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RESEARCH ARTICLE

Fine mapping of an *Arabidopsis thaliana* male sterile mutant *EC2-157*

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Abstract An *Arabidopsis thaliana* male sterile mutant *EC2-157* has been isolated using an EMS mutagenesis strategy. Genetic analysis indicated that it was controlled by a single recessive gene called *ms157*. No pollen grains have been observed in mutant anthers. *ms157* Has been mapped to a region of 74 kb located in BAC clone T6K22 on chromosome IV using a map-based cloning strategy. As no male sterile genes have been reported in this region, *ms157* could be a novel gene related to fertility. The further molecular cloning and functional analysis on this gene should facilitate our understanding of *A. thaliana* anther development.

Keywords *EC2-157*, anther development, male sterile, map-based cloning, *Arabidopsis thaliana*

1 Introduction

Anther and pollen development is an important aspect of plant functional genomics project. Mutations of genes necessary for anther and pollen development would result in male sterility. Thus, the investigation of anther and pollen development has biological importance and significant commercial interest to produce hybrid seeds. The screening of male sterile mutants and isolation of genes from them have been used as a tool to investigate the processes of anther and pollen development.

Male sterile (ms) phenotype has been observed in many species of higher plants (Rick, 1948; Van and Wirtz, 1968; Albertson and Phillips, 1982; Kaul, 1988). It is related with anther structural aberrations, defect in dehiscence, functional defects associated with gametogenesis and specifically meiosis (Aarts et al., 1993; Zoe, 2001). Most of reported male sterile genes are cloned from *Arabidopsis* for the advantage of its small genome, short life cycle, easy screening and identification of mutants (Feldmann, 1991; Forsthoefel et al., 1992; Chandhury, 1993). It is estimated that about 3 500 genes specifically are expressed in the anther (Sanders et al., 1999), of which 60–100 genes have a specific effect on male fertility. Mutations in any of theses genes cause male sterility (Wilson, 2001). More than 20 male sterile genes have been cloned from *Arabidopsis* and these can be classified into three groups. The first is related with jasmonic acid biosynthesis. The second encodes transcription factors. The third is associated preferentially with male meiosis. There are also some other genes related with male sterility such as *EMS1/EXS*, *TPD1*, *APT1* (Zhao et al., 2002; Canales et al., 2002; Yang et al., 2003; Gailard et al., 1998).

Map-based cloning is one of the important gene cloning methods used in *Arabidopsis*, and it has become a common tool in *Arabidopsis* molecular genetics. In this study, we screened a novel *Arabidopsis thaliana* male sterile mutant *EC2-157* using ethyl methane sulfonate (EMS) mutagenesis strategy. It shows short siliques and has a defect in pollen development. Genetic analysis and cytological observation have been done and a map-based cloning strategy was used to clone the corresponding gene.

2 Materials and methods

2.1 Plant materials

Plants of *A. thaliana* (ecotype Landsberg erecta and Columbia) were used. The *EC2-157* mutant was provided by Professor Huang Hai of the Institute of Plant Physiology and Ecology, Chinese Academy of Sciences. Before phenotypic analysis, *ms157* had been backcrossed to wild-type *Ler* 3–4 times. Seeds were sown on vermiculite and allowed to imbibe for 3 days at 4℃. Plants were grown at 22℃ under a 16-hour-light/8-hour-dark photoperiod.

2.2 Molecular markers

The (In/Del), single-sequence length polymorphism (SSLP)

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and single-nucleotide polymorphism (SNP) molecular markers of FCA7, F21C20, T13K14, F7K2, T10C21, T16L1, F7J7, F17L22, T6K22 (HindⅢ) and T6K22 (*Eco*RⅤ) were designed based on Cereon database (http://www.arabidopsis.org/). All primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd.

2.3 Cytological observations

Flower buds at different developmental stages were fixed overnight in FAA (ethanol 50% (v/v), acetic acid 5.0% (v/v), and formaldehyde 3.7% (v/v)), dehydrated in a graded ethanol series (50% × 2, 60%, 70%, 80%, 90%, 95% and 100% × 2), transferred to xylene, ultimately embedded in paraffin. Anther transverse section were then stained in toluidine blue at 42ºC for 30 minutes and observed under an Olympus BX51 microscope. The images were taken with the Olympus DX51 digital camera.

