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ORIGINAL PAPER

High-resolution fine mapping of *ps-2*, a mutated gene conferring functional male sterility in tomato due to non-dehiscent anthers

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Abstract Functional male sterility is an important trait for the production of hybrid seeds. Among the genes coding for functional male sterility in tomato is the positional sterility gene *ps-2*. *ps-2* is monogenic recessive, confers non-dehiscent anthers and is the most suitable for practical uses. In order to have tools for molecular-assisted selection (MAS) we fine mapped the *ps-2* locus. This was done in an F_2 segregating population derived from the interspecific cross between a functionally male sterile line (*ps-2/ps-2*; *Solanum lycopersicum*) and a functionally male fertile line (*S. pimpinellifolium*). Here we report the procedure that has led to the high-resolution fine mapping of the *ps-2* locus in a 1.65 cM interval delimited by markers T0958 and T0635 on the short arm of Chromosome 4. The presence of many COS markers in the local high-resolution map allowed us to study the synteny between tomato and *Arabidopsis* at the *ps-2* locus region. No obvious candidate gene for *ps-2* was identified among the known functional male sterility genes in *Arabidopsis*.

Introduction

In plants, pollen maturation is normally followed by its release by dehiscence of the anthers (reviewed by Goldberg et al. 1993). This normal process of pollen formation and release may be hampered, resulting in male sterility. This phenomenon has been, for a long time, recognized as an important trait for hybrid seed production as self-pollination of male sterile plants is prevented. Male sterility can be divided into two groups: pollen sterility and functional male sterility. In tomato (*Solanum lycopersicum*, formerly *Lycopersicum esculentum*) more than 40 genes coding for pollen sterility have been identified (Georgiev 1991); however, the use of this trait in tomato breeding programs is restricted due to the difficulties of maintaining the pollen sterile lines, as restorer genes have not been identified yet. Functional male sterility is characterized by a normal development of viable pollen that is not released for natural pollination due to abnormal morphology and functioning of the anthers (Georgiev 1991). One of the best characterized functional male sterility genes in tomato is *positional sterility 2* (*ps-2*), originating from a spontaneous mutation in the Czech tomato cultivar "Vrbicanske nizke", which is characterized by non-dehiscent anther bags (Atanassova 1999). Its low level of self-pollination and the possible use of the viable pollen by manual anther opening render the *ps-2* gene the most suitable male sterility gene for tomato hybrid seed production. At least 19 tomato hybrid cultivars have been developed with the use of male sterility, mainly in Eastern Europe. Seventeen of them were based on the *ps-2* gene (Atanassova 1999).

The molecular mechanism of *ps-2* in tomato is unknown, but similar mutant phenotypes have been observed and studied mainly in *Arabidopsis*, such as the mutant *myb26* (Steiner-Lange et al. 2003), the mutants *coi1* (Feys et al. 1994; Xie et al. 1998) and *dad1* (Ishiguro et al. 2001), the double mutants *opr3*/*dde1* (Sanders et al. 2000; Stintzi and Browse 2000) and *aos*/*dde2-2* (von Malek et al. 2002; Park et al. 2002) and

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the triple mutant *fad3/fad7/fad8* (McConn and Browse 1996). They are characterized by defects in filament elongation, timing of anther dehiscence and often show reduced pollen viability. All, except the mutant *myb26*, are in one way or another involved in the jasmonic acid pathway. The mutant *coi1* (Xie et al. 1998) is insensitive to jasmonic acid, while the others are defective in jasmonic acid synthesis (Feys et al. 1994; Ishiguro et al. 2001; McConn and Browse 1996; Park et al. 2002; Sanders et al. 2000; Stintzi and Browse 2000; von Malek et al. 2002).

Atanassova (1999) observed up to 26% self-pollination in the most extreme cases, on cultivar “Vrbicanske nizke” (*ps-2/ps-2*) under field conditions. In contrast, no self-pollination was found in a *ps-2* advanced breeding line (*ps-2*ABL) bred for this trait (data not shown), which means that the problem of self-pollination in *ps-2/ps-2* lines can be overcome by breeding selection. Because the expression of the *ps-2* gene depends on the genetic background, the availability of molecular markers closely linked to this gene is essential for breeding purposes.

Previously, Atanassova (1991) demonstrated a close linkage between the recessive gene *ps-2* and the *ful* gene on the short arm of tomato Chromosome 4. However, this *ful* gene cannot be used as a marker in practical breeding programs. The availability of molecular markers closely linked to the *ps-2* gene is relevant for functional studies as well as for breeding purposes. The marker-assisted introduction of the *ps-2* allele in parent lines would greatly enhance the use of this trait for the production of modern hybrid seeds.

In order to accurately map the *ps-2* gene on the tomato genome, we generated an interspecific F_2 population originating from a cross between a *ps-2* ABL (*S. lycopersicum*) and *S. pimpinellifolium* as male fertile parent. Here we report the high-resolution fine mapping of the *ps-2* gene in a window of 1.65 cM on tomato Chromosome 4. The syntenic relationship between tomato and *Arabidopsis*, at the *ps-2* locus region, was determined and the knowledge of functional male sterility genes in *Arabidopsis* was used to search for candidate gene for *ps-2*. The possibility of map-based cloning of the *ps-2* gene is discussed.

Materials and methods

Plant material

We developed an F_2 mapping population from a single cross between the tomato (*S. lycopersicum*) true breeding line *ps-2*ABL and *S. pimpinellifolium* accession GI.1554. The *ps-2*ABL is homozygous for the *ps-2* mutation. *S. pimpinellifolium* accession GI.1554 is a close wild relative of *S. lycopersicum* and homozygous for *ps-2/ps-2* and hence functionally male fertile. The obtained F_2 population, composed of about 3,070 plants, was used for the high-resolution fine mapping of the *ps-2* locus. Plants were grown on rock wool, under controlled environment, in greenhouses in The Netherlands.

Another 176 ABLs, among which seven were *ps-2/ps-2*, were used to test the association between microsatellite markers and the *ps-2* locus.

A population of 98 recombinant inbred lines (RILs) generated by four generations of single seed descent (F_6), originating from the cross between *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium* accession GI.1554, was used to map AFLP markers on the tomato genome. This population has previously been generated and used as mapping population. A genetic linkage map composed of 323 AFLP markers and 69 RFLP markers was already available with this population (Finkers and van Heusden 2002).

Genomic DNA extraction and molecular markers development

Total DNA was isolated by two different methods, following the NaOH extraction protocol (Wang et al. 1993) or the CTAB extraction protocol (Steward and Via 1993):

The NaOH DNA extraction was performed essentially as described by Wang et al. (1993). The protocol was slightly modified and up-scaled to a 96 wells COSTAR plate format: only one plastic ball was placed in each COSTAR tube (1 ml) before collecting fresh leaf samples of about 0.5 cm^2. NaOH (0.5 N), 20 μl, was added to the leaf material and the samples were crushed using a Retsch and 80 μl of Tris buffer (100 mM) was added after crushing. Five microliters of that mix was transferred to 100 μl of Tris (100 mM). One microliter of this final mix was used for PCR amplification.

The CTAB DNA extraction protocol was performed mainly as described by Steward and Via (1993) up-scaled to a 96 wells COSTAR plate format. Leaf samples were crushed using a Retsch.

AFLP products were prepared essentially as described by Vos et al. (1995). *Eco*RI and *Pst*I primers were labeled with IRD700 or IRD800. AFLP fragments were separated on LI-COR 4200 DNA sequencer essentially as described by Myburg and Remington (2000). AFLP markers were named following the nomenclature presented by Haanstra et al.

Table 1 Details on AFLP adaptors and primers giving linked AFLP markers to the *ps-2* locus

Primer and adaptor name	Use	Primer or adaptor sequence
*Mse*I adaptor	Adaptor	GAC GAT GAG TCC TGA G TA CTC AGG ACT CAT
M00	Universal primer	GAT GAG TCC TGA GTA A
M02	1 selective base	M00 + C
M47	3 selective bases	M00 + CAA
M51	3 selective bases	M00 + CCA
M52	3 selective bases	M00 + CCC
M54	3 selective bases	M00 + CCT
M60	3 selective bases	M00 + CTC
*Eco*RI adaptor	Adaptor	CTC GTA GAC TGC GTA CC CTG ACG CAT GGT TAA
E00	Universal primer	GAC TGC GTA CCA ATT C
E01	1 selective base	E00 + A
E32	3 selective bases	E00 + AAC
E33	3 selective bases	E00 + AAG
E36	3 selective bases	E00 + ACC
*Pst*I adaptor	Adaptor	CTC GTA GAC TGC GTA CAT GCA CAT CTG ACG CAT GT
P00	Universal primer	GAC TGC GTA CAT GCA G
P13	2 selective bases	P00 + AG
P15	2 selective bases	P00 + CA
P19	2 selective bases	P00 + GA

(1999). Positive AFLP primer combinations detected by BSA are described in Table 1.

To perform a bulked segregant analysis (BSA, Michelmore et al. 1991), two pools of ten functionally male sterile plants and two pools of ten functionally male fertile plants were made for AFLP screening by mixing equal amounts of AFLP pre-amplification products of the individual plants. After identification, informative AFLP markers were tested on 50 functionally male sterile F_2 (*ps-2/ps-2*) plants in order to determine the genetic distance between the identified AFLP markers and the *ps-2* locus.

To develop locus-specific PCR markers, primers were designed based on the RFLP sequences publicly available in the SGN database (Mueller et al. 2005) and amplification products were generated from genomic DNA of the two parent lines. None of the PCR amplification products showed length polymorphism between *ps-2*ABL and *S. pimpinellifolium* GI.1554. Therefore, the PCR fragments of both parents were sequenced (BaseClear BV, The Netherlands). When polymorphisms were found between the sequences of PCR products of the two parental lines, appropriate restriction enzymes were selected to develop CAPS markers (Table 2). When no appropriate restriction enzyme was found, dCAPS markers were developed if possible (Neff et al. 2002). The sequences of the PCR products of *ps-2*ABL were compared to the marker sequences of the SGN database in order to validate the primer specificity, using the SGN web BLAST interface (Mueller et al. 2005) (http://sgn.cornell.edu/tools/blast/simple.pl).

In order to increase the rate of polymorphisms between the two parental lines, primers of converted COS markers were specifically designed to amplify putative intron regions. The putative intron regions were detected with the "intron finder tool" (http://sgn.cornell.edu/tools/intron_detection/find_introns.pl; Mueller et al. 2005).

PCR mix for CAPS and dCAPS markers was prepared as follows: per reaction about 100 ng of DNA was mixed, in a total volume of 15 µl, with 10 ng of each primer, 1 µl of dNTPs (5 mM), 0.5 U *Taq* polymerase (SuperTaq, Enzyme Technologies Ltd, UK) and 1 × superTaq PCR reaction buffer. The PCR reaction started with a hot step at 94°C for 5 min, followed by 39 cycles of 30 s at 94°C, 30 s at appropriate annealing temperature (Table 2) and 30 s or 1 min (depending on the expected PCR product size) at 72°C. The PCR reaction ended with a final extension of 7 min at 72°C. About 3 µl of the amplified product was digested for 3 h with 1–2 U of the appropriate restriction enzyme (Table 2) and appropriate restriction buffer, in a total volume of 15 µl. DNA fragments of CAPS and dCAPS markers were separated on a 1.5 and 2% agarose gel, respectively, stained with ethidium bromide and visualized by UV light.

The PCR mix for microsatellite markers was prepared in a total volume of 10 µl per reaction, by mixing 50 ng of DNA with 1 ng of each primer, 1 µl of dNTPs (5 mM), 0.5 U *Taq* polymerase (SuperTaq, Enzyme Technologies Ltd) and 1 × superTaq PCR reaction buffer. PCR profiles were as described by Areshchenkova and Ganal (1999, 2002), Suliman-Pollatschek et al.

Table 2 PCR markers used in the genetic linkage maps

Marker name	Use	Origin	Primer sequence	Annealing *T* (°C)	Size (bp)	Restriction enzyme
Primers and enzymes used on *ps-2* mapping sub-population						
TG483	CAPS	RFLP (SGN database)	Fw: CACTCCCATGGCAGATAAAA Rv: AGTGAAGTAAAACAAAGCCAAAAT	59	334	*HPY*CH4IV
TG339	CAPS	RFLP (SGN database)	Fw: AACATAGTAGCGTAATCCCACAGT Rv: ATTTATTTTCACGAAGCAAGTAGT	50	360	*Hin*fI
CT175	CAPS	RFLP (SGN database)	Fw: CAGCTAAGCGTTGACAGTTGAGAA Rv: ATGGCCGCGGTTTGAGC	54	339	*Alu*I
CT192	CAPS	RFLP (SGN database)	Fw: AGGGTCCTGTTGTCACTGTC Rv: CAATTGCCATCTCACCTAAA	55	200	*Hpy*188I
TG609	dCAPS	RFLP (SGN database)	Fw: GATATGACTAGGAGGCAATGACTGA Rv: TGATAGTCAAAGATCACAGACATTTAGATT	54	200	*Hin*fI
TMS26	SSR	Areshchenkova and Ganal (1999)	Fw: TTCGGTTTATTCTGCCAACC Rv: GCCTGTAGGATTTTCGCCTA	55	270	
TOM316	SSR	Suliman-Pollatschek et al. (2002)	Fw: GAGTTGTTCTTTGGTTGTTT Rv: TAGATTTTTCGTGTAGATGT	46	215	
SSR94	SSR	SSR (SGN database)	Fw: AATCAGATCCTTGCCCTTGA Rv: AGCTGAGAAAGAGCAGCCAT	55	187	
TMS22	SSR	Areshchenkova and Ganal (1999)	Fw: TGTTGGTTGGAG AAACTCCC Rv: AGGCATTTAAACCAATAGGTAGC	55	160	
EST259379	SSR	Areshchenkova and Ganal (1999)	Fw: TTGGTCTCCCTCTTTATGCC Rv: GGCTTCATTGATGAACCCAT	55	150	
SSR450	SSR	SSR (SGN database)	Fw: AATGAAGAACCATTCCGCAC Rv: ACATGAGCCCAATGAACCTC	58	270	
TOM160	SSR	Suliman-Pollatschek et al. (2002)	Fw: TGCTGAAGAATACAATGTTACC Rv: ATTGTTGGATGCTCAGTTTG	48	210	
TOM268	SSR	Suliman-Pollatschek et al. (2002)	Fw: AGGGTATGAGATGAGACAAT Rv: TTTTACCTTCTTTACTTGGA	48	195	
Primers and enzymes used on recombinant sub-population						
TG182	dCAPS	RFLP (SGN database)	Fw: CGAACTGATCCTAATGCCCTGGTA Rv: CAGTTAAGAGAAGAACTGTCACTCA	55	200	*Dde*I
T1070	dCAPS	COS (SGN database)	Fw: AATGGAGTTTCCAGTTGTAGA Rv: TGAACACAAAGACGATACCA	55	160	*Acc*I
T0958	dCAPS	COS (SGN database)	Fw: GTGTCGAACCCTTGGCAACAAT Rv: AGTAAACTGTAGCTGACATTGGG	54	200	*Hph*I
T0953	CAPS	COS (SGN database)	Fw: AAGTTCTTCACAATGAAACTTAC Rv: ACTTTCATTAATGGTCCTTAGGTC	55	350	*Sec*I
T0891	CAPS	COS (SGN database)	Fw: GACCGCTACCTCAACTTCT Rv: CACTCTAATACTCCACTCAACATA	55	1200	*Ssi*I
cLED-7-G23	CAPS	COS (SGN database)	Fw: GGATGAAGTTAGGGATCGTGTTA Rv: GCACACTGTATAAATCCATAGGT	55	250	*Mse*I
T0635	dCAPS	COS (SGN database)	Fw: AATCCGAAAGTTTTGCCTACAG Rv: CAAGTGTGTCTGCAACTGGCTT	55	200	*Mse*I

(2002) and the SGN database (Mueller et al. 2005). Reverse primers were labeled with IRD700 or IRD800. PCR products were run on a LI-COR 4200 DNA sequencer.

Evaluation of functional male sterility and map construction

Two types of phenotypic observation were performed to evaluate functional male sterility in the F_2 segregating population: (1) the ability of the anthers to release pollen; (2) the presence of seeded fruits:

To evaluate the ability of the anthers to release pollen, several flowers were collected per plant. Flowers were collected at anthesis and pollen release was tested by gently shaking the anther cones manually. Anthers classified as functionally male fertile could easily release pollen while the functionally male sterile anthers remained closed.

When the presence or absence of seeded fruits was used as phenotypic observation, plants with less than 10% of flowers setting seeded fruits were considered as functionally male sterile while plants with more than 50% of flowers setting seeded fruits were scored as functionally male fertile. Plants with an intermediate phenotype, between 10 and 50% of flowers setting seeded fruits, were considered unknown.

We used JoinMap 3.0 (van Ooijen and Voorrips 2001) to generate the genetic linkage maps presented in this study, applying the Kosambi mapping function.

Microsynteny between tomato and *Arabidopsis* and candidate gene analysis

All COS marker sequences mapped in this study were scanned against the *Arabidopsis* genome sequence and *Arabidopsis* transcripts sequences from TAIR (http://www.Arabidopsis.org; Huala et al. 2001) using TBLASTX interface, in order to identify orthologs in *Arabidopsis*. The significance thresholds were identical to the ones set by Fulton et al. (2002). Positions and order of these orthologous genes in *Arabidopsis* were subsequently compared to the positions of the functional sterility genes identified in *Arabidopsis*.

Results

To perform the fine mapping of the *ps-2* locus, we developed an F_2 segregating population from the cross between *ps-2*ABL (*S. lycopersicum*) and accession GI.1554 (*S. pimpinellifolium*). *S. pimpinellifolium* was used as wild-type donor because the level of DNA polymorphism may be substantially higher in this interspecific mapping population, which is essential for the development of molecular markers. In addition, we preferred to use *S. pimpinellifolium* as a close relative of the cultivated tomato as this enabled us to work with a segregating population with vigorous and fertile progeny plants in order to maximize the accuracy of the phenotyping in the segregating population. The experimental approach followed in this study is presented in Fig. 1.

Identification of AFLP markers by BSA

The BSA subpopulation was used to identify AFLP markers closely linked to the *ps-2* locus. This subpopulation was composed of 530 F_2 plants. DNA of these F_2 plants was extracted according to the CTAB protocol. Functional male sterility was evaluated on these plants by testing the ability of the anthers to release pollen. Out of the 530 F_2 plants, 85 were clearly characterized as functionally male sterile, representing 16% of the total population, which is significantly lower than the expected 25% of the Mendelian ratio ($X^2 = 22.7$). This low percentage is likely to be due to a skewed segregation in the direction of *S. pimpinellifolium* alleles.

Two bulks of ten functionally male sterile plants and two bulks of ten functionally male fertile plants were formed and 220 AFLP primer combinations were tested in a BSA approach. The use of two times two bulks reduced the chance to find false positive markers. The AFLP primer combinations used in the BSA were *Pst*I/*Mse*I and *Eco*RI/*Mse*I. Because the functional male sterility is a recessive trait, a BSA approach only allowed us to find markers in repulsion of the *ps*-2 allele. Therefore the identified AFLP bands linked to the *ps-2* locus were specific to *S. pimpinellifolium* accession GI.1554. On average we observed eight AFLP bands specific to *S. pimpinellifolium* per *Eco*RI/*Mse*I primer combination and four bands per *Pst*I/*Mse*I primer combination.

Ten positive AFLP markers present in the functionally male fertile bulks and absent in the functionally male sterile bulks were identified and tested on 50 functionally male sterile plants of the BSA subpopulation. Three AFLP markers showed more than three recombinations on the 50 tested plants and were therefore not used in further analysis. The seven remaining AFLP markers, considered closely linked to the *ps-2* locus, were: P19M54-90, P19M51-185, E32M60-180, E36M47-240, E33M51-240, P15M52-270/272 and P13M47-156. The final number indicated after each primer combination represents the size of the AFLP fragment. AFLP

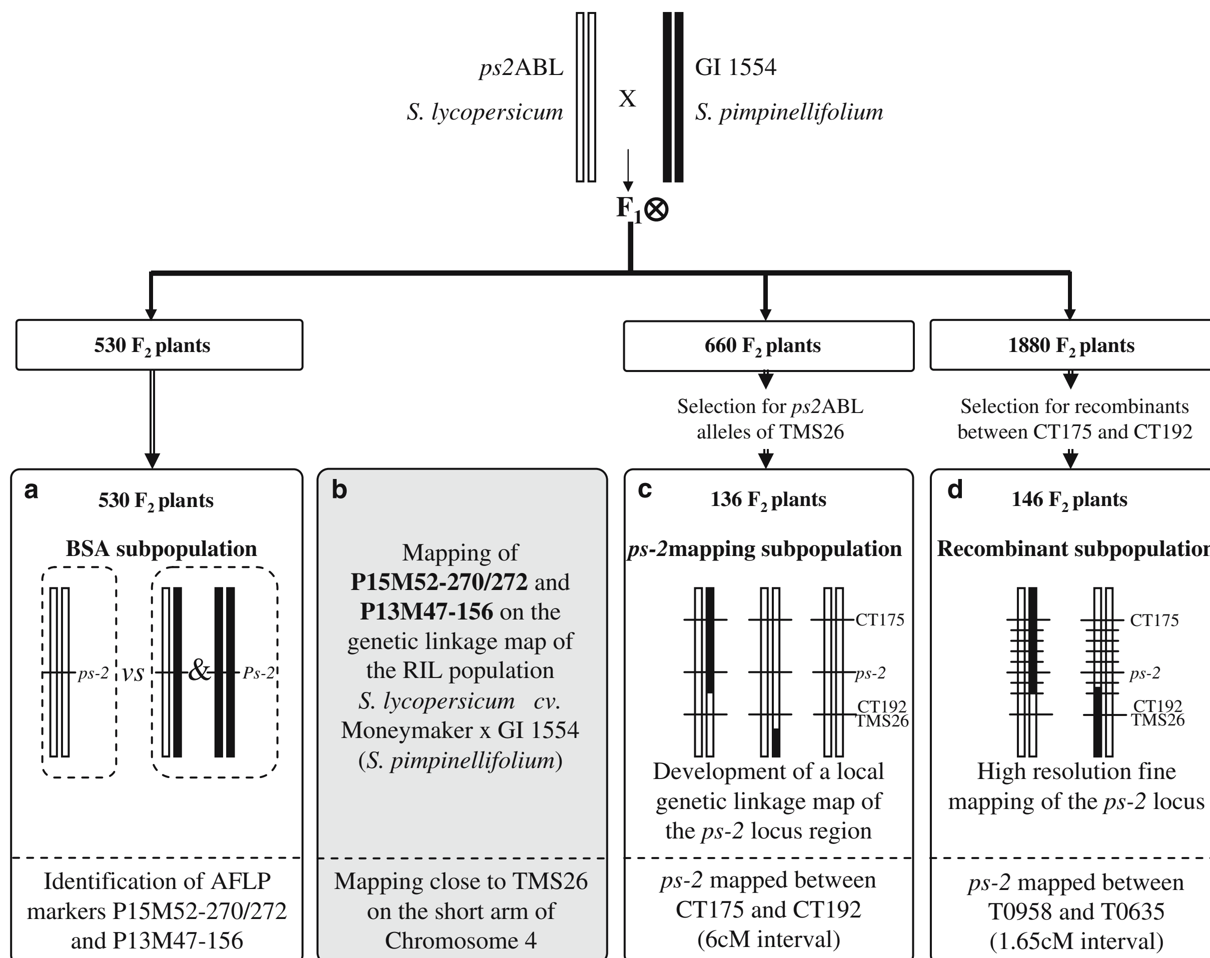

Fig. 1 Representation of the populations and subpopulations used to fine map the *ps-2* gene (see also text). F_2 plants originate from the cross between *ps-2*ABL, functionally male sterile (*S. lycopersicum*) and accession GI.1554 (*S. pimpinellifolium*). F_2 plants are divided into three subpopulations: the BSA subpopulation, the *ps-2* mapping subpopulation and the recombinant subpopulation. The fine mapping procedure was as follows: **a** the BSA subpopulation was used for the identification of AFLP markers closely linked to the *ps-2* locus; **b** these AFLP markers were mapped close to TMS26 on the short arm of Chromosome 4 in the RIL population *S. lycopersicum cv*. Moneymaker × *S. pimpinellifolium* (accession GI.1554); **c** the *ps-2* mapping subpopulation, obtained by screening F_2 plants for the presence of the *S. lycopersicum* alleles of the TMS26 locus, was used for the construction of a linkage map; **d** the recombinant subpopulation, composed of F_2 plants recombinant between CT175 and CT192, was used for high-resolution mapping

marker P15M52-270/272 was a co-dominant marker in which the fragments of *S. lycopersicum* and *S. pimpinellifolium* were, respectively, 270 and 272 bp long.

Mapping of P15M52-270/272 and P13M47-156 in the RIL population

In order to determine the genetic position of the *ps-2* locus in the tomato genome, two of the seven AFLP markers detected by BSA were mapped in the RIL population. A genetic linkage map mainly based on AFLP markers has been developed on this later population and the chromosome numbers and orientation of the chromosomes have been assigned. Both markers, P15M52-270/272 and P13M47-156, co-segregated and mapped on the short arm of Chromosome 4 between TG483 and the putative centromere (Fig. 2a). TG483 was at a distance of 20 cM from the putative centromere in the RIL linkage map. The position of the putative centromere was suggested by the clustering of the *Eco*RI/*Mse*I AFLP markers (Haanstra et al. 1999) on the RIL genetic linkage map (Fig. 2a).

Linkage map of the *ps-2* locus region

In order to accurately generate a local genetic linkage map of the *ps-2* locus region, we developed the *ps-2* mapping subpopulation, enriched for functionally male sterile plants. For this purpose, about 660 F_2 plants were screened at an early plant stage with the

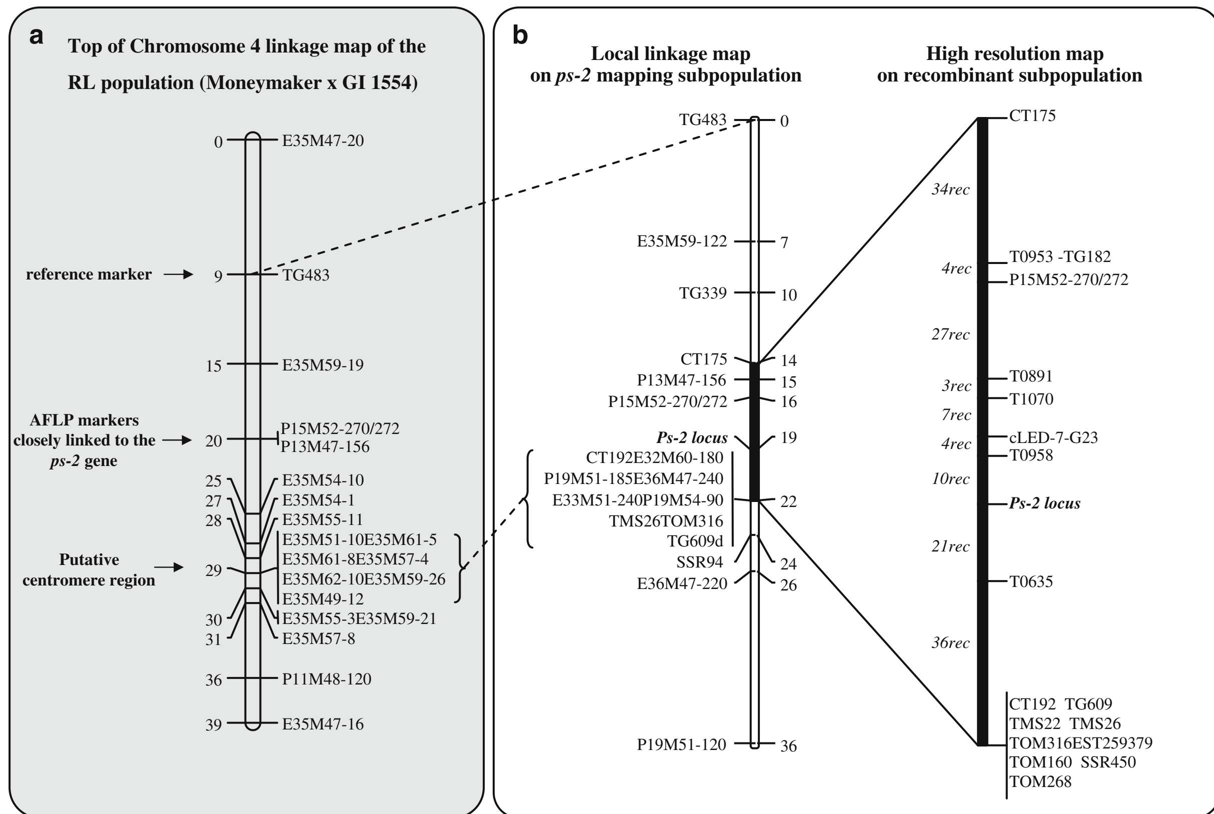

Fig. 2 **a** Genetic linkage map of the top of Chromosome 4 in the RIL population (Moneymaker [*S. lycopersicum*] × GI.1554 [*S. pimpinellifolium*]). AFLP markers P15M52-270/272 and P13M47-156 were mapped on this linkage map (LOD > 8). The other AFLP markers of this linkage map do not follow the same nomenclature as presented in Materials and Methods. *Numbers* along the linkage map indicate the position of the markers, in cM, relative to E35M47-20. **b** On the *left*, the local linkage map of the *ps-2* region developed on the *ps-2* mapping subpopulation (LOD > 6). The clustering of AFLP and microsatellite markers corresponds to the putative centromere of Chromosome 4. *Numbers* along the local linkage map indicate the positions of the markers, in cM, relative to TG483. On the *right*, the high-resolution map of the *ps-2* region developed on the recombinant subpopulation, delimited by CT175 and CT192. *Numbers* along the map indicate the number of recombinant plants identified in the respective intervals

microsatellite marker TMS26, located in the centromeric region of Chromosome 4 (Areshchenkova and Ganal 1999), which is at a distance of 9 cM to P15M52-270/272 and P13M47-156 on the RIL genetic linkage map (Fig. 2a). DNA of these F_2 plants was extracted following the CTAB protocol. We used the microsatellite marker TMS26 to perform the selection for the *ps-2* mapping subpopulation, rather than P15M52-270/272 or P13M47-156, because simple co-dominant PCR markers are more accurate and easier to use than AFLP markers.

A total of 136 plants were homozygous for the *ps-2*ABL allele of TMS26. These plants were maintained in the greenhouse and allowed to grow further. Ten F_2 plants carrying at least one allele of *S. pimpinellifolium*, ten plants of both parents and of the F_1 were included as controls. The selected plants were scored for functional male sterility. These selected plants are hereafter denoted as the *ps-2* mapping subpopulation.

By selecting for the *ps-2*ABL allele of TMS26, we enriched the F_2 population with functionally male sterile plants and discarded most of the homozygous *ps-2/ps-2* F_2 plants. We could therefore perform a more efficient phenotyping because the functionally male fertile plants, among the selected F_2 plants, were in most of the cases heterozygous at the *ps-2* locus.

Out of the 136 plants homozygous for the *ps-2*ABL allele of TMS26, 117 could be clearly phenotyped: The phenotype was based on the presence/absence of seeded fruits on the plants: 103 F_2 plants were scored as functionally male sterile and 14 as functionally male fertile. Nineteen plants had an intermediate phenotype and were therefore discarded for further analysis. To accurately map the *ps-2* locus, we developed a local genetic linkage map in the *ps-2* mapping subpopulation, with AFLP, RFLP-derived CAPS markers and microsatellite markers according to the following approach: the seven linked AFLP markers detected by

BSA were mapped on the *ps-2* mapping subpopulation. Six of the seven AFLP markers were dominant and one marker, P15M52-270/272, was co-dominant. The six dominant AFLP markers were specific to *S. pimpinellifolium* accession GI.1554. However, with the TMS26 selection performed to develop the *ps-2* mapping population, most plants were homozygous for the *S. lycopersicum* alleles in this region and, consequently, only few plants showed the presence of *S. pimpinellifolium* alleles near TMS26. Therefore, any *S. pimpinellifolium* allele in the *ps-2* plants reflected a recombination between this marker and TMS26. This greatly enhanced the resolution of the mapping of the AFLP markers as well as the *ps-2* locus.

Microsatellite markers known to be located in or distal to the centromere of Chromosome 4 were tested for polymorphisms between the *ps-2*ABL and the wild-type *S. pimpinellifolium* parent (GI.1554). In total 15 microsatellite markers from Areshchenkova and Ganal (1999, 2002), Suliman-Pollatschek et al. (2002) and the SGN database were tested. Thirteen of them resulted in an amplification product on both parents. Four were not polymorphic between *ps-2*ABL and accession GI.1554: SSR603, Tom89, Tom95 and Tom332. One marker, SSR86, did not map in the *ps-2* locus region. In total eight microsatellite markers were mapped in the *ps-2* mapping subpopulation: EST259379, TMS22, TMS26, SSR94, Tom316, SSR450, Tom160 and Tom268. Seven of them co-segregated in the putative centromere region and one, SSR94, mapped 2 cM distal to the putative centromere on the long arm of Chromosome 4 (Fig. 2b).

In addition, some RFLP markers located on the RFLP map of Tanksley et al. (1992) between TG483 and the centromere of Chromosome 4 were converted into PCR markers. The RFLP markers TG483, TG339, CT175, CT192 and TG609 were successfully converted into CAPS or dCAPS markers and mapped on the *ps-2* mapping subpopulation. The position of these markers near the *ps-2* locus was relatively well conserved in comparison to the RFLP map of Tanksley et al. (1992) except for CT192, which mapped in the centromeric region of Chromosome 4 in the *ps-2* mapping subpopulation, though it has been mapped at 5 cM above the centromere in the RFLP map of Tanksley et al. (1992).

Eventually a local genetic linkage map was generated, with the *ps-2* gene mapped between the AFLP marker P15M52-270/272 and the cluster of markers (one genetic locus) corresponding to the centromere, in a window of 6 cM (Fig. 2b).

