Theor Appl Genet (2006) 113:673–683
DOI 10.1007/s00122-006-0332-0

ORIGINAL PAPER

Genetic characterization of the *polycotyledon* locus in tomato

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Received: 6 February 2006 / Accepted: 16 May 2006 / Published online: 29 June 2006

Abstract Developmental mutants serve as a useful material to unravel the mechanisms necessary for organ development. The *polycotyledon (poc)* mutant of tomato, with multiple cotyledons in the seedling and varied phenotypic effects in the adult plant is one such mutant. Studies using physiological and anatomical methods in our lab suggest that POC is involved in the negative regulation of polar auxin transport, which is likely the reason for the pleiotropic phenotype in the mutant. Because of the physiological significance of the polycotyledon mutant described in this paper and also being first of its kind in tomato and also other plant species, we are using a map-based cloning approach to map the *polycotyledon* gene. Molecular mapping of this locus using segregating interspecific F_2 mapping population localized *polycotyledon* gene close to TG424 marker on the long arm of chromosome 9. The closest marker mapped was a PCR marker identified in this study, E8A2 at a distance of 7.4 cM from the *poc* locus. The absence of tightly linked RAPD markers and the non-availability of more mapped markers in this region led us to initiate chromosome walk to *polycotyledon* gene. Both the flanking markers TG248 and E8A2 were used to screen the BAC library and a contig was developed for TG248 marker. The BAC-end sequences were analyzed for their use as RFLP markers to enrich this region for markers. Analysis of the BAC-end sequences revealed that *poc* is localized in the region surrounded by copia-like retrotransposon elements explaining the absence of markers in the euchromatin region on long arm of chromosome 9. Further studies identified two BAC-end sequences which mapped around the *poc* locus and also indicated very low physical versus genetic distance ratio in this region. The double mutant analyses of *poc* with the other two known polycotyledon mutants of tomato, *pct* and *dem* revealed allelism with *pct*; therefore, the *poc* mutant was named as *pct1-2*, and also the original *pct* mutant was renamed as *pct1-1*.

Communicated by Y. Xue

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Introduction

Cotyledon is the first organ to form during embryogenesis of plants. After germination, the cotyledon sustains seedling growth till the seedling becomes photosynthetically competent. The number of cotyledons is also the basis of classification of angiosperms into monocotyledons and dicotyledons. Given the fact that cotyledon is the site for storage of proteins and oil in dicots, the identification of genes involved in cotyledon development may help to improve the seed quality. The normal formation of cotyledons is usually

disrupted in *Arabidopsis* mutants defective in polar transport of auxin. The *pinoid1* (*pid1*) mutant of *Arabidopsis* that has a defect in polar auxin transport, shows altered number (1–4), size and shape of the cotyledons (Bennett et al. 1995). The *pin-form1 (pin1)* mutant shows fused or deformed cotyledons, which arise due to reduction in polar auxin transport (Liu et al. 1993). The molecular cloning of *PID1* and *PIN1* genes has revealed that these encode a serine-threonine protein kinase (Christensen et al. 2000) and auxin efflux carrier (Gälweiler et al. 1998; Furutani et al. 2004), respectively. In mutants such as *xtc1*, *xtc2*, and *amp1*, the polycotyledon phenotype arises from the transformation of first leaves into cotyledons (Conway and Poethig 1997). The *AMP1* gene encodes for a protein significantly similar to glutamate carboxypeptidases that may modulate the level of a small signaling molecule regulating development (Helliwell et al. 2001). The mutations at two loci *hyd1* and *hyd2* cause seedling lethality and pleiotropic phenotypes such as defective cell shape, multiple cotyledons, and short roots and hypocotyls (Topping et al. 1997). The *HYDRA1* gene encodes a Δ8-Δ7 sterol isomerase, whereas *HYDRA2* encodes a sterol C14 reductase that was earlier identified as the *FACKEL* gene product (Souter et al. 2002). Seedlings of the *cuc1cuc2* (*cup-shaped cotyledon*) double mutant have no shoot apical meristem, and two cotyledons are fused along both edges to form one cup-shaped structure (Aida et al. 1997). All the three CUC1, CUC2, and CUC3 genes encode NAC domain-containing proteins and are essential genes for embryonic shoot apical meristem formation and cotyledon separation (Takada et al. 2001; Vroemen et al. 2003). Given the diversity of mutations affecting polycotyledon phenotype, it is apparent that multiple processes are regulating cotyledon numbers in *Arabidopsis*.

Compared to *Arabidopsis* only few mutants are described in tomato or other plant species that display altered number of cotyledons. The *Lanceolate* (*La*) mutation is incompletely dominant (Caruso 1968; Kessler et al. 2001), which in homozygous condition is profoundly affected with extremely lanceolate leaves and producing seedlings with either one cotyledon and a SAM or those that entirely lack both cotyledons and a SAM. The transposon-induced recessive *defective embryo meristem* (*dem*) mutant seedlings bear 1–5 cotyledons, lack both root, and shoot apical meristems (Keddie et al. 1998). The *DEM* gene encodes a novel protein of 72 kD with significant homology to YNV2, a yeast hypothetical protein of unknown function. The *La* and *dem* being seedling lethal mutants do not enable characterization of the mutant beyond seedling stage. The polycotyledon mutant *pct* with 3–4 cotyledons in the seedling stage was mapped morphologically to chromosome 9 (Rick et al. 1992), but was not analyzed further. Based on the analysis of these mutants it is likely that tomato may have genes that affect the initiation and development of cotyledon in a manner different from that observed in *Arabidopsis*.

