halfman, an *Arabidopsis* male gametophytic mutant associated with a 150 kb chromosomal deletion at the site of transposon insertion.

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Abstract

To identify genes that play an important gametophytic role during pollen development, an *Arabidopsis* transposon (DsE) mutagenised population was screened for marker segregation ratio distortion. We report the characterisation of a male gametophytic mutant termed *halfman* (*ham*) that results in an aborted pollen phenotype in mature anthers. The genetic transmission efficiency of DsE was 6.6% through the male and 98.4% through the female, which suggested that *HAM* may encode essential male-specific component(s) required for pollen development. Molecular analysis of the insertion site revealed a single copy of the DsE element inserted into the second exon of the *RLK5* gene that was adjacent to a large (~150 Kb) genomic deletion. The deleted region is predicted to encode 38 genes and to include one or more genes with important function(s) during pollen maturation and seed development.

Keywords

Introduction

The mature male gametