RESEARCH ARTICLE

Mapping of the *multifoliate pinna* (*mfp*) leaf-blade morphology mutation in grain pea *Pisum sativum*

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Abstract

The *multifoliate pinna (mfp)* mutation alters the leaf-blade architecture of pea, such that simple tendril pinnae of distal domain are replaced by compound pinna blades of tendrilled leaflets in *mfp* homozygotes. The *MFP* locus was mapped with reference to DNA markers using F_2 and $F_{2:5}$ RIL as mapping populations. Among 205 RAPD, 27 ISSR and 35 SSR markers that demonstrated polymorphism between the parents of mapping populations, three RAPD markers were found linked to the *MFP* locus by bulk segregant analyses on *mfp/mfp* and *MFP/MFP* bulks assembled from the $F_{2:5}$ population. The segregational analysis of *mfp* and 267 DNA markers on 96 F_2 plants allowed placement of 26 DNA markers with reference to *MFP* on a linkage group. The existence of common markers on reference genetic maps and *MFP* linkage group developed here showed that *MFP* is located on linkage group IV of the consensus genetic map of pea.

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Introduction

Pea (*Pisum sativum* L.) is a grain legume which is widely cultivated for use as an ingredient of human foods and animal feed. Improvement of its allometry for high-grain yield and diversification of genetic tolerance for pests and diseases are the main breeding objectives in this crop. On account of its architectural features, pea plant is proving to be an useful model system for studying the developmental biology of leaf and flower (Gourlay *et al*. 2000; Kumar *et al*. 2004; Wang *et al*. 2008).

The leaves of pea, into which more than one third of biomass is partitioned, comprise a target for improvement of harvest index. Each node produces a compound leaf blade and a peltate simple-stipule blade on either side of leaf blade. The leaf blade rachis bears up to three pairs of simple leaflets on the petiole proximal side; up to four pairs of simple tendrils on the petiole distal side, and terminates into a simple tendril. Mutations in the *multifoliate pinna* (*MFP*), *afila* (*AF*), *tendril-less* (*TL*), *unifoliata*[(*UNI*) or *unifoliata-tendrilled acacia* (*UNI-TAC*)], *stamina pistilloida* (*STP*) and *crispa* (*CRI*) genes/loci alter the morphology of one or more domains of leaf blade (de Vilmorin and Bateson 1911; Lamprecht 1933; Kujala 1953; Goldenberg 1965; Taylor *et al*. 2001; Kumar *et al*. 2004; Tattersall *et al*. 2005). Stipule morphology is altered by the mutations in *stipule-reduced* (*ST*) and *cochleata* (*COCH*) genes (Pellew and Sverdrup 1923; Blixt 1972). The *UNI*, *STP* and *CRI* genes of pea have been identified as orthologues of *leafy* (*LFY*), *unusual floral organs* (*UFO*) and *asymmetric leaves 1* (*AS1*) of *Arabidopsis thaliana* (Hofer *et al*. 1997; Gourlay *et al*. 2000; Taylor *et al*. 2001; Champagne *et al*. 2007). The counterparts of *MFP, AF, TL, COCH* and *ST* remain unknown in *A. thaliana* and other plant systems. To increase the understanding of genetic control of leaf blade, stipule blade and of links in the development of the two organs, interactions among different leaf-blade mutations, stipule-blade mutations and mutations affecting the two organs are being investigated. It has been observed that pea plant typifies a unique group of plant species in which, unlike in *Zea mays, A. thaliana* and *Solanum lycopersicon* and many other

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plants, leaf blade development is not controlled by *KNOX-1*-like genes (Hofer *et al*. 2001; Hake *et al*. 2004; Champagne *et al*. 2007), but is an outcome of interactions of *AF, TL* and *MFP* genes with *UNI* and *STP* genes. The *COCH* gene excludes the functioning of *UNI–STP* pathway from stipules and directs the *COCH–ST* pathway of stipule development.

Mapping of classical mutations is a means for their positional cloning and, thereupon, sequencing and genetic engineering of the concerned genes. However, the *uni, stp* and *cri* genes of pea could be cloned, characterized and molecularly deployed on the basis of their orthology with already characterized genes in heterologous plants like *A. thaliana*. The *uni* (*uni-tac*), *stp, cri, af, tl, coch* and *st* mutations have been placed on the molecular genetic linkage map of pea (Ellis and Poyser 2002). Here, we report about the (i) application of bulk segregant analysis to identify DNA markers linked to the *MFP* locus, (ii) subsequent analyses of a mapping population to place the *MFP* on molecular map of linkage group IV, and (iii) use of certain common markers of the present study and earlier studies to align the present map of linkage group IV with earlier reported maps of this linkage group.

Materials and methods

Plant materials

The origin of *mfp* mutation has been described earlier (Kumar *et al*. 2004). The *mfp/mfp* line SKP-351a was crossed with multi-marked inbred line RMP-1 (*A, MFP, gp, R*) and a population consisting of 96 F_2 plants were developed. The F_2 was used as the mapping population and was also the progenitor of $F_{2:5}$ RIL population deployed for bulk segregant analysis. The *MFP/MFP, MFP/mfp* or *mfp/mfp* genotypic identities of F_2 individual plants were confirmed by phenotyping of F_2 derived F_3 families. The phenotypes of members of F_4 families were used to select *MFP/MFP* and *mfp/mfp* lines for the syntheses of phenotypic bulks. The MFP and mfp bulks comprised of plants from 11 and eight $F_{2:5}$ lines, respectively. The MFP or mfp phenotype of individual plants was assessed after the onset of flowering. In this regard, each plant was scored at least twice. The second scoring was done after one week interval. The leaves of a single F_2 plants were harvested occasionally and plant-wise pools of leaves were stored at −80°C and samples were drawn for DNA extraction as and when required. For preparing MFP and mfp bulks, equal amount (by weight) of fresh leaves from selected lines were cut into small pieces, mixed and stored at −80°C to serve as DNA resource.

Growth conditions

The parental lines and filial generations were grown in field plots of the experimental farm of the institute (NIPGR) at New Delhi, India, during the winter rabi season (October/November to March/April) of the years 2003 to 2008. The cultivation and seed preservation were done as described earlier (Prajapati and Kumar 2001, 2002).

DNA isolation and marker analysis

The method of Doyle and Doyle (1990) was used for the extraction of DNA. DNA amplifications were carried out in a iCycler Thermal Controller (Bio-Rad, Hercules, USA). In all 337 RAPD (Operon Technologies, Alameda, USA), 50 ISSR (Bioneer Corporation, Daedeok-gu, Korea) primers, 97 pea SSR (Agrogene Company Moissy Cramayel, France) and 51 *Medicago truncatula* EST-SSR primer pairs (Eujayl *et al*. 2004; Gutierrez *et al*. 2005) were deployed to map the *mfp* mutation. The PCR reactions with RAPD primers were performed in 25 μL volume containing 2.5 μL of 10x PCR reaction buffer (200 mM Tris- HCl (pH 8.4), 500 mM KCl) and 3 mM $MgCl_2$, 0.1 mM of each dNTP, 0.8 μM of primer, 25 ng of genomic DNA and 1 unit *Taq* polymerase (Invitrogen, Carlsbad, USA). Amplification was performed in an iCycler Thermal Controller (Bio-rad, Hercules, USA). PCR conditions were: one cycle of 60 s at 94°C, 30 s at 36°C and 60 s at 72°C followed by 45 cycles of 5 s at 94°C, 15 s at 36°C and 60 s at 72°C and a final cycle of 7 min at 72°C. The amplified products were separated by electrophoresis in 1.2% agarose gels, stained with ethidium bromide and visualized under UV light. The bands were photographed using gel documentation system (Alpha Innotech Corporation, San Leandro, USA). The amplification and visualization of products with ISSR primers were similar except that PCR conditions included a preliminary cycle of 120 s at 94°C, followed by 35 cycles of 20 s at 94°C, 50 s at 50°C and 90 s at 72°C and final cycle of 7 min at 72°C. The amplification of the pea SSR and *M. turncatula* EST-SSR markers was done in 20 μL volume containing 2.0 μL of 10x PCR reaction buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl) and 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.8 μM of each primer, 25 ng of genomic DNA and 1 unit of *Taq* polymerase (Invitrogen, Carlsbad, USA). DNA amplifications were carried out in a iCycler Thermal Controller (Bio-rad, Hercules, USA). The annealing temperature of each primer was different. The general amplification conditions were 3 min at 94°C, followed by 45 cycles of 60 s at 94°C, 60 s at 50°C and 120 s at 72°C and final step of 10 min at 72°C. The amplification products were resolved in 3% metaphore agarose gels in TBE (45 mM Tris borate and 1 mM EDTA) (Cambryx Bioscience, Rockland, USA) and PAGE gels and visualized like for RAPD and ISSR products.

Data analysis and genetic mapping

For linkage analysis, the markers were analysed by a chi-square test for goodness-of-fit to the expected Mendelian segregation ratio (3 : 1) of a dominant locus in F_2 population. Linkage groups were built using the group assign order and RIPPLE command of MAPMAKER/EXP version 3.0 (Lander *et al*. 1987; Lincoln *et al*. 1992). LOD score of 3.0 and maximum distance 30 cM were chosen for establishing the marker order on each linkage group. The order of loci within the linkage group was refined by the RIPPLE command. The

Kosambi (1944) mapping function was used to calculate genetic distances in centimorgans.

Results

Phenotype and inheritance of mfp

The *mfp* trait is inherited as a single gene effect (Kumar *et al*. 2004). It was imperative to note the *mfp* phenotype and its inheritance in the mapping population(s). The phenotypic screening of F_2 derived F_3 progenies permitted unambiguous identification of *mfp/mfp* genotypes (figure 1). The genotype of each F_2 plant was inferred by phenotyping of 15–20 progeny plants of each of F_2 derived F_3 family. In *mfp/mfp* plants, the first flowering node, and the nodes above and below it bore leaf blades that had mfp phenotype. The *mfp* leaf blades produced pairs of compound pinna blades in place of tendrilled pinnae in their distal domain. Each pinna blade comprised of three or more highly tendrilled leaflets borne on rachide. The petiole proximal domain of *MFP* and *mfp* leaf blades had the same phenotype by producing pairs of simple leafleted pinnae.

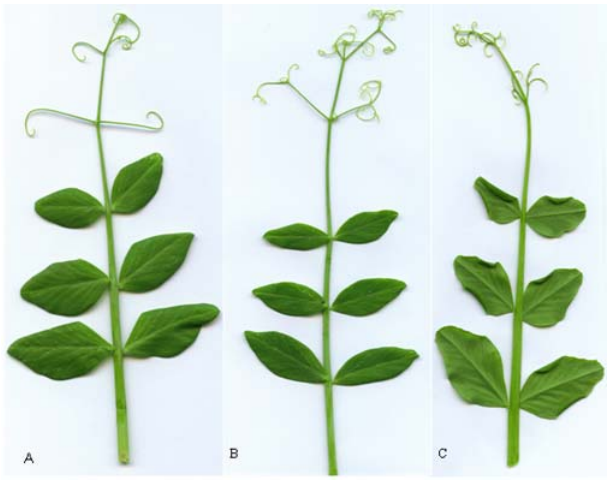

Figure 1. Leaf-blade morphology of (A) wild type *MFP/MFP*, (B) heterozygote *MFP/mfp*, and (C) *multifoliate-pinna* (*mfp*) mutant homozygote, in grain pea *Pisum sativum*.

The F_2 generation obtained from the cross between SKP-351a and RMP-1 segregated into 23 *mfp/mfp* and 73 *MFP/mfp* + *MFP/MFP* plants, a pattern that gave a good-fit-to 1 : 3 ratio expected of single-gene inheritance ($\chi^2 = 0.03$, $P = 0.81$). In the RIL $F_{2:5}$ of the cross, the number of *mfp/mfp, MFP/MFP* and *MFP/mfp* lines were 40, 46 and 10, respectively. The segregation pattern of the homozygous lines fitted the 1 : 1 ratio expected for a single-gene model of segregation ($\chi^2 = 0.42$, $P = 0.52$). These results are in agreement with those reported earlier on the segregation analyses of *mfp* in different crosses.

Bulk segregant analysis of mfp/mfp and MFP/MFP RILs

Bulks were made of randomly selected plants of 11 *MFP/MFP* and eight *mfp/mfp* lines present in the RIL ($F_{2:5}$) population of the cross SKP-351a × RMP-1. The DNA of bulks were amplified by use of 337 RAPD and 50 ISSR primers and 148 SSR primer pairs. Initially, 12 RAPD, three ISSR and five SSR primers were observed to have generated polymorphism between the bulks. However, upon further testing by amplification of DNA of individual members of the two bulks with the above putatively identified primer(s), only three RAPD markers were found to discriminate all the *mfp/mfp* lines from *MFP/MFP* lines, of the two bulks. These markers were OPH2a (600 bp), OPB5a (600 bp) and OPB5b (850 bp).

Linkage mapping of mfp

The mapping population comprised of 96 F_2 generation plants of the cross SKP-351a × RMP-1. The DNA of the parental lines were amplified by use of all the 535 primers or primer pairs used above to distinguish the *mfp* and *MFP* bulks. A total of 125 RAPD, 12 ISSR primers and 20 SSR primer pairs were found to generate polymorphism between the parents of the mapping population. In all, 205 RAPD, 27 ISSR and 35 SSR bands were polymorphic between SKP-351a and RMP-1. Segregational analyses of all 267 DNA markers were carried out on the 96 F_2 generation mapping population plants. The linkage analyses on the phenotypic and DNA marker segregational data by use of MAPMAKER/EXP version 3.0 produced a linkage group on which the *mfp* locus was located in between H2a-600 and B5a-600 markers. The distance between H2a-600 and *mfp* was estimated as 6.9 cM and that between *mfp* and B5a-600 as 15.1 cM, the distance between H2a-600 and B5a-600 being 22.0 cM. In all, 26 markers could be placed on the *mfp* carrying linkage group which measured 285.5 cM in length (figure 2, B). The average distance of markers on this linkage group was 10.9 cM. In this map, OPJ14 and OPH14 on one hand, and OPG12 and OPL18 on the other, showed no recombination.

Location of mfp on the linkage group IV

To determine the location of *mfp* gene in relation to the published and consensus linkage maps of pea, linkage groups of two reference maps on which some of the markers were common with the *mfp* carrying linkage groups were aligned with the latter. The *mfp* linkage group developed in this study has two RAPD markers; namely OPJ14a and OPG12, that are placed in the linkage group IV of the map generated by Irzykowska *et al*. (2001). Further, two RAPD markers; namely J14a-850 (or OPJ14a) and L19-700, of the linkage group IV of the map of Laucou *et al*. (1998) are present in the *mfp* linkage group constructed in this study. One marker J14a-850 is common to the linkage group IV of Irzykowska *et al*. (2001) and Laucou *et al*. (1998) and the presently

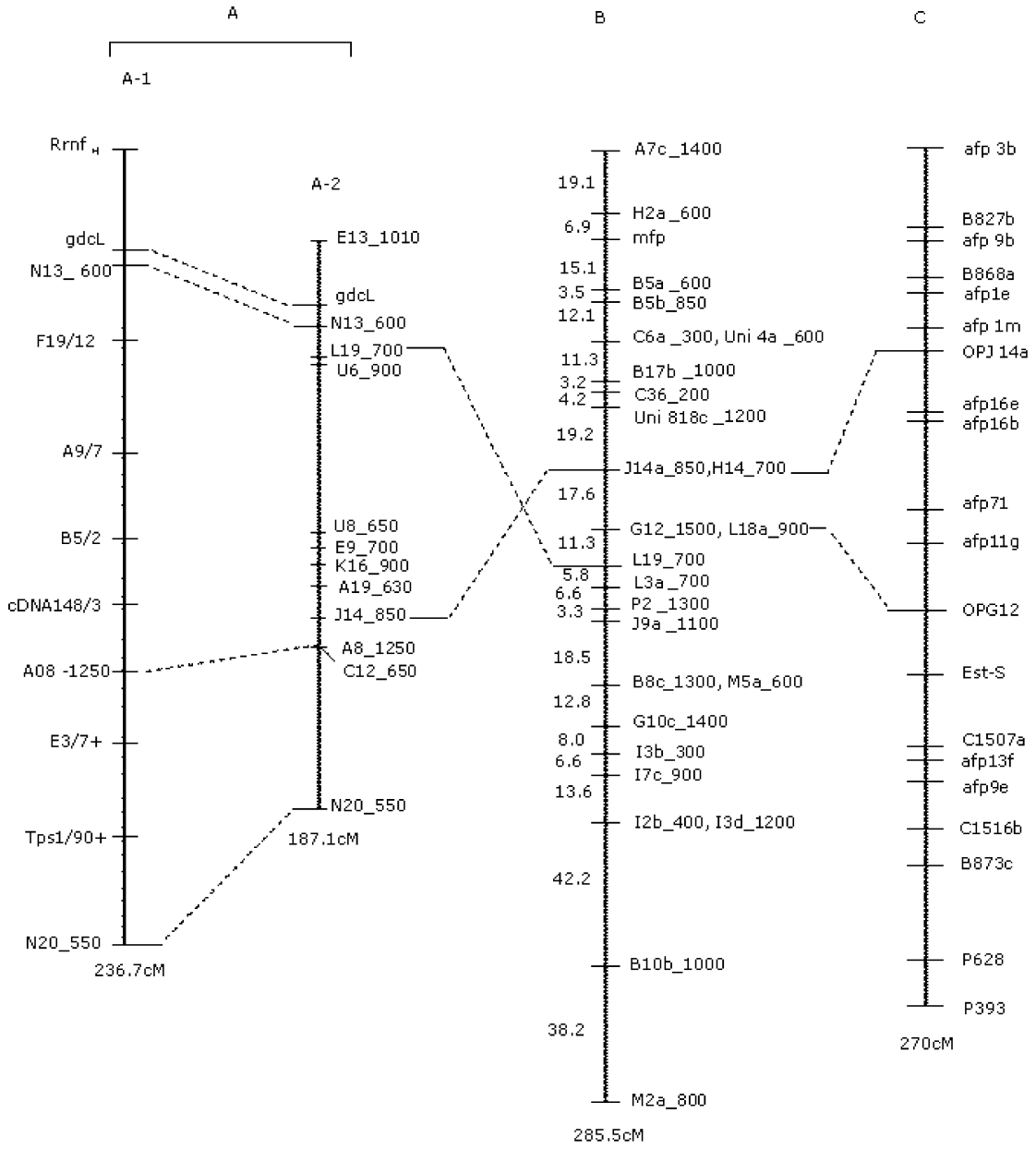

Figure 2. Location of the *multifoliate-pinna* (*mfp*) locus on linkage group IV of grain pea *Pisum sativum*. (A_1 and A_2) Reference linkage group IV maps from the work of Laucou *et al*. (1998), (B) linkage group developed in this study, and (C) reference linkage group IV of Irzykowska *et al*. (2001).

developed *mfp* linkage group. These results suggest that *mfp* is located on the linkage group IV of the conventional genetic map of pea.

Discussion

In grain pea, *P. sativum, mfp* is a dominant mutation responsible for gain of a function. In the leaf blades of *mfp/mfp* plants, the simple tendrils of the distal domain are replaced by compound pinna blades of tendrilled leaflets. Some of the distal tendrils are branched in the distal domain of *MFP/mfp* leaf blades. The *mfp* mutation increases the compoundness of leaf blades in the backgrounds of *af* and *tl* mutations such that *af tl mfp* homozygotes produce leaf blades bearing several hundred leaflets. The mfp function appears to enlarge the size of leaf-blade primordium and subprimordia leading to extensive ramification of the leaf blade.

The experiments described above have localized *mfp* mutation on the linkage group IV of pea. Using bulked segregant analysis (Michelmore *et al*. 1991), three RAPD markers (OPH2a-600, OPB5a-600 and OPB5b-850) that are linked to *mfp* were identified. Based on segregation of these markers, *mfp* and additional 264 DNA markers in 96 F_2 progeny

plants of a suitable cross, the genetic distance between *mfp* and H2a, B5a and B5b was estimated as 6.9, 15.1 and 18.6 cM, respectively and the loci were arranged in the order H2a-*mfp*-B5a-B5b. Further, 23 additional DNA markers could be placed with respect to each other, *mfp* and markers revealed to be linked with *mfp* by BSA, on the *mfp* bearing linkage group.

Pea genetic linkage maps have been constructed using mapping populations from a variety of crosses and markers, including classical mutations, allozymes, RAPDs, RFLPs, SSRs, AFLPs, retrotransposons, and gene anchored ESTs and SNPs (Weeden and Marx 1987; Ellis *et al*. 1992; Dirlewanger *et al*. 1994; Gilpin *et al*. 1997; Laucou *et al*. 1998; Remeau *et al*. 1998; Weeden *et al*. 1998; Irzykowska *et al*. 2001; Ellis and Poyser 2002; Loridon *et al*. 2005; Aubert *et al*. 2006). Because some of the markers were common, the maps developed for different mapping populations or marker sets could be integrated to give rise to reference or consensus maps. The current genetic linkage map of pea has seven linkage groups corresponding to its $n = 7$ chromosomes and is a result of integration of different maps on account of common markers. Some of the common markers in the integrated maps are RAPD markers (Gilpin *et al*. 1997; Laucou *et al*. 1998; Irzykowska and Wolko 2004). Carefully selected RAPD markers give reproducible results and have proved valuable in map construction and mapping of specific traits.

The *mfp* linkage group of 26 DNA markers constructed in the present study comprises of one SSR, two ISSR and 23 RAPD markers. Because it has the RAPD markers L19-700 and J14-850 mapped by Laucou *et al*. (1998) and Remeau *et al*. (1998) on linkage group IV, RAPD marker L18-900 mapped by Gilpin *et al*. (1997) on linkage group IV and RAPD markers OPJ14a and OPG12 placed on linkage group IV by Irzykowska *et al*. (2001) and Irzykowska and Wolko (2004), *mfp* linkage group of the present work must be linkage group IV of the consensus genetic map of pea genome.

The linkage map positions of the leaf-blade-architectural mutations *af, tl, uni, stp* and *cri* and stipule morphology mutations *st* and *coch* are known; they map on linkage groups I, V, III, VII, V, III and V, respectively (Ellis and Poyser 2002). In the present study, the *mfp* mutation has been mapped on the linkage group IV. Among the listed genes, *UNI, AF, TL* and *MFP* determine leaf blade ramification and *ST* and *COCH* control stipule growth and development, yet no two members of these two groups map on the same linkage groups. It appears that in *P. sativum*, the genes whose functions are inter-related in leaf blade or stipule development are not closely clustered but widely dispersed in the genome.

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