To develop more markers closer to the *ps-2* locus we performed another BSA with *Pst*I/*Mse*I AFLP primer combinations. Contrasting bulks from the *ps-2* mapping subpopulation were constructed for the region between P15M52-270/272 and CT192 in such a way that only AFLP markers located between P15M52-270/272 and CT192 could be identified (data not shown). More than 180 AFLP primer combinations were tested, but no positive marker was found.

High-resolution mapping of *ps-2* in a recombinant subpopulation

As we wanted to increase the genetic resolution of the *ps-2* locus, more than 1,880 F_2 plants were screened at the early plant stage for a recombination event between CT175 and CT192. To save time, DNA of these 1,880 F_2 plants was extracted following the NaOH protocol. CT175 and CT192 were used for the recombinant screening because they were the closest flanking PCR markers and could easily be scored on agarose gels. None of the microsatellite markers in the centromere of Chromosome 4 could be scored on agarose gels. In total, 146 F_2 plants were selected. DNA of those 146 F_2 plants was extracted following the CTAB protocol in order to obtain stable high-quality DNA. The selected recombinant plants were homozygous *S. lycopersicum* for either CT175 or CT192 while heterozygous for the other marker. In this way all recombinants were informative for the *ps-2* locus.

The recombinant subpopulation was evaluated for functional male sterility based on the ability of the anthers to release pollen and based on the presence/absence of seeded fruits. In a first scoring, 54 plants were scored as functionally male sterile, 79 as functionally male fertile and 13 as ambiguous. These 13 last plants were maintained by cuttings and evaluated a second time. Nine were functionally male sterile, three were functionally male fertile and one remained ambiguous. The combination of both evaluations, ability of the anthers to release pollen and presence of seeded fruits, improved the accuracy of the scoring of these plants.

To develop a high-resolution linkage map, CT175, CT192, TG609, P15M52-270/272 and the microsatellite markers used for the *ps-2* mapping subpopulation, EST259379, TMS22, TMS26, SSR94, Tom316, SSR450, Tom160 and Tom268, were screened on the recombinant subpopulation. In addition, we intended to convert every COS markers (Fulton et al. 2002) located between TG182 and T0635 on the Tomato-Expen 2000 map (SGN database) into CAPS or dCAPS markers. We successfully converted six out of eight COS markers: T0953, T0891, T1070, cLED-7-G23, T0958 and T0635. No polymorphism was found for T1571 and T0964. The RFLP marker TG182 was also converted

into a dCAPS marker. Polymorphisms between the parents were generally found in the intron regions, and it was often necessary to sequence several introns to find a polymorphism. The newly developed CAPS and dCAPS markers were mapped on the recombinant subpopulation. Eventually, we developed a high-resolution map of the *ps-2* locus region, in which distances between loci are indicated in terms of the number of recombinant plants (Fig. 2b).

The order of the COS-derived CAPS markers of the recombinant subpopulation was not entirely similar to the order in the Tomato-Expen 2000 map. Eventually the *ps-2* locus mapped in a 1.65 cM window delimited by T0958 and T0635 at about 0.53 cM (10 recombinants) and 1.12 cM (21 recombinants), respectively.

Microsynteny between tomato and *Arabidopsis* at the *ps-2* locus region

The presence of six COS markers in the high-resolution map developed in this study allowed us to analyze the syntenic relationship between tomato and *Arabidopsis* at the *ps-2* locus region. Orthologs of the tomato COS markers, in *Arabidopsis*, and their corresponding bacterial artificial chromosome (BAC) clones were identified using a TBLASTX interface. Five of the six COS markers had only one significant *Arabidopsis* BAC clone match. COS marker T0958 matched to two unrelated *Arabidopsis* BAC clones (Table 3). When compared to the *Arabidopsis* gene database, only one corresponding ortholog was identified in *Arabidopsis*, for all six markers (Table 3). Four of the six identified orthologs were located on Chromosome 5 of *Arabidopsis*. Among them, the orthologs of the two closest markers flanking the *ps-2* locus, T0958 and T0635, were separated by 1.6 megabases (mb).

The positions of the functional male sterility genes in *Arabidopsis* were determined (Table 4) and compared to the position of the six *Arabidopsis* orthologs previously identified. Two of these candidate genes are located on Chromosome 5 of *Arabidopsis* (Fig. 3). Among them, *fad8* is at a distance of 1.2 mb from the ortholog of T0958.

Association of the microsatellite markers with the *ps-2* allele in a population of advanced breeding lines

Microsatellite markers have a relatively high level of polymorphism among cultivated tomato lines (Vosman et al. 1992). Therefore in order to test whether the eight microsatellite markers, located near the *ps-2* locus (Fig. 2b), could be used for molecular-assisted selection (MAS) of the *ps-2* allele, we studied the association of alleles of those microsatellite markers with the *ps-2* allele on a collection of ABLs. Among those ABLs, the functionally male sterile lines (including the

Table 3 Sequence similarities between tomato COS markers and *Arabidopsis* BAC clones and genes

Tomato	*Arabidopsis* BAC clones					*Arabidopsis* genes			
	Accession no.	Chr.	AGI coordinates (bases)	TBLASTX		Accession no.	AGI coordinates (bases)	TBLASTX	
Marker				*E* value	Score			*E* value	Score
T0953	AB017069.1	5	14748652–14846779	3E-78	144	BT008856	14829915–14833750	3E-96	357
T0891	AB016886.1	5	19339994–19432496	8E-83	196	AY091394	19365145–19367895	2E-88	322
T1070	AC013483.5	3	2448977–2570030	7E-30	104	BT015904	2552483–2554927	7E-35	144
cLED	AC004512	1	24237602–24324793	5E-41	122	BT012855	24252515–24254065	3E-50	188
T0958	AB010077.1	5	15964645–16052024	3E-38	158				
	AB006697	5	2732479–2813448	2E-34	69	AY128719	2806324–2813227	1E-56	217
T0635	AB006704.1	5	4356798–4446056	1E-50	198	AY078971	4387337–4392230	3E-51	198

Table 4 Position of the functional male sterility genes in *Arabidopsis*

Gene name	Accession no.	Reference	Chr.	AGI coordinates (bases)
MYB26	Z95749	Steiner-Lange et al. (2003)	3	4576751–4578034
COI-1	AF036340	Xie et al. (1998)	2	16679040–16682826
DAD-1	AB060156	Ishiguro et al. (2001)	2	18486128–18487268
OPR3	AF132212	Sanders et al. (2000), Stintzi and Browse (2000)	2	2359111–2362176
AOS	AB007647	von Malek et al. (2002), Park et al. (2002)	5	17114823–17116623
FAD3	L22931	McConn and Browse (1996)	2	12788668–12792129
FAD7	AC073395	McConn and Browse (1996)	3	3499807–3502458
FAD8	AF056565	McConn and Browse (1996)	5	1664148–1666891

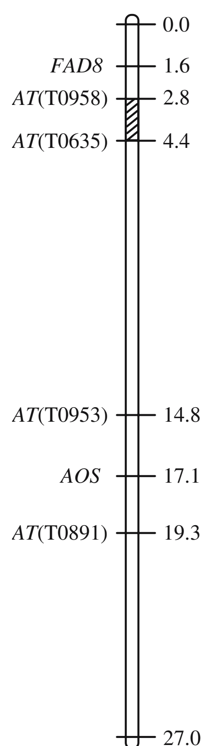

Fig. 3 *Arabidopsis* Chromosome 5 represented with the orthologs of the tomato markers T0958, T0635, T0953 and T0891 and the functional male sterility genes of *Arabidopsis* identified on that chromosome. *Numbers* along the map indicate the position of the genes in megabases (mb) relative to the top of the chromosome. Orthologs of the tomato markers are indicated by "*AT*" followed by the name of the marker within *parentheses*

*ps-2*ABL used to develop the mapping population in this study) were all developed from the same donor line with the *ps-2* trait. Therefore all the lines were most likely in Hardy Weinberg disequilibrium and the chance to identify association between the microsatellite markers and the *ps-2* allele was rather high.

The eight microsatellite markers present in the *ps-2* local map were screened on a subset of eight randomly selected ABLs (*S. lycopersicum*): four functionally male sterile lines and four male fertile lines. Two out of eight microsatellite markers, TOM316 and TOM268, showed polymorphism among this subset of ABLs. These two microsatellite markers were then screened on a collection of 176 ABLs, in which seven were functionally male sterile. Eight different alleles were identified with TOM268 and four alleles with TOM316 over the set of ABLs. We did not identify one common allele that discriminated the seven functionally male sterile lines from the non-male sterile plants. In conclusion, no association was found between the microsatellite marker alleles of the centromere of Chromosome 4 and the *ps-2* allele.

Discussion

In this study we developed a high-resolution linkage map of the *ps-2* locus region and localized the *ps-2* gene in a window of 1.65 cM on the short arm of Chromosome 4, between T0958 and T0635. Only 1.65 cM separates the two flanking markers T0958 and T0635 in the present study, though these two markers are at a distance of 4 cM in the Tomato-Expen 2000 map (Fulton et al. 2002). The order of the COS markers was rather in accordance with the Tomato-Expen 2000 map, except for T0958. The present map was built with a resolution of 1,880 F_2 plants. By contrast, the Tomato-Expen 2000 map has been generated using only 80 F_2 plants, which may explain the differences in positions of some markers within a small genetic interval. In addition, the use of a different wild species, in the present study, can explain the differences of genetic distances.

The accuracy of the phenotyping was the most important obstacle for the high-resolution fine mapping of the *ps-2* locus. Though the expression of the *ps-2* gene in an *S. lycopersicum* genetic background can prohibit completely the dehiscence of the anthers and therefore the development of seeded fruits (data not shown), it was rather common, in this experiment, to observe seeded fruits on homozygous *ps-2/ps-2* F_2 plants. The *S. pimpinellifolium* genetic background may be the reason for the incomplete positional sterility observed in those cases. Influence of the genetic background for the expression of *positional sterility 2* has already been observed by Atanassova (1999) within *S. lycopersicum*, where up to 26% self-pollination was observed in the most extreme cases, on cultivar "Vrbicanske nizke" (*ps-2/ps-2*) under field conditions during a hot summer. The most accurate phenotyping was performed on the recombinant subpopulation, by combining the observation of the anther appearance and its ability to release pollen, and the presence/absence of seeded fruits.

We chose *S. pimpinellifolium* as a wild-type donor in order to still have a good phenotypic expression of the trait in the segregating population. However, this has cost us significant extra work in comparison to other wild relatives such as *S. pennellii*, for the development of markers polymorphic in the segregating F_2 population due to the rather low level of DNA polymorphism between *S. lycopersicum* and *S. pimpinellifolium*, as found by Chen and Foolad (1999). This problem was first encountered with the AFLP-BSA, where the amount of absence/presence polymorphism of AFLP bands between the two parents was rather low, 14 polymorphic bands per primer combination of *Eco*RI/*Mse*I, including the bands specific to *ps-2*ABL and the bands specific to *S. pimpinellifolium*, and eight polymorphic bands per *Pst*I/*Mse*I primer combination. In comparison, an average of 42 polymorphic bands was identified per *Eco*RI/*Mse*I primer combination and

27 per *Pst*I/*Mse*I primer combination, between *S. lycopersicum* cv. Allround and *S. pennellii* LA716 (Haanstra et al. 1999). Also for the conversion of COS markers into CAPS or dCAPS markers, many intron sequences had to be compared between the two parents before identifying polymorphism. This low level of DNA polymorphism may explain why we could not identify closer AFLP markers to the *ps-2* gene during the second BSA procedure. In addition, this level of polymorphism will probably be one of the major obstacles during the map-based cloning of the *ps-2* gene, which will require frequent CAPS development.

The selection for *ps-2*ABL (*S. lycopersicum*) alleles of TMS26, to develop the *ps-2* mapping subpopulation, helped us considerably to develop an accurate linkage map composed mainly of dominant AFLP markers specific to *S. pimpinellifolium*. With such a selection, the limitation in the mapping accuracy conferred by dominant markers was overcome.

The syntenic relationship between tomato and *Arabidopsis* at the *ps-2* locus region was investigated thanks to the presence of COS markers in the high-resolution map. No global synteny was found between tomato and *Arabidopsis* over the entire *ps-2* locus region. However, the orthologs of the two closest markers flanking the *ps-2* gene were only separated by 1.6 mb on Chromosome 5 of *Arabidopsis*. The foreseen map-based cloning of the *ps-2* gene will provide us extra sequence information to study this syntenic relationship in depth. Microsynteny between tomato and *Arabidopsis* has already been proven to be of great help for the development of molecular markers in tomato (Ku et al. 2001).

No association was found between two microsatellite markers, TOM316 and TOM268, and *ps-2*, in the 176 ABLs. This finding confirms the need for extremely tight linkage to the locus of interest to get significant association between a marker and the *ps-2* gene, for practical use in applied breeding, as described in maize by Remington et al. (2001). In addition the development of markers for MAS will also be limited by the extremely low level of DNA polymorphism within *S. lycopersicum*. The cloning of the *ps-2* gene will provide us the sequence information necessary for the development of a universal marker to assist the introduction of this gene into breeding lines. In addition the sequence of the *ps-2* gene will give us more insights into the physiology of anther dehiscence in tomato. Similar phenotypes have been observed in some *Arabidopsis* mutants where, in most of the cases, the jasmonic acid pathway was involved (Feys et al. 1994; Ishiguro et al. 2001; von Malek et al. 2002; McConn and Browse 1996; Park et al. 2002; Sanders et al. 2000; Stintzi and Browse 2000; Xie et al. 1998). Once the *ps-2* gene is cloned, it will be interesting to know whether this gene is ortholog to one of those genes already identified in *Arabidopsis*. None of the known candidate genes in *Arabidopsis* is located between the orthologs of T0635 and T0958. However, the gene *fad8* and the ortholog of T0958 were only separated by 1.2 mb, which makes it a possible candidate gene for *ps-2*.

Better insights into the physiological process responsible for the non-anther dehiscence of *ps-2* could also be obtained by testing whether application of jasmonic acid on the mutated flowers would restore the wild phenotype and trigger anther dehiscence, as observed in some *Arabidopsis* mutants.

With the availability of the high-resolution map of the *ps-2* locus, developed on a recombinant subpopulation (this study), a map-based cloning procedure can be initiated, which will lead us to the cloning of the *ps-2* gene. Assuming an even distribution of the recombination events between T0958 and T0635 in the recombinant population and considering an average physical distance of 750 kb/cM (Tanksley et al. 1992), we may have a physical interval of 35 kb between each recombination event. This physical distance may even be smaller as it is usually observed in the genes-rich regions (Peters et al. 2003). Sequences of the COS markers closely linked to the *ps-2* locus in this study have been used as probes for the hybridization of BACs, in the frame of the tomato sequencing project (Mueller et al. 2005). Markers T0891, T1070 and T0635 have successfully matched BAC clones: BAC clones linked to T0635 and T1070 were not part of the same contig indicating that the physical distance separating T1070 and T0635 is larger than an average BAC clone size. Markers T0891 and T1070 are linked to BAC clones of the same contig though these markers are separated by three recombination events in the high-resolution linkage map. This may indicate that the recombination frequency in the region of the *ps-2* locus is high enough to intend to clone the *ps-2* gene by map-based mean.

References

Areshchenkova T, Ganal MW (1999) Long tomato microsatellites are predominantly associated with centromeric regions. Genome 42:536–544

Areshchenkova T, Ganal MW (2002) Comparative analysis of polymorphism and chromosomal location of tomato microsatellite markers isolated from different sources. Theor Appl Genet 104:229–235

Atanassova B (1991) Localization of tomato *ps-2* gene (functional sterility). Rep Tomato Genet Coop 41:12

Atanassova B (1999) Functional male sterility (*ps-2*) in tomato (*Lycopersicon esculentum* Mill.) and its application in breeding and hybrid seed production. Euphytica 107:13–21

Chen FQ, Foolad MR (1999) A molecular linkage map of tomato based on a cross between *Lycopersicon esculentum* and *L. pimpinellifolium* and its comparison with other molecular maps of tomato. Genome 42:94–103

Feys BJF, Benedetti CE, Penfold CN, Turner JG (1994) *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell 6:751–759

Finkers R, van Heusden AW (2002) Express QTL: a way to genetical genomics. In: Proceedings of PAG X, San Diego, p 201

Fulton TM, van der Hoeven R, Eannetta NT, Tanksley SD (2002) Identification, analysis, and utilization of conserved ortholog set markers for comparative genomics in higher plants. Plant Cell 14:1457–1467

Georgiev H (1991) Heterosis in tomato breeding. In: Kalloo G (ed) Genetic improvement of tomato. Monograph on Theor Appl Genet 14. Springer, Berlin Heidelberg New York, pp 83–98

Goldberg RB, Beals TP, Sanders PM (1993) Anther development: basic principles and practical applications. Plant Cell 5:1217–1229

Haanstra JPW, Wye C, Verbakel H, Meijer-Dekens F, van den Berg P, Odinot P, van Heusden AW, Tanksley S, Lindhout P, Peleman J (1999) An integrated high density RFLP-AFLP map based on two *Lycopersicon esculentum* × *L. pennellii* F2 populations. Theor Appl Genet 99:254–271

Huala E, Dickerman AW, Garcia-Hernandez M, Weems D, Reiser L, LaFond F, Hanley D, Kiphart D, Zhuang M, Huang W, Mueller LA, Bhattacharyya D, Bhaya D, Sobral BW, Beavis W, Meinke DW, Town CD, Somerville C, Rhee SY (2001) The *Arabidopsis* Information Resource (TAIR): a comprehensive database and web-based information retrieval, analysis, and visualization system for a model plant. Nucleic Acids Res 29:102–105

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The DEFECTIVE IN ANTHER DEHISCENCE1 gene encodes a novel phospholipase a1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. Plant Cell 13:2191–2209

Ku H-M, Liu J, Doganlar S, Tanksley SD (2001) Exploitation of *Arabidopsis*-tomato synteny to construct a high-resolution map of the ovate-containing region in tomato Chromosome 2. Genome 44:470–475

von Malek B, van der Graaff E, Schneitz K, Keller B (2002) The *Arabidopsis* male-sterile mutant *dde2-2* is defective in the ALLENE OXIDE SYNTHASE gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta 216:187–192

McConn M, Browse J (1996) The critical requirement for linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis* mutant. Plant Cell 8:403–416

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by Bulked Segregant Analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Mueller LA, Solow TH, Taylor N, Skwarecki B, Buels R, Binns J, Lin C, Wright MH, Ahrens R, Wang Y, Herbst EV, Keyder ER, Menda N, Zamir D, Tanksley SD (2005) The SOL genomics network. A comparative resource for *Solanaceae* biology and beyond. Plant Physiol 138:1310–1317

Myburg AA, Remington DL (2000) Protocol for high-throughput AFLP analysis using LI-COR IR2 automated sequencer. NC State Forest Biotech AFLP protocol, September 2000

Neff MM, Turk E, Kalishman M (2002) Web-based primer design for single nucleotide polymorphism analysis. Trends Genet 18:613–615

van Ooijen JW, Voorrips RE (2001) JoinMap 3.0, software for the calculation of genetic linkage maps. Plant Research International, Wageningen

Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J 31:1–12

Peters JL, Cnudde F, Gerats T (2003) Forward genetics and map-based cloning approaches. Trends Plant Sci 8:484–491

Remington DL, Thornsberry JM, Matsuoka Y, Wilson LM, Whitt SR, Doebley J, Kresovich S, Goodman MM, Buckler ES IV (2001) Structure of linkage disequilibrium and phenotypic associations in the maize genome. Proc Natl Acad Sci USA 98:11479–11484

Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, Goldberg RB (2000) The *Arabidopsis* DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell 12:1041–1062

Steiner-Lange S, Unte US, Eckstein L, Yang C, Wilson ZA, Schmelzer E, Dekker K, Saedler H (2003) Disruption of *Arabidopsis* thaliana MYB26 results in male sterility due to non-dehiscent anthers. Plant J 34:519–528

Steward CN, Via LE (1993) A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques 14:748–750

StintzI A, Browse J (2000) The *Arabidopsis* male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci USA 97:10625–10630

Suliman-Pollatschek S, Kashkush K, Shats H, Hillel J, Lavi U (2002) Generation and mapping of AFLP, SSRs and SNPs in *Lycopersicon esculentum*. Cell Mol Biol Lett 7:583–597

Tanksley SD, Ganal MW, Prince JP, de-Vicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Roder M S, Wing RA, Wu W, Young ND (1992) High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141–1160

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper J, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Vosman B, Arens P, Rus-Kortekaas W, Smulders MJM (1992) Identification of highly polymorphic DNA regions in tomato. Theor Appl Genet 85:239–244

Wang H, Qi M, Culter AJ (1993) A simple method of preparing plant samples for PCR. Nucleic Acids Res 21:4153–4154

Xie D-X, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) COI1: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science 280:1091–1094