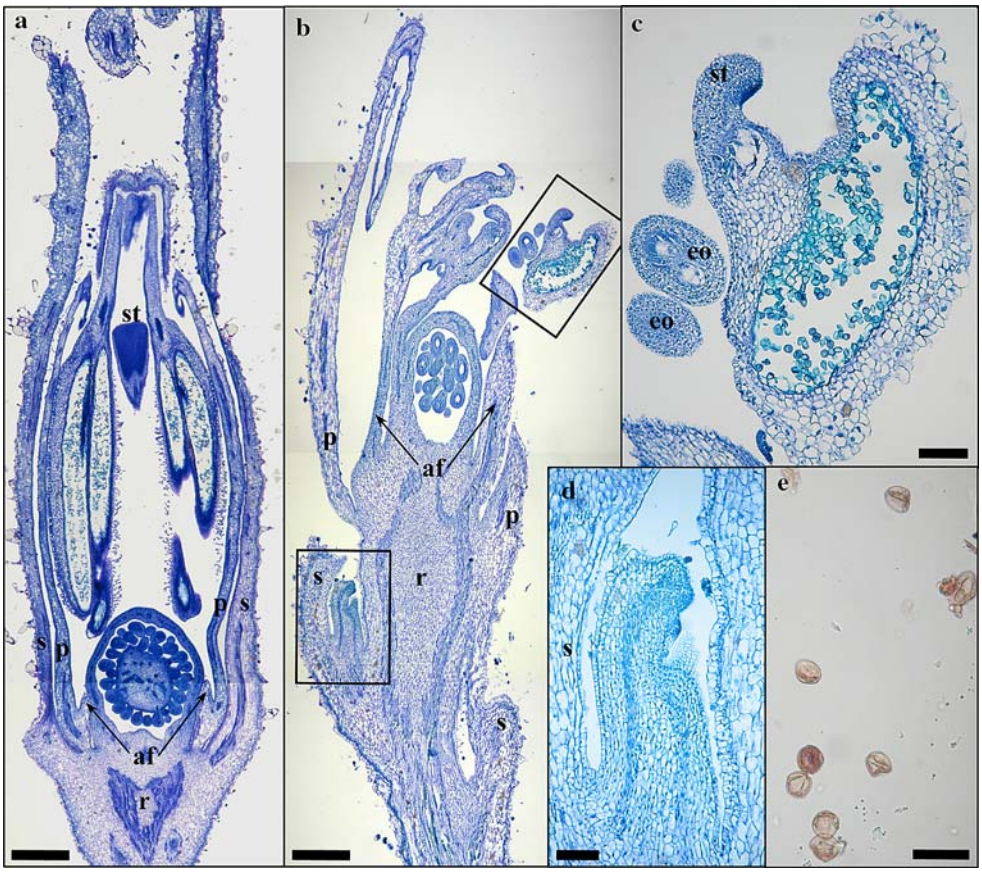

Fig. 2 Toluidine blue-stained sections of wild type (**a**) and *pi* (**b**–**e**) flowers at stages 2–3 (flower bud 9.0–12.0 mm-long or opening flower according to Mazzucato et al. 1998). **b** General, axial view of a *pi* flower bud, and enlarged particular of the tip of a chimaeric anther (**c**) and of an axillary shoot meristem (**d**). **e** Pollen grains stained during gametogenesis. *Bars* are 1 mm in **a**, **b**, 0.2 mm in **c**, **d** and 30 μm in **e**. *af* Anther filament, *eo* external ovules, *p* petal, *r* receptacle, *s* sepal, *st* stigma or stigma-like tissue

foot cells were present (Fig. 3h, j, arrows), that were not observed in the WT (Fig. 3i). On the adaxial petal surface, both genotypes were almost devoid of hairs (Fig. 3k). Petals that were seldom staminoid (with techae) or petals adnate to the (rudimentary) anthers were also found (not shown). Rarely, these chimaeric structures beard on the adaxial surface carpel-like outgrows with EOs (Fig. 3l).

Wild-type abaxial (Fig. 3m) and adaxial (Fig. 3n) normal stamen morphology was almost never found in the *pi* mutant; mutant stamens were homeotically transformed into carpelloid organs with a variety of features; the most common was a styliform shape, usually adnate to the true pistil (Fig. 3o, arrowheads). Partially staminoid anthers showed carpelloid tissue on the adaxial surface bearing EOs (Fig. 3o, arrow). Such chimerism was usually supported by the morphology of epidermal tissue which shared anther and style-like cells (Fig. 3p). Long hairs were seen on the basis of the more or less modified anthers (Fig. 3q, arrows), a feature never observed in the WT.

Whereas the WT style beard elongate, non-glandular hairs in the basal third in this genetic background (Fig. 3r, s), the *pi* true style showed also glandular hairs, that were often dispersed along the entire pistil length (Fig. 3t, u). SEM analysis confirmed that *pi* ovules have normal appearance (not shown).

Mapping of the *PI* locus

The map position of the *PI* locus was first indicated when it was attempted to obtain double mutants bearing the *pi* and the *pat* mutations. Out of 126 F_2 plants obtained from the cross between the *pat* and the *pi* mutant, 65 WT, 30 *pi*, 31 *pat* and no clear double mutant plant were observed (Mazzucato et al. 2008). Thus, the population segregated as a single locus (χ^2 0.071, $P > 0.90$), supporting the hypothesis of tight linkage between the two loci (Mazzucato et al. 2008). Because *PAT* was located in the distal end of the long arm of tomato chromosome 3 (Beraldi et al. 2004), an F_2 population segregating *pi* after cross with the introgression line IL3-5 (Eshed and Zamir 1994) was produced for molecular mapping. After having progeny tested all the WT individuals, the 91 plants of the mapping population were

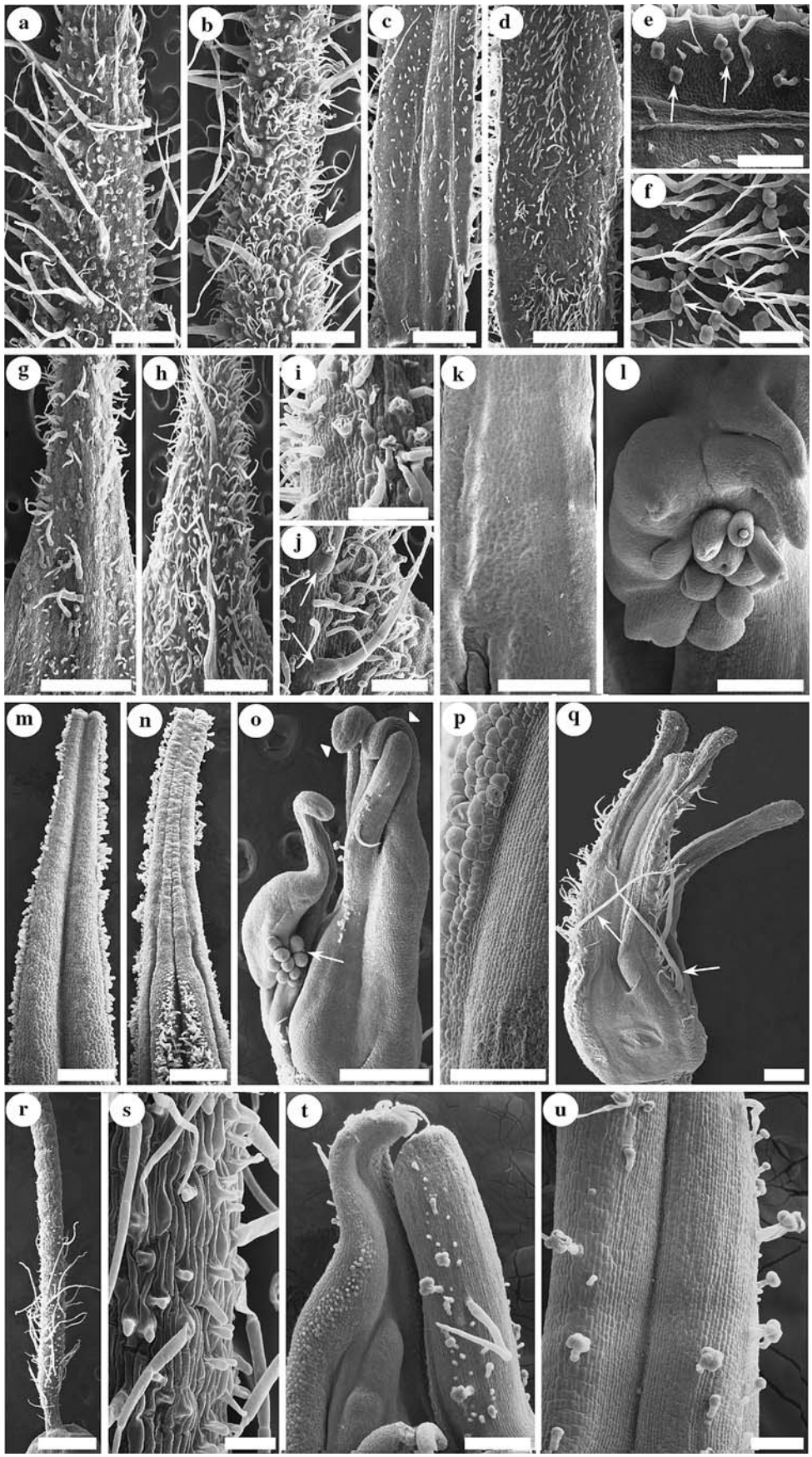

Fig. 3 Scanning electron microscope (*SEM*) analysis of floral organs from wild-type (*WT*) and *pi* mutant plants. All photographs are taken from organs of flowers at stage 2 (flower bud longer than 9.0 mm according to Mazzucato et al. 1998) except for **t** and **u** which are from a stage 1 (flower bud from 6.0 to 8.9 mm-long) flower. **a** WT and **b** *pi* sepal abaxial surface (*arrows* point to hairs with "foot cells"). **c** WT and **d** *pi* sepal adaxial surface, proximal half. Details of **e** WT and **f** *pi* sepal adaxial surface showing differences in the distribution of glandular hairs (*arrows*). **g** WT and **h** *pi* petal abaxial surface, distal half (*arrow* points to a hair with "foot cells"). Details of **i** WT and **j** *pi* petal abaxial surface. **k** WT petal adaxial surface and **l** *pi* petal adaxial surface showing an ectopic carpelloid structure bearing external ovules (EOs). WT **m** abaxial and **n** adaxial anther structure, distal half. **o** modified androecium of a *pi* mutant flower, with a partially carpellized anther with EOs (*arrow*) and at least two completely carpellized anthers fused to the true pistil (*arrowheads*). **p** chimaeric epidermal tissue on a *pi* anther showing anther (*top-left*) and style-like (*bottom-right*) cell types. **q** Anthers fused to the pistil of a *pi* flower with long, sepaloid hairs at the basis (*arrows*). **r** Complete view and **s** detail of a WT style showing hairs on the basal third, **t** complete view and **u** detail of a *pi* style showing long, non-glandular and glandular hairs distributed along the entire organ. *Bars* are 500 μm in **a**–**d**, **g**–**h**, **k**, **m**–**o**, **q**–**r**; 200 μm in **e**–**f**, **i**–**j**, **l**, **p**, **t**; 50 μm in **s** and 100 μm in **u**

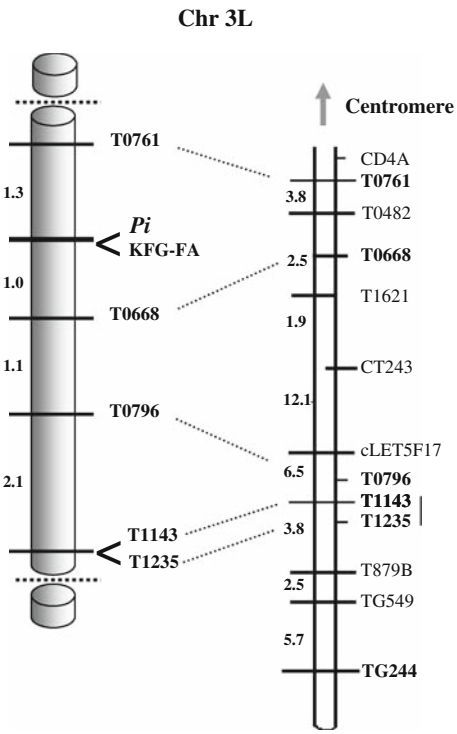

Fig. 4 Genetic linkage map surrounding the *Pi* locus based on segregating COS markers (*left*) and its integration with the published genetic map of the long arm of tomato chromosome 3 (*right*; http://www.sgn.cornell.edu/). Genetic distances are expressed in centimorgans

classified as 21 *pi* mutants, 62 heterozygotes and 8 WT homozygotes (χ^2 15.68, $P \leq 0.001$), showing a significant defect of the *S. pennellii* WT allele.

Whereas marker T0482 and T0581 were not informative (see "Materials and methods"), the other six markers gave codominant polymorphisms that could be easily scored. All the tested markers confirmed their position on the long arm of chromosome 3 and showed in the F_2 population a segregation distortion similar to that reported for *PI*.

Association analysis positioned *PI* inside a genomic window of 2.3 cM flanked proximally by the COS marker T0761 (position 133.00, http://www.sgn.cornell.edu/) and distally by T0668 (position 137.50; Fig. 4). *PI* was not separated from the marker locus KFG-FA which mapped inside the above mentioned window (position 135.00). As expected, markers T0796, T1143 and T1235 mapped distal to T0668. The two genomic windows containing *PI* (T0761-T0668) and *PAT* (T0796–T1143, Beraldi et al. 2004) were separated in our local map by 1.1 cM (Fig. 4).

Genetic relationship between *PI* and *PI-2*

pi-2 mutant plants were easy to score in the original segregating stock because of the homeotic conversion of petals to sepals (Fig. 1g, arrows) and of stamens to carpels (Fig. 1h). The F_2 population segregating after the cross with IL3-5 produced 10 mutant and 19 WT plants (χ^2 1.39, $P > 0.05$) in agreement for a single locus with two alleles and dominance (Rick 1993). Markers T0761, T0668 and T1143 also showed a Mendelian segregation and, as expected, tight linkage. However, association analysis showed that *PI-2* was independent from this region, thus excluding allelism with *PI* (data not shown).

Search for a gene candidate for the *PI* locus

The homeotic conversions detected on flowers of the *pi* mutant first suggested that the mutation could involve one of the MADS-box TFs involved in the specification of the stamens. Tomato members belonging to the B and E classes of MADS-box have recently been listed (Hileman et al. 2006; Leseberg et al. 2008; Mazzucato et al. 2008), as well as those belonging to the *AP1/FUL* clade that interact with the former and exert roles in the induction of flowering (Pelaz et al. 2001; Leseberg et al. 2008). According to the information available, none of these TFs showed a map position compatible with *PI* (Table 4).

At the same time, a deeper investigation was carried out on the KFG-FA marker, that mapped inside the genetic window containing *Pi*. The description for this marker referred to loci that are known-function genes but it did not report its identity (http://www.sgn.cornell.edu/). After sequencing the PCR product amplified from the DNA of a SM plant and blasting the sequence against the tomato unigene database (http://compbio.dfci.harvard.edu/), we could ascertain that the KFG-FA marker actually corresponded to the *FALSIFLORA* locus. A candidature of *FA* for the *PI* locus is plausible because *fa* and *pi* phenotypes for flowering induction and inflorescence development are in good agreement.

Analysis of sequences obtained from the KFG-FA primers (position 1023–1880 of the deposited genomic sequence AF197934, encompassing a portion of the second and third exon and the entire second intron, Molinero-Rosales et al. 1999) in two homozygous F_2 plants (one for the *PI* and one for the *pi* allele), in their parental lines (a SM-*pi* plant and the IL3-5) and in a wild-type SM plant confirmed the lack of the *EcoRI* and the presence of the *SstI* recognition sequence in SM. Although several polymorphisms were found in the intron between the original *FA*, the IL3-5 and the SM background sequences, no difference was found between the WT and *pi* SM plants indicating that the eventual mutation underlying *pi* was not located in this region (not shown).

Discussion

Confirming the early description (Rick and Robinson 1951), the present work showed that *pi* is a completely

Table 4 Map position of tomato MADS box genes belonging to the class B and E subfamilies and to the *AP1/FUL* clade

Gene clade	Gene name	Synonymises	TC/EST identifier	Map location		Reference[b]
				Chromosome	Position[a] (cM)	
DEF	*SlDEF*	*LeAP3*, *TAP3*	TC154541	4	121.00	(3) (5)
	TM6	*TDR6*	TC185418	2	87.00	(2) (5)
PI	*SlGLO*	*LePI*, *SlMBP2*	TC182347	8	30.00–50.00	(3)
	SlGLO-2	*LePI-B*, *SlMBP1*, *TPI*	TC171847	6	38.00	(5)
SEP	*TM5*	*TDR5*	TC163381	5	67.50	(2) (5)
	MADS-RIN		TC156089	5	35.10–49.00	(1) (4) (5)
	MADS1		TC155511	3	104.50	(5)
	TM29		TC154219	2	110.00	(5)
	SlMBP21		EST244410	12	57.20	(5)
AP1/FUL	*MC*		TC156088	5	35.10–49.00	(4) (5)
	SlMBP20		EST313420	2	111.30	(5)
	TM4	*TDR4*	TC158381	6	52.50	(2) (5)
	SlMBP7	*TFUL-like*	TC187494	3	104.00	(5)