2.4 Mapping of male-sterility gene

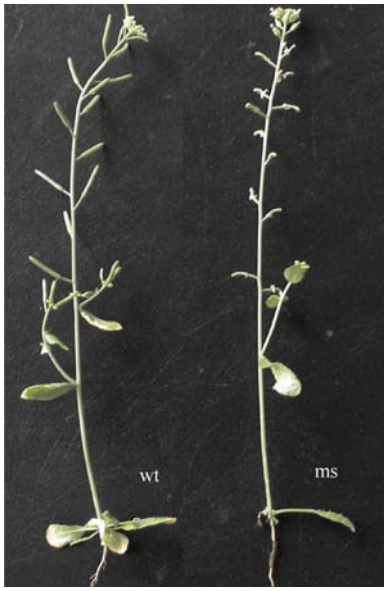
In order to map the MS157 gene, an F2 population from a cross between MS157 (ecotype Ler) and Col was used. For first-pass mapping, a total of 50 F2 mutant plants were pooled to prepare DNA for polymerase chain reaction (PCR)-based mapping with In/Del markers. DNA was isolated from lyophilized tissue as described by Reiter et al. (1992). PCR amplifications were carried out in 30-μL reactions containing 100 ng genomic DNA, 0.15 mL of each primer, 1.5 mL MgCl2, 10 mL Tris-HCl (pH 8.3), 50 mL KCl, 200 mL of each deoxyribonucleoside triphosphate, and 2.5 units of Taq DNA polymerase (TaKaRa). The standard thermal profile included an initial denaturation for 5 minutes at 95°C followed by 45 cycles at 94°C for 20 seconds, 52°C for 20 seconds, and 72°C for 20 seconds.

3 Results

3.1 Isolation and identification of the *ms157* mutant

The male sterile mutant *EC2-157* was screened using the EMS mutagenesis strategy in *Arabidopsis* ecotype Landsberg erecta. It showed short siliques and there was no pollen in the developed anther (Fig. 1). Although the *EC2-157* mutant was completely male sterile, it exhibited normal vegetative and floral development.

To elucidate the biological functions of *ms157* in anther development, the anther of both *ms157* and wild type were examined under light microscope. In *Arabidopsis*, anther development has been divided into 14 stages based on morphological landmarks of cellular events under the light microscope (Sanders et al. 1999). From stage 1 to 5, anther primordia underwent cell division to establish lateral structure, and microsporocytes were formed. No detectable



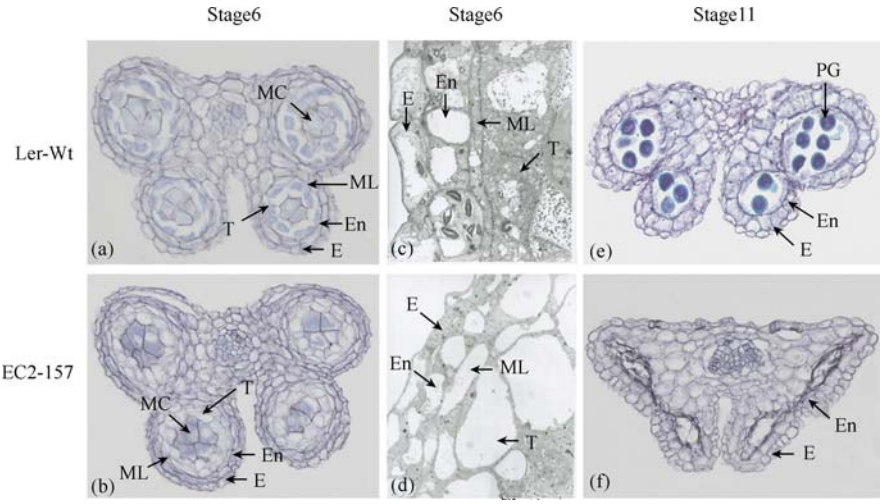
Left: wild-type plant with normal fertility (wt); Right: *ms157* mutant with small and short siliques without seeds inside (ms)

Fig. 1 Male sterile mutant *ms157*

difference between the *ms157* mutant and wild type was observed during these stages (data not shown). At stage 6, the middle layer in wild-type anthers degenerated during meiosis and it was left only as a crushed remnant between the tapetal and endothecial cell layers at stage 7 (Fig. 2a). There are few small vacuoles in middle layer cells. By contrast, the middle layer of *ms157* was not degenerated at stage 6 and 7. On the other hand, there were large vacuoles in the middle layer cells (Fig. 2d). In the wild-type plant, the tapetal cells become vacuolated and the microsporocytes further underwent meiosis (Fig. 2a). The *ms157* tapetum enlarged significantly with large vacuoles at the onset of meiosis (Fig. 2b, 2d). In wild-type anthers, individual microspores were released from tetrads after degeneration of the callose cell walls by callase and developed further into pollen grains during stages 8 to 12. From stages 12 to 14, dehiscence occurred and pollen grains were released. Although tetrads could be observed in *ms157* anther locules, there were no free microspores in the anther of the mutant after stage 7 (Fig. 2f). These microspores were degraded thereafter. The cytological observation of *ms157* showed that *ms157* regulated middle layer and tapetum cell development and might play an important role in pollen development.

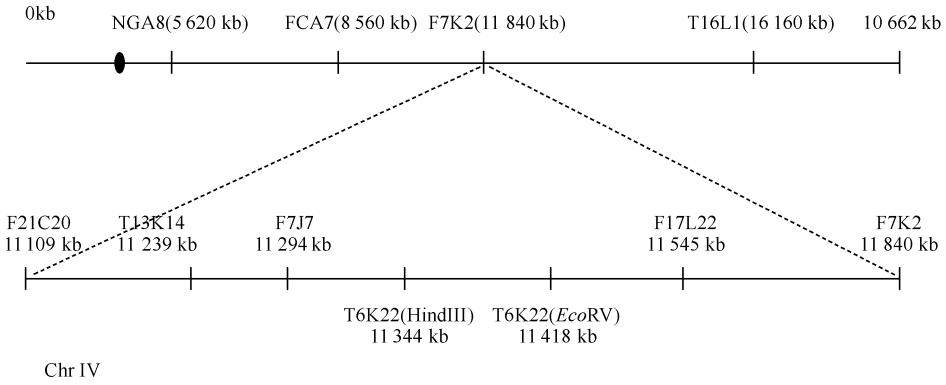
3.2 Genetic analysis

In order to examine the segregation ratio of the male-sterility phenotype, the mutant plant was backcrossed with wild-type pollen (ecotype Landsberg erecta) and self-pollinated. The F1 plants from a cross between the sterile mutant and the wild-type had normal fertility, and the fertility and sterility plants in the F2 population segregated at 3:1. This suggested that the male sterile phenotype of *ms157* inherited as a single recessive mendelian locus.



(a, c): Wild-type anther at stage 6 showing the collapse middle layer and vacuolated tapetum;
(b, d): *ms157* anther at stage 6 showing the middle layer and tapetum cells with large vacuoles;
(e): Wild-type anther at stage 11 with the pollen mitotic division occurred and tapetum degenerated;
(f): *ms157* anther at stage 11 without pollen grains formed in the locule;
E: Epidermis; En: Endothecium; MC: Meiocyte cell; ML: Middle layer; PG: Pollen grains; T: Tapetum

Fig. 2 Cytological observation of anther development of wild-type (a, c, e) and *ms157* (b, d, f) plants



The *ms157* gene was mapped to a region of 74kb between SNP markers T6K22 (HindIII) and T6K22 (*Eco*RV) on chromosome 4

Fig. 3 Fine mapping of the *ms157* gene

3.3 Molecular mapping of the *ms157* gene

A genetic population was generated from a cross between *ms157* and an *Arabidopsis* ecotype, Columbia, and a map-based cloning approach was used to identify the *ms157* gene. For the first-pass mapping, a total of 24 In/Del and SSLP markers were used (Table 1) and 50 individual F2 mutant plants were used for initial linkage analysis. The *ms157* gene was linked to In/Del marker F7K2 on chromosome 4. For further fine mapping, we developed four new markers near the marker of F7K2 (Table 1) based on Cereon database (http://www.arabidopsis.org/), and a population containing more than 2 000 male sterile progeny was used. Finally, *ms157* was mapped to a region of 74kb between SNP markers T6K22 (HindIII) and T6K22 (*Eco*RV) on chromosome 4 (Fig. 3).

4 Discussion

Anther and pollen development are an area of major biological importance in plant developmental biology. It can be divided into 2 phases and 14 stages (Goldberg et al., 1993; Sanders et al., 1999). The first phase (stages 1–7) includes cell specification, tissue differentiation, meiosis and spore formation. The second phase includes pollen grain and sperm cell differentiation, anther enlargement, filament extension, dehiscence and pollen release. Cytological observation of *ms157* shows that *ms157* regulates middle layer and tapetum development at the first phase, subsequently affecting pollen development at the second phase. During anther development, both middle layer and tapetal cells undergo programmed cell death, and middle layer degenerates earlier than tapetal cells. The tapetal cells are the

Table 1 Molecular markers used for first-pass mapping

Chromosome	Primer	Location	Primer sequences
Chr. 1	T28K15	4 083 880 bp	F: TGCTACGGAAGAAAAAGAGTGA R: TCGGATTAGCGCAGAGGG
	F13K23	4 382 009 bp	F: CTTGATAGTCATTAATAC R: AGAACTTGCTTGCTATGC
	F26F24	8 304 550 bp	F: TTGGTTGCTCTAAATCAC R: TTGCCAATAATAAGAAGG
	F5J5	13 533 310 bp	F: TTGTCTTCTTCATGGCTTACCT R: TCTTTTACATTTGTATCCGAAC
	F7A10	20 680 791 bp	F: TTCTTCCACCGCAAATCAGT R:TTCAACCAAGGACGCCATCT
	NGA111	27 356 874 bp	F: TGTTTTTTAGGACAAATGGCG R: CTCCAGTTGGAAGCTAAAGGG
Chr. 2	F5G3	1 944 525 bp	F: CTTGGCAACACGCTTTCT R: GGGGTGATGCAGCTAAAA
	T24I21	7 290 768 bp	F: TTCCCGCCAAAAATACACAG R: AGGCCCATAAACTCAAAACCA
	F3N11	10 963 408 bp	F: CACGCACCCTTCTACTCTTT R: AACTATATTGGAATTTGACACAT
	F12L6	16 473 094 bp	F: GGAAATGAAACCAGAAGAAGATAA R: GAGATAGGAAGAAAGGGAAAGACC
	MAG2	4 694 414 bp	F: CTTCCATGGCGTCAGAT R: TTACCGAAAGAACAAAAG
Chr. 3	CIW11	9 775 545 bp	F: CCCCGAGTTGAGGTATT R: GAAGAAATTCCTAAAGCATTC
	CIW4	18 901 818 bp	F: GTTCATTAAACTTGCGTGTGT R: TACGGTCAGATTGAGTGATTC
	NGA6	23 042 025 bp	F: ATGGAGAAGCTTACACTGATC R: TGGATTTCTTCCTCTCTTCAC
	NGA8	5 628 810 bp	F: TGGCTTTCGTTTATAAACATCC R: GAGGGCAAATCTTTATTTCGG
	FCA7	9 595 870 bp	F: CATAGCGTATGGTGGACC R: ATATCGGGTCAATTTCCG
Chr. 4	F21C20	11 158 482 bp	F: TATGCCAACCTCCAAAAC R: AACATAGCTAGCGCTACG
	F17L22	11 545 273 bp	F: CCACGGGTTTATCACATC R: ATTCCTGAAGACAATCCAT
	T10C21	14 985 540 bp	F: ATGGCTGCAGCGAATAAC R: GCAACGATGTGGTGCTGG
	T16L1	16 168 316 bp	F: GTCATTCGCCAAGATTCC R: TTGGTTTTCTAGCCCATG
	T22D6	2 584 878 bp	F: TGAACACTTTGTGATGAC R: GGATTATTCCATCGTTGC
Chr. 5	NGA139	8 428 136 bp	F:GGTTTCGTTTCACTATCCAGG R:AGAGCTACCAGATCCGATGG
	CIW9	17 061 229 bp	F: CAGACGTATCAAATGACAAATG R: GACTACTGCTCAAACTATTCGG
	MCO15	22 416 234 bp	F: CGAGGGCTACAGGTAAAGGAAT R: AATGTAACGACTAACGAGGTAAAA
	MHJ24	25 655 260 bp	F: GTTCCGCGTGCTTTCTAC R: TCTGCATGGCTTACCCTC
Molecular markers used for fine-scale mapping			
Chr. 4	T13K14	11 238 967 bp	F:ATTTGATTGATAACCCTTTG R: CTTCCACTACTGCTGGCTTT
	F7J7	11 293 774 bp	F: GCTTAGTTGATGATTCCC R: AGTGGCTGAAAAGGAGAA
	T6K22(HindIII)	11 343 831 bp	F:TCAAGTATCATCCACGGC R: GTCAAGGGTAGTCGCAGC
	T6K22(*Eco*RV)	11418021bp	F:CATTAATACCTTTTGGGAAA R: CAGCACTGAGGATGAAATAGA

innermost of the four sporophytic layers of the anther wall that comes in direct contact with the developing gametophyte. Therefore, it has been considered to play an essential role in pollen production, which provides nutrients for the developing microspores and secretes material that forms the pollen exine as well as pollen coat (Pacini et al., 1985; Mascarenhas, 1990; Goldberg et al., 1993). Several genes are reported to regulate tapetal cell development (Wilson et al., 2001; Sanjay et al., 2002; Shu et al., 2003). In this work, tapetal development was also affected by the *ms157* gene. However, very few genes are reported to regulate middle-layer development. As *ms157* also affects middle-layer

development, *ms157* may play an important role in anther development.

ms157 has been mapped to a region of 74kb on chromosome 4 using a map-based cloning strategy (Fig. 3). BAC clone T6K22 covers this region. The mapped region contains about 12 genes and none of them is reported to be necessary for anther development. Thus, ms*157* should be a novel gene regulating anther development. Further cloning and functional analysis of this gene is being undertaken.

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