In our studies, the recessive *polycotyledon* mutant plants were selected as tricot and tetracot seedlings after germination from an EMS mutagenesis experiment in *L. esculentum* cv Ailsa Craig background (Al-Hammadi 2000). The *poc* mutation is strongly pleiotropic, affecting all the stages of plant development. The morphological effects of *poc* are tri- or tetracot seedlings, splitting of the stem, altered phyllotaxy, differences in leaf and flower morphology, development of epiphyllous leaves, production of more number of flowers per inflorescence, structural male sterility, and development of shoots and whole inflorescences from determined flowers. Though the *poc* mutants are structurally male sterile, anthers of mutants make viable pollen; however, they do not open to release pollen. Unlike *Arabidopsis*, where cotyledon mutants show low penetrance (20–30%) of polycotyly, in case of *poc* mutant 98.5% seedlings were polycotyledon showing high degree of penetrance for the multiple-cotyledon phenotype. Studies using physiological and anatomical methods in our lab suggest that POC is involved in the negative regulation of polar auxin transport, which is likely, the reason for the pleiotropic phenotype in the mutant (Al-Hammadi et al. 2003). The molecular characterization of the tomato *poc* mutant may provide the information on genes regulating auxin transport during plant development. Hence, to study this gene further and identify its role in plant development, we intend to map the POC gene and ultimately identify the gene by map-based cloning. Map-based cloning has been successfully used to clone a number of genes including genes for disease resistance and for biochemical metabolism, such as the *Pto* gene conferring resistance to *Pseudomonas syringae* pv tomato (Martin et al. 1993), *jointless* and *jointless-2* genes that suppress the formation of flower and fruit pedicel abscission zones (Mao et al. 2000; Zhang et al. 2000), *fer* and *chln* genes involved in iron uptake in tomato (Ling et al. 1999, 2002).

Here the complementation analyses of the *poc* mutant with the two tomato polycotyledon mutants' *dem* and *pct* are described. We also report the genetic mapping of the *poc* locus to the long arm of tomato chromosome 9 using the existing RFLP markers and PCR-based markers in contrast to the position of allelic *pct* locus as marker on the short arm of chromosome

9. The *poc* locus is in the region devoid of molecular markers and the closest marker identified is at a distance of 7.4 cM. Initial chromosome walking using the two closest markers to identify the BAC clones and mapping the BAC-end sequences derived from them indicates this region to have low physical/genetic distances.

Materials and methods

Plant materials

The *polycotyledon* mutant in *Lycopersicon esculentum* cv Ailsa Craig was described in Al-Hammadi et al. (2003). *L. pennellii* [LA 716] and *L. esculentum (pct)* [LA 2896] seeds were obtained from Tomato Genetics Resource Center (UC, Davis, USA). The *dem* mutant plants are described in Keddie et al. (1998). Double mutants were generated by crossing the *poc* mutant with the two mutants, *dem* and *pct*, as pollen donors and by reciprocal crosses. A F_2 mapping population was generated, segregating for polycotyledony, from an interspecific F_1 hybrid (*L. esculentum poc*/*poc* × *L. pennellii POC*/*POC*). Owing to self-incompatibility, the F_1 plants were selfed by manual sib mating. The F_2 seedlings were scored for altered cotyledon number and size after 1 week of germination under continuous white light (Fig. 1).

RFLP and COS marker analysis

Tomato genomic DNA was isolated following the CTAB extraction method (Bernatzky and Tanksley 1986). Southern blot analysis was performed using restriction enzymes *Eco*RI, *Eco*RV, *Dra*I and *Hin*dIII according to standard procedures. RFLP probes were derived from cDNA and genomic DNA fragments from chromosome 9 (Tanksley et al. 1992) and the COS markers were obtained from Fulton et al. (2002).

RAPD analysis

Genomic DNA was isolated following Messeguer et al. (1991). RAPD analysis was performed with 1,000 different random decamer primers obtained commercially from Operon Technologies (Alameda, USA) (Giovannoni et al. 1991). RAPD fragments were amplified using DNA from the two parents (P_1 and P_2) and two bulks composed of five F_2 wild type and five F_2 mutant plants, respectively, as templates (Michelmore et al. 1991; Williams et al. 1993). The reproducible RAPD bands that were polymorphic between the two bulks were excised from agarose gel, radiolabeled and analyzed in RFLP experiments. The polymorphic bands generated from RAPD fragments were subcloned into the plasmid pCRII (Invitrogen, USA) and sequenced.

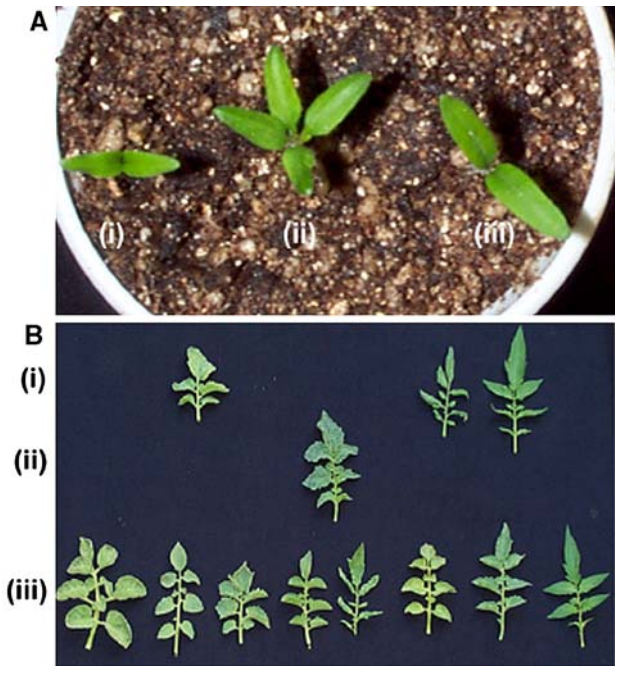

Fig. 1 **a** One-week-old seedlings of *L. pennellii* (wild type) (i), *L. esculentum* (*poc*) (tetracot) (ii) and *L. esculentum* (wild type) (iii). **b** The phenotypes of the leaves from Row (i) *L. pennellii*, *L. esculentum* (*poc) and L. esculentum* (dicot) from which *poc* was derived (*left* to *right*). Row (ii) F_1 hybrid from cross between *L. pennellii* and *L. esculentum* (*poc*). Row (iii) F_2 population showing the leaf phenotypes ranging from *L. pennelli* leaf to *L. esculentum* leaf (*left* to *right*)

Design of primers and analysis of SCAR markers

The primer sequences used to amplify the *E8* gene as E8A1 and E8A2 markers were E8A1F, 5′-AATCGG CCCTTATTCAAAAAT-3′, E8A1R, 5′-CTGATTA AGAATAGA AAAATA-3′ and primer E8A2F, 5′- T GCGGGTAAGTATTTTCATTTCT-3′, E8A2R, 5′-AGCCCTCCCATATCATCTTGTA-3′. For each of the cloned RAPD amplification products, a pair of 21–24-mer oligonucleotide primers was designed to be used as SCAR primers by extending the original 10-mer RAPD primer plus the next 14 nucleotides at the 3′ end (Paran and Michelmore 1993). Amplification of genomic DNA of F_2 plants was executed in 25 µl of the same reaction mixture as applied for RAPD reaction but with 1 pmol of each SCAR forward and reverse primer each. Each PCR consisted of 35 cycles of 1 min at 94°C, 1 min at annealing temperature for respective SCAR markers, and 2 min at 72°C. The amplified

products were separated by electrophoresis on a 2% (w/v) agarose gel.

Conversion of SCAR to cleaved amplified polymorphic sequences markers

The SCAR markers were converted to co-dominant PCR based molecular markers, using the procedures previously described for cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993). Amplification reactions were done using 30 ng genomic DNA in 25 µl of reaction mix programmed for 45 cycles with a denaturing temperature of 94°C for 1 min, an annealing temperature specific for each reaction, for 1 min, and an extension temperature of 72°C for 2 min. Following PCR, the amplified genomic DNA was digested with a series of restriction enzymes to find a restriction endonuclease that was unique to one parent but not the other, thus yielding co-dominant markers that were resolved by agarose gel electrophoresis. The enzymes used in this analysis were *Alu*I, *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hin*fI, *Mbo*I, *Rsa*I, *Ssp*I and *Xba*I. SCAO18a, SCAO18b and SCP17 markers were thus converted to co-dominant CAPS (Table 1).

Linkage analysis

Genetic maps were generated using the MAPMAKER program (Lander et al. 1987). The genetic distances in centiMorgans (cM) were calculated using the Kosambi function of the program (Kosambi 1944). For RFLP mapping on to the tomato genome, 43 F_2 plants of the Cornell reference mapping population were used (Tanksley et al. 1992). For fine mapping in the *poc* region, 74 F_2 plants were used.

Screening the tomato BAC library

E8A2, a co-dominant PCR marker and the RFLP marker TG248 were used as probes. One BAC filter for each probe was used to screen the BAC library consisting of seven full size filters (22.5 × 22.5 cm, 129,024 clones) representing 15 genome equivalents following the method of Budiman et al. (2000). All the identified BAC clones were purified using Qiagen BAC DNA isolation and purification kit and sequenced on both the ends. All the BAC clones were analyzed by *Hin*dIII digestion. PCR primers were also designed from BAC-end sequences and their amplification products were analyzed and hybridized to a survey filter containing restricted DNA of both mapping parents (*L. esculentum poc*/*poc* × *L. pennellii POC*/*POC)* in order to determine their copy number and identify their usefulness as co-dominant RFLP markers.

Results

Double mutant analysis of *poc* and *dem*

poc is a novel mutation and in order to distinguish it from known polycotyledon mutants, *poc* was crossed to *dem* and *pct*. *dem* and *pct* are the two mutants identified in tomato, which show polycotyledony in the seedling stage. The *dem* mutant seedlings develop as tricots and tetracots, which do not develop further as they lack functional apical meristems (Keddie et al. 1998). Mutant plants with genotypes *poc/poc* and *dem/DEM* were crossed and the resulting F_1 seedlings (49 in number) were all wild type (dicot). These data were further confirmed by analysis of the F_2 individuals derived from a double heterozygous (*Dem/dem; POC/poc*) F_1 plant. The data show a ratio of 9:3:4 (WT: *poc*: *dem*) (Table 2), a ratio indicating the epistatic nature of *dem* mutation over *poc*. The *dem/dem* homozygotes were lethal in the single homozygous state and in the double mutant in combination with *poc/poc*. Therefore, the plant phenotype could not be analyzed beyond seedling stage. These F_1 and F_2 results demonstrate the non-allelic nature of *dem* and *poc* mutations, confirming their different gene status.

Table 1 CAPS markers linked to *poc*

SCAR marker	Primer	Sequence (5′–3′)	Annealing temp. (°C)	Phenotype
SCAO18a (KK2)	$OPAO18_{800}$	gggagcgcttACCAGCCCCTTTAC gggagcgcttGGTGGTCTTAAGAA	60	Co-dominant with *Hae*III and *Rsa*I
SCAO18b (KK4)	$OPAO18_{550}$	gggagcgcttGGAATTTCAGTCCT gggaAgcgcttACCCCTATCGACC	60	Co-dominant with *Hae*III
SCP17 (KK10)	$OPP17_{340}$	tgacccgcctACGATTGATTGGAG tgacccgcctGCTTCACACCTTCT	60	Co-dominant with *Eco*RI

The RAPD primer sequences are given in small letters

Table 2 Double mutant analysis data of *poc* with the *dem* mutant presenting the non-allelic nature of these two mutants with a segregation in the F_2 generation showing epistatic ratio of *dem* over *poc*

Generation	Expected ratio	Observed ratio	Wild type	*poc*	*dem*	χ^2	Probability
poc	–	–	0	10	0	–	–
dem	–	–	0	0	10	–	–
F_1	All WT or all polycots	All wild type	49	0	0	–	–
F_2	9:3:3:1	9:3:4	118	43	66	2.76	0.2–0.3

A total of 227 plants were screened in the F_2 generation

Allelism of *poc* with *pct* mutant

pct is a polycotyledon mutant identified spontaneously and has a phenotype and morphological characters similar to *poc* (Fig. 2). This mutant was previously mapped morphologically using markers *ah* and *marm* to the short arm of chromosome 9 closer to marker *ah* by Rick et al. (1992). The *pct* mutant is a highly pleiotropic mutant and has several features similar to *poc*, like the tri- or tetracot seedlings (polycotyledony), profuse branching of the root, differences in leaf and flower morphology to different extents in each, production of more number of flowers per inflorescence and male sterility. In spite of these basic similarities, the extent of phenotypic variation differs in both *poc* and *pct*. These are the hypocotyl length, with the seedling height being higher in *pct* than in *poc* and wild type, the visual anthocyanin content being increased in *poc*, and profuse branching of the root in *pct* compared to *poc*, larger flowers with a stout pistil in *pct* compared to *poc*. The fruits of *pct* mutants are abnormal with structural deformities (Fig. 2).

The F_1 seedlings (98 in number) from both the reciprocal crosses show polycotyledon phenotype and hypocotyl length longer than both *poc* and *pct.* The double mutants are indistinguishable from the parents in the

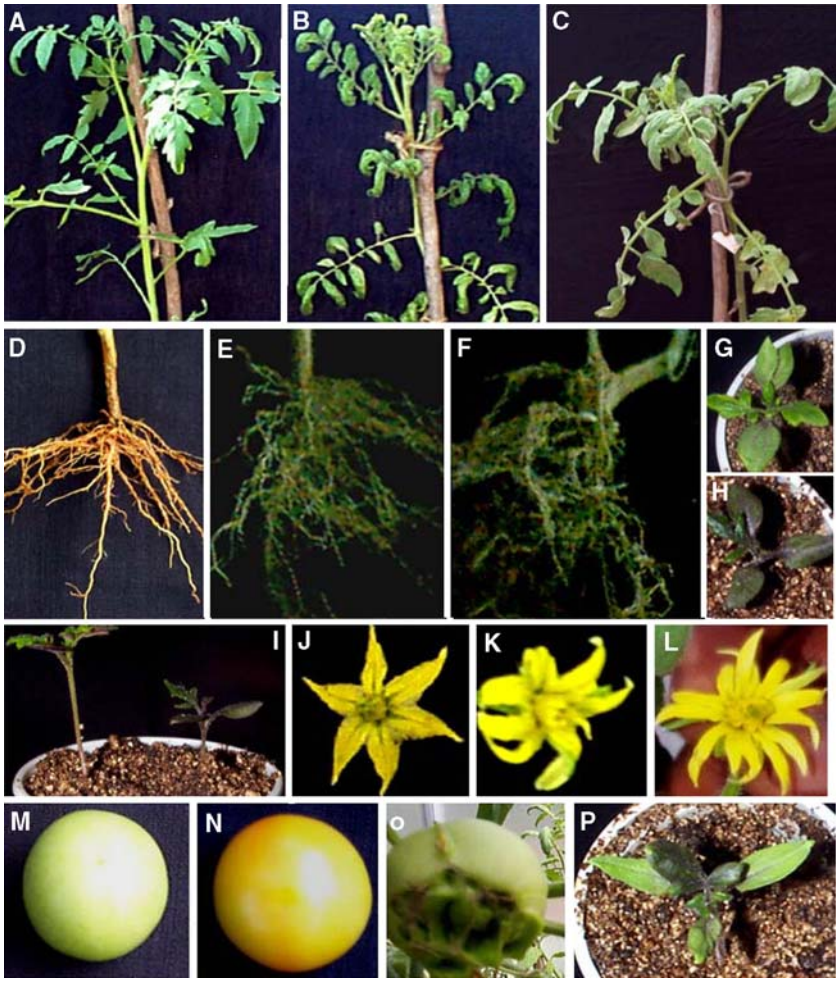

Fig. 2 One-month-old plants of **a** *L. esculentum* (wild type), **b** *L. esculentum* (*poc*), and **c** *L. esculentum* (*pct*). Root morphology of **d** *L. esculentum* (wild type), **e** *L. esculentum* (*poc*) and **f** *L. esculentum* (*pct*) showing profuse branching in *poc* and *pct*. Visual anthocyanin content was less in **g** *pct* compared to **h** *poc*. **i** Longer hypocotyl was observed in *pct* than in *poc.* Variation in the wild type (**j**), *poc* (**k**) and *pct* (**l**) flowers showing increased number of petals in *pct.* Fruits in wild type (**m**), *poc* (**n**) and *pct* (**o**) plants displaying structural deformity in *pct*. **p** *poc–pct* double mutant seedling

adult stages as all the morphological characteristics are intermediate with inflorescences having flowers typical to both *poc* and *pct*. The F_2 data showed the polycot phenotype in all the 199 seedlings of the F_2 population (Table 3). These data show the allelic nature of *poc* and *pct*. The linkage between the morphological markers *ah, marm, pct* and *poc* was also confirmed by the polycot phenotype in all seedlings of the F_1 and the F_2 generations of the cross *ah, marm, pct* × *poc* (unpublished data).