[a] Map positions refer to the TomatoEXPEN 2000 molecular map (http//:www.sgn.cornell.edu/); the interval is given where the existing literature does not allow more precision

[b] (1) Giovannoni et al. (1995), (2) Lifschitz et al. (1993), (3) Mazzucato et al. (2008), (4) Vrebalov et al. (2002), (5) http//:www.sgn.cornell.edu

recessive mutation segregating in a Mendelian fashion after intraspecific crosses. The homeotic nature of the *pi* mutation was evident to the first describers, that adopted a name recalling the prevalence of female over male floral organs (Rick and Robinson 1951). Our present investigation expanded the first analysis and showed that *pi* flowers present homeotic transformation in all whorls except sepals; such transformation consisted in a sepalization of the three inner whorls, that was revealed by hair distribution. In addition, all floral organs except sepals showed a tendency to adnation, that was the basis for frequent tissue chimaerism. Finally, the flower meristem sometimes showed indeterminacy and the *pi* ovary revealed ectopic outgrowth of leaf- or sepal-like structures.

Many of the traits reported in the *pi* floral syndrome recall the phenotype of mutants affected in homeotic MADS box TFs of the B or E class, whose products function, in combination with other MADS box proteins, in specifying the identity and development of the three inner floral whorls organ and in maintaining floral meristem determinacy (Pelaz et al. 2001; Theißen 2001; Malcomber and Kellogg 2005). However, the information on the map position available for all the known tomato members of these classes of TFs excluded a direct involvement of one of them in the *pi* mutation. This was actually in agreement with a thorough consideration of the complex phenotype described for the *pi* mutant. In general agreement with class B and E mutants or knock-outs described in other species, down-regulating the activity of class B members in tomato usually did not cause the defects on the fourth whorl reported in *pi* (sepal-like hair patterning on the pistil and indeterminacy, Lifschitz et al. 1993; de Martino et al. 2006), whereas down-regulating members of the E class caused a much more severe sepallization on the three inner whorls than that seen in *pi* (Pnueli et al. 1994b; Ampomah-Dwamena et al. 2002).

Differently, the map position of *PI* was compatible with that of the *FA* locus. Strong evidence was produced that *FA* represents a flower meristem identity gene in tomato (Molinero-Rosales et al. 1999) that is orthologue to the *Arabidopsis thaliana LEAFY* (*LFY*, Schultz and Haughn 1991; Weigel et al. 1992) and *A. majus FLORICAULA* (*FLO*, Coen et al. 1990; Carpenter et al. 1995) genes. Actually, many of the defects reported in *pi* mutants paralleled those described in *fa* plants, including delayed flowering, reversion of the flower (and sometimes the inflorescence) meristem identity and altered sympodial meristem (Allen and Sussex 1996; Molinero-Rosales et al. 1999). Such defects were also described in *flo/lfy* mutants of several species (Coen et al. 1990; Schultz and Haughn 1991; Hofer et al. 1997; Souer et al. 1998),

Differently from *pi*, *fa* mutants never showed the development of flowers (Allen and Sussex 1996; Molinero-Rosales et al. 1999), a severe phenotype also reported for *flo* (Coen et al. 1990). An allele weaker than *fa* has been described in tomato under the name *leafy inflorescence* (*lfi*); also such genotype did not produce complete flowers, but only some leafy organs on the inflorescence stem that turned into fleshy carpelloid organs able to ripen to a red color (Kato et al. 2005). Differently, in *pi* flowers all floral organs were developed, although with the described degree of aberrancy. Thus, if the allelism between *PI* and *FA* will

be confirmed, *pi* should represent a leaky allele of *FA*, where the residual protein activity is permissive for the differentiation of four-whorled flowers. A similar phenotype was described for the *lfy-13* weak allele of Arabidopsis (Huala and Sussex 1992). Also considering the delayed flowering and modified sympodial segment phenotype *pi* appeared weaker than *fa* (Molinero-Rosales et al. 1999), although these differences could be accounted for by the different genetic background.

The *pi* flower phenotype is in agreement with the role of *LFY* as a transcriptional activator of the genes involved in the ABC model (Huala and Sussex 1992; Weigel and Meyerowitz 1993; Parcy et al. 1998; Lamb et al. 2002). In addition to the model species, upstream regulation exerted by *LFY* orthologues on the ABC floral organ identity genes has been also reported in *Zea mais* (Bomblies et al. 2003) and in tomato (Kato et al. 2005). Thus, the *pi* floral phenotype indirectly and partially phenocopies tomato knockouts of class B (*TM6*, Lifschitz et al. 1993; *SlDEF* and *TM6*, de Martino et al. 2006) and E MADS box genes (*Tomato MADS box 5*, *TM5*, Pnueli et al. 1994b; *TM29*, Ampomah-Dwamena et al. 2002).

Although the positive regulation of *FLO/LFY* is reportedly exerted on the whole panel of ABC(DE) genes (Huala and Sussex 1992; Parcy et al. 1998), general evidence suggests that the activating mechanisms are different, and more restrictive, for class B genes than for members of other subfamilies (Lamb et al. 2002). When features of flower development are retained in *FLO/LFY* mutants, the second and third whorls are more severely affected than the first or fourth. In *lfy*, sometimes poorly developed, late flowers form that show carpel-like or sepal-like cell types, but petals and stamens are never produced (Schultz and Haughn 1991). In the tomato leaky allele *lfi*, carpelloid structures are formed along the inflorescences (Kato et al. 2005) and in Arabidopsis alleles weaker than *lfy-1*, the inflorescence ends in a number of partially fused carpelloid organs (Weigel et al. 1992; Schultz and Haughn 1993). Aberrant flowers mainly composed of sepals and carpels have also been reported in *aberrant leaf and flower* (*alf*), a *lfy* orthologue in Petunia (Souer et al. 1998) and in weak alleles of *flo* (Carpenter et al. 1995). In legumes, *FLO/LFY* orthologues have been implicated in the control of leaf compoundness; however, the flower phenotype of the corresponding loss-of-function mutants is in agreement with that reported in other families. In the *Medicago truncatula single leaflet1* (*sgl1*) and in the pea *unifoliata* (*uni*) mutants, the flower lacks petals and stamens and consists of an incomplete sepal whorl and an open gynoecium, where often 'flowers within flowers' are produced (Hofer et al. 1997; Wang et al. 2008). *uni* flowers also share with *pi* the characteristic of bract-like laminae often present in the sepal whorl and the common fusion and/or mosaicism of floral organs (Hofer et al. 1997). Leaf-like bract organs subtending the flower or leafy sepals are also found in *lfy* mutants (Schultz and Haughn 1991; Blázquez et al. 1997).

Lack of activation of class E genes depending on defective *LFY* activity should be cause of the phenotype of indeterminate flower, the tendency to parthenocarpy and the subtle sepalizzation of the three inner whorls reported in *pi* flowers. These phenotypes have been described in knockouts of class E genes in tomato (Pnueli et al. 1994b; Ampomah-Dwamena et al. 2002) and in other related species (Ferrario et al. 2003; Vandenbussche et al. 2003). The heavy occurrence of hairs typical of sepals in the three inner whorls of the *pi* mutant flower could also be interpreted as a leafy phenotype, because such trichome types are also largely distributed in leaves surfaces. The stronger presence and distribution of glandular hairs on the adaxial surface of the mutant sepal supports this hypothesis, because such trichomes are generally widely distributed on tomato adaxial leaf surfaces (Simmons et al. 2003). Accordingly, the late flowers produced by *lfy* are structures that combine characteristics of both flowers and shoots (Blázquez et al. 1997).

Lack of allelism between *PI* and *PI-2* is in agreement with the reported analysis; the *pi-2* mutant shows a clear-cut homeotic conversion of petals into sepals and of stamens into carpels, directly recalling a B-function mutation. As such, *pi-2* is better compared to the *sl* mutant series, which is a candidate to represent loss-of-function phenotypes of these genes (Gómez et al. 1999; Mazzucato et al. 2008). However, lack of definite information on the map position of *PI-2* hinders the candidature of a gene for this mutation.

The sequencing of the whole *FA* genomic region in *pi* mutant and WT siblings, allelism test between *FA* and *PI* and complementation analysis will definitely address the functional involvement of the *FA* locus in the *pi* mutation. If this will be the case, the increased sequence knowledge of the tomato homeotic TFs and the availability of a mutant were all floral organs are differentiated, although aberrantly, will improve the possibility of studying the functional relationship between the activity of floral meristem identity genes and that of floral organ identity genes. Because *Lfy* is a master gene, that regulates flowering time and floral meristem and floral organ identity, the dissection of its functions in a species other than the most studied models will contribute important information on the degree of conservation of reproductive mechanisms in flowering plants.

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