Molecular mapping of the *poc* locus on long arm of chromosome 9

For molecular mapping of the *poc* locus, we developed a high-resolution molecular map around the *poc* gene. First, we developed a segregating F_2 mapping population with 74 F_2 individuals derived from the cross of *L. esculentum* (*poc*) × *L. pennellii* (Materials and methods, Fig. 1). Detailed mapping was initiated with 16 molecular markers (RFLP and COS) of chromosome 9 of the high-density molecular linkage map of tomato, Tomato-EXPEN 1992 (Tanksley et al. 1992) and Tomato-EXPEN 2000 (Fulton et al. 2002). Since the morphological mapping gives only an approximate position for the gene on the genetic map, all the RFLP and COS markers in the region of markers *ah* and *marm* on chromosome 9 were used to determine the precise mapping position. Four RFLP markers, CT17, TG424, TG248 and TG421, identified polymorphic bands between the two mapping parental lines that were useful for mapping in the present population. Interestingly, the *poc* locus was mapped on the long arm and not on the short arm of chromosome 9 as tentatively mapped earlier for its allele *pct* (Rick et al. 1992) using morphological markers and was not confirmed by molecular mapping. Our studies using molecular markers linked the *poc* gene with the RFLP markers TG248 and TG421 located on the long arm of chromosome 9 at a distance of 11.2 and 7.1 cM respectively. Similar results were also reported in case of *jointless-2* mutant which was tentatively mapped to chromosome 11 by classical methods and was later mapped to chromosome 12 using bulk segregant analysis (BSA) with RAPDs and genetic mapping (Zhang et al. 2000). Apart from the markers mapped, all of the available and previously mapped markers in Tomato-EXPEN 1992 and Tomato-EXPEN 2000 maps, like the EST-by-clone markers (cLET-7-D17 and cLED-42-O2), RFLP markers from potato (CP115, GP94, and GP129 from potato chromosome IX) (Gebhardt et al. 1991) and tomato (CT177 and CT198) were not polymorphic in the present mapping population and/or there was no sequence or marker information for these. We have also tried Conserved Ortholog Set (COS) markers (T0732, T0393 and T1190), these were assigned to chromosome 9 with a very low LOD score of < 2 or less, majority of the COS markers gave multiple bands (more than four) which were very difficult to analyze and assign polymorphism to any set of bands.

Identification of linked SCAR and CAPS markers based on RAPD fragments

In order to identify new markers in the *poc* region, BSA using RAPD primers was initiated. For this, DNA from the parents and the pooled samples of five wild type and five *poc* plants from the same F_2 segregating mapping population were used. One thousand random decamer primers were tested on parents and bulks by PCR. Depending on the primer, 1–8 DNA fragments were amplified from a given template DNA. Screening revealed that many primers gave polymorphic DNA fragments between the parents, and only 15 primers generated polymorphic DNA fragments that were observed in both the parents and the two bulks for two different phenotypes of the F_2 population.

Thirteen polymorphic RAPD fragments obtained from 11 primers and co-segregating with wild type but not with the mutant phenotype were isolated and used

Table 3 Complementation test data of *poc* with the *pct* mutant showing the allelic nature of these two mutants resulting in all polycotyledon seedlings in the F_1 generation; this result was confirmed by the F_2 ratio also showing all polycotyledon seedlings without any segregation

Generation	Cross	Expected phenotype	Obtained phenotype	Wild type	Mutant
P_1 (*poc*)	–	–	–	0	6
P_2 (*pct*)	–	–	–	0	6
F_1	*poc* × *pct*	All WT or all polycots	All polycots	0	42
F_1'	*pct* × *poc*	All WT or all polycots	All polycots	0	56
F_2	*poc* × *pct*	All polycots	All polycots	0	96
F_2'	*pct* × *poc*	All polycots	All polycots	0	103

A total of 199 plants were analyzed in the F_2 generation

as RFLP markers on the F_2 mapping population. Six of the 13 RAPD fragments identified low copy hybridization signals but no polymorphisms (not shown). Five RAPD fragments resulted in smears or high copy number, indicating repetitive sequences. Two DNA fragments recognized single or low copy number sequences and displayed a RFLP. These were hybridized to genomic DNA derived from the standard mapping population of tomato (Tanksley et al. 1992) in order to map these new markers, but these revealed no polymorphism on these mapping filters. Since the RAPD markers were not useful for mapping as RFLP markers, they were converted into SCAR markers (Materials and methods, Table 1). We expected to amplify a unique band in the wild type *L. pennellii* parent and no band in *L. esculentum* (*poc*) parent, instead we found single bands of the same size corresponding to the RAPD fragment to be present in both the parents and all the F_2 individuals.

In order to detect amplified length polymorphism (co-dominant) if any, the SCAR primer amplification products were converted to CAPS markers. Three of the five primers revealed polymorphism between the parents. The SCAR product KK2 obtained using primer pair SCAO18a, displayed a polymorphic pattern with two enzymes, *Hae*III and *Rsa*I. The SCAO18b primer product KK4 showed polymorphism with enzyme *Hae*III. The third amplicon KK10, from primer pair SCP17 resulted in polymorphism with enzyme *Eco*RI (Table 1). KK2 and KK4 were mapped onto chromosome 9, with KK2 placed at a distance of 21.4 cM from the *poc* locus and KK4 much farther. KK10 was not linked to *poc* (not shown). Thus, RAPD analysis and modifications revealed two additional markers on chromosome 9, and one in the vicinity of *poc* (Fig. 4).

E8A2, a new co-dominant PCR marker linked to *poc*

The high-density molecular linkage map of tomato (Tanksley et al. 1992) reveals marker E8A, co-segregating with RFLP marker CT218. *E8* is a gene involved in ethylene responsive fruit development in tomato (Lincoln et al. 1987). We utilized the E8A sequence to develop two PCR-based genetic markers at the E8A locus (Materials and methods). PCR analysis with these two markers revealed that E8A1 produced a dominant phenotype with amplification in *poc* parent (~900 bp) and no amplification from the wild type *L. pennellii* parent. The E8A2 primer pair resulted in a co-dominant polymorphism with different sized amplification products from the parents. The amplified product from wild-type parent P_2 (*L. pennellii POC*/*POC*) was ~500 bp and that from the mutant parent P_1 (*L. esculentum poc*/*poc*) was around 800 bp (Fig. 3). Amplification results were scored and analyzed to produce the map shown on Fig. 4. E8A2 was shown to be linked to *poc*, as it was mapped 7.4 cM away from the *poc* locus.

Initial chromosome walking to the *poc* locus

None of the available maps (http://www.sgn.cornell.edu) reveals more markers around the *poc* locus indicating this region to be devoid of any low copy sequences. The non-identification of any closely linked markers by RAPD using BSA also confirms that this region to be highly repetitive. The fact that this region has no more markers led us to initiate a chromosome walk to the *poc* locus. To begin chromosome walking to *polycotyledon* gene, the two closest flanking markers E8A2 and TG248 were used for screening the first two BAC filters from *L. esculentum* Heinz *Hin*dIII library (CUGI, http://www.genome.clemson.edu/). Three clones 3M20, 9I11, 48I08, were identified with E8A2 and four BAC clones 54P14, 63B20, 74G09, 90C08, were identified with TG248 confirmed by southern hybridization (data not shown). Restriction digestion analysis of these BACs using *Hin*dIII revealed different restriction patterns indicating that all the seven BACs were dissimilar (not shown). Both the ends of the seven BACs were sequenced and PCR primers were designed from 12 end sequences to identify BAC-end sequence markers closer to the *poc* gene. Amplification using the 12 PCR primer pairs on all the seven BACs resulted in aligning four TG248 BACs into a contig, and almost all of the BAC-end sequences of the three BAC clones selected with E8A2 contained

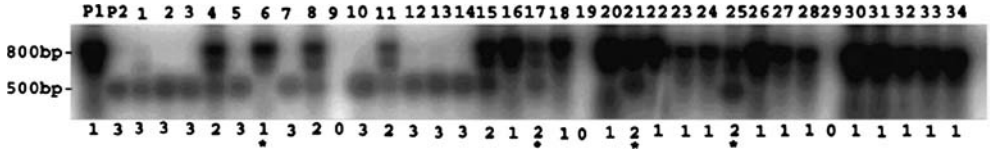

Fig. 3 PCR-based mapping of *E8A2* using the *poc* mapping population. *Lanes 1–34* are F_2 plants from the cross *L. esculentum (poc)* P_1 × *L. pennellii* P_2. *Lanes 1–14* are dicot individuals and *lanes 15–34* are polycot plants. Parent P_1 genotype, heterozygous condition, parent P_2 genotype and missing data are denoted by 1, 2, 3 and 0, respectively. Recombinant plants are denoted by an *asterisk*

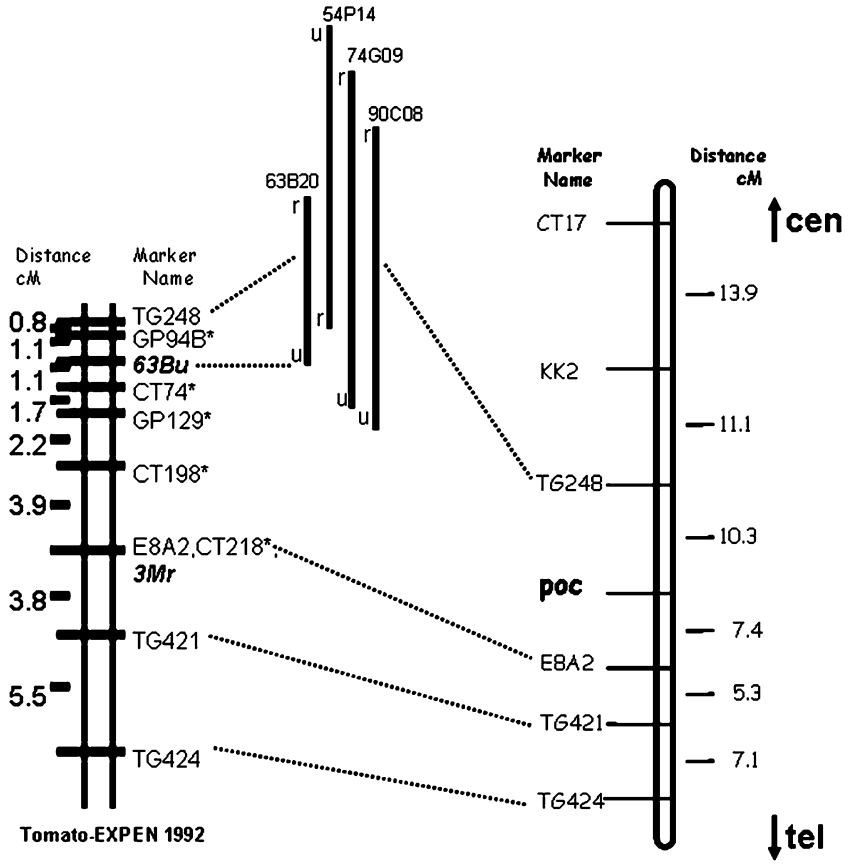

Fig. 4 Genetic linkage map of *poc* region on long arm of chromosome 9 is shown to the *right*. The *thick vertical lines* represent the BAC contig spanning the marker TG248 region. The Tomato-EXPEN 1992 map is to the *left* showing the map positions of BAC-end sequence markers, 63Bu and 3Mr. *Asterisks* denote the markers that were either not reproducible or not polymorphic in the present mapping population. The telomere is to the *bottom* and the centromere to the top, as indicated by the *arrows*

repeat DNA sequences, it was impossible to analyze or align them into a contig.

Among the 12 BAC-end sequences, two RFLP markers were mapped in the standard mapping population (Tanksley et al. 1992), 3Mr derived from clone 3M20 (size not estimated) identified using E8A2 was mapped between markers CT198 and TG421 and co segregating with CT218. Marker 63Bu originating from BAC 63B20, with an estimated length of 34,077 bp, identified using TG248 mapped at a distance of 1.9 cM from TG248 (Fig. 4) and closer to *poc* gene. However, we failed to walk further toward the *poc* locus because of the repetitive nature of the BAC-end sequences. The end-sequencing project of BAC clones of the 'Heinz 1706' library is underway and the end sequencing data available for all these clones (http://www.genome.clemson.edu/) agreed well with the BAC-end sequencing results of our chromosome walking.

Discussion

In plants, there are very few polycotyledon mutants identified and in tomato, there are only three mutants: *La* (Caruso 1968), *dem* (Keddie et al. 1998), and *pct* (Rick et al. 1992) that show variation in the cotyledon number. Interestingly, *poc* was found to be allelic to *pct* and the degree of phenotypic variation from the wild type could be attributed to the nature of the mutations. *pct* was a spontaneous mutation whereas *poc* was obtained by EMS mutagenesis, which usually results in point mutations such as stop codons or transitions. The *pct* mutation could be a deletion or an addition mutation where a small segment is either inserted or deleted, which may result in enhanced effects of its morphological characteristics compared to *poc*. Further, the localization of the *poc* on chromosome 9 clearly differentiates it from *dem* mutation, which maps to chromosome 4 as known by transposon tagging (Keddie et al. 1998). In view of allelism between *poc* and *pct*, in accordance to established convention of naming mutants, we have now redesignated *pct* mutant as *pct1-1* and *poc* mutant as *pct1-2*. Henceforth in the following text *poc* mutant is referred as *pct1-2*.

This gene *pct1-2*, for polycotyledony, is first of its kind in tomato and also other plant species that has been mapped using molecular markers. *pct1-2* is both physiological and developmentally significant mutant, and understanding the molecular events underlying polycotyledony would provide information on factors regulating the polar auxin transport in tomato as well

as the genes involved in the development of the SAM in the early seedling stages.

In the present work we have mapped the *pct1-2* locus to the long arm of chromosome 9 of tomato and also identified RFLP markers closely linked to this gene. Though morphological mapping of *pct1-1* placed it closer to the marker *ah* on short arm of chromosome 9 (Rick et al. 1992), the present genetic linkage map comprising molecular markers (RFLP, CAPS and PCR) confirmed the relative position of the *pct1-2* locus flanked by markers TG248 and E8A2. The current map is based on the F_2 population of *L. esculentum* (*pct1-2*) and *L. pennellii* and is comparable to the previously published tomato high-density linkage map of tomato (Tanksley et al. 1992). While the linear order of the common markers was confirmed to remain constant between the maps, the genetic distances differed slightly. For the interval spanning the markers TG248 and E8A, the distance on the Tomato-EXPEN 1992 map was 10.8 cM, whereas in the current study we found that these markers are 17.7 cM apart. Similar results were also observed while fine mapping the chromosome 12 *Mi-3* region conferring root-knot nematode resistance (Yaghoobi et al. 1995). Possible explanation for the discrepancy with the map distances could be the differences in the backgrounds of the parental lines. Further fine mapping the *pct1-2* locus was limited because the markers mapped on other mapping populations failed to be polymorphic in our mapping population. The conserved orthologous markers in this region from the F2-EXPEN 2000 map, which are a good source of candidate gene strategy by utilizing the chromosomal microsynteny between tomato and *Arabidopsis*, were also not mappable in our mapping population.

The microsatellite markers, though generally highly polymorphic in nature, are of limited use in tomato as there are few microsatellite markers found which are clustered around the centromeres showing uneven distribution (Areshchenkova and Ganal, 2002). The mapping data with microsatellite markers also show that a large number of alleles in *L. esculentum* are often associated with null alleles in *L. pennellii*. Like microsatellites, AFLP markers were also not randomly distributed over the genome. Even if AFLP markers may appear more interesting because of the large number of bands detected on a single gel, they showed a high level of co-segregation and clustering (Saliba et al. 1998; Haanstra et al. 1999). RAPD analysis which we have opted for is a PCR-based marker system which is non-radioactive, easy to handle, and requires only small amount of DNA. In particular, RAPD analysis involving the use of a single DNA primer to direct amplification of discrete random sequences (Klein-Lankhorst et al. 1991) was previously used successfully in tomato and other crops (Ling et al. 1996; Ohmori et al. 1996, 2000; Dixon et al. 1995; Huang et al. 2000). There are though few limitations with RAPD markers, especially the use of these markers across the laboratories may not be reproducible. This problem may be overcome by converting the RAPD markers to more locus specific SCAR markers (Paran and Michelmore 1993) or CAPS markers.

In BSA, pools are homogeneous for all genomic sequences except those within the target interval. Polymorphisms result if the primers amplify fragments within the target interval. Thus in the case of dominant traits, such as most disease resistance genes, polymorphic fragments should be tightly linked to the trait. Whereas for *pct1-2*, which is a recessive mutant, we targeted for polymorphic fragments linked to the wild type *L. pennellii PCT* allele and therefore not segregating with the *pct* phenotype. With a small pool size of the samples such as five as in the present case, there is a significant chance that the pools will share areas of homozygosity other than the targeted interval, thus resulting in increased number of false positives during PCR screening (Giovannoni et al. 1991). In spite of using 1000 RAPD primers in BSA we could not obtain by this method a single tightly linked marker to *pct1-2*. However, we were able to obtain two new markers for chromosome 9 after conversion of RAPD fragments into CAPS markers. PCR using whole genomic DNA as template and an oligonucleotide primer tends to amplify middle- or high repetitive sequences predominantly (Williams et al. 1990). Similarly in the present situation, all the RAPD markers were either part of repetitive sequences or could not be mapped as RFLP markers.

This region on chromosome 9 long arm around the *pct1-2* locus appears to be in the region devoid of markers as shown in the latest maps of tomato (Tomato-EXHIR 1997, Tomato-EXPEN 2000, Tomato-EXPIMP 2001, and Tomato-EXPIMP 2005) as well as from related potato, pepper and eggplant maps (Eggplant-LXM 2002) show long gaps in the range of ~12–20 cM between the markers in this region. Hence chromosome walking to the *pct1-2* gene with the closest linked flanking markers was initiated. To generate a physical map in the region around *pct1-2*, chromosome walking using a genomic BAC screen was performed. Flanking markers, E8A2 and TG248 probes were used to screen the BAC library resulting in detection of seven BAC clones lodging the marker sequences. The repetitive sequences of the end-clones of almost all BAC clones screened led to many difficulties in construction of a

BAC contig harboring the *pct1-2* gene. However, analysis of the BAC-end sequences resulted in identifying a BAC contig of four clones in the TG248 region that maps closer to the *pct1-2* locus. Simultaneously, the end-sequencing project of BAC clones of the *L. esculentum* Heinz *Hin*dIII and *Mbo*I library is underway, and publicly available information was very useful for the confirmation of the end sequences and the construction of BAC contigs. BAC 63B20 has an estimated length of 34,077 bp and one of its BAC-end sequence, 63Bu mapped 1.9 cM away from the marker TG248 using which this BAC was identified. Hence, the physical/genetic distance for this region is deduced at around 17.9 kb/cM. The other BAC-end sequence marker 3Mr from the BAC clone 3M20 cosegregated with the marker E8A2, using which it was identified. The length of the BAC clone 3M20 is not yet estimated. These findings indicate that the physical versus genetic distance is very low in that region, and suggest that even though TG248 and *pct1-2* locus are genetically mapped far apart, their physical distances are quite low. On average, 1 cM in tomato corresponds to about 750 kb (Tanksley et al. 1992); the mapping data with TG248 and 63Mu indicate that the *pct1-2* region appears to lie in an area of the genome with a high recombination rate at least on one side. The physical to genetic distance ratio varies greatly in different regions of the tomato genome. For example, a ratio of 25 Mb/cM was found for the *jointless-2* region, which maps near the centromere of chromosome 12 (Budiman et al. 2004), whereas for *jointless* the corresponding value is < 50 kb/cM (Mao et al. 2001).

The sequence annotations for all the BAC-end sequences of other clones show that these ends have significant alignments to putative retroelement 'gag-pol' polyprotein sequences from *Solanum tuberosum* and *Oryza sativa* and one BAC 74G09 has sequence similarity to a polyprotein from tomato. A BAC-end sequence of 90C08 has high sequence similarity to TGRII tomato repeat element. The high repetitive sequence content on the distal ends of the most BACs explains the lack of closely linked markers to the *pct1-2* locus in that region.

The tomato genome is composed of approximately 950 Mb of DNA, more than 75% of which is heterochromatin and largely devoid of genes. *pct1-2* appears to be one gene which is located in euchromatin region on long arm of chromosome 9, with the presence of active genes and transposable elements. Hence this region devoid of markers serves as useful model to explore in detail the composition and organization of such euchromatin regions and the surrounding anchored BACs as seed BACs where the physical map can be linked to genetic map, facilitating in the whole genome sequencing of tomato.

Acknowledgments We thank Dr. M. Röder (IPK, Gatersleben) for discussions on the work and manuscript. Dr. M. Ganal kindly provided tomato mapping filters and RAPD primer sets. The research fellowship support from Government of Yemen (ASAA), CSIR (MK), ICMR (MSS) is gratefully acknowledged. This work was supported by research grants of CSIR (RS), IAEA (RS) and DST-DAAD (RS, PB). We thank B Carroll, University of Queensland, Australia for providing *dem* mutant seeds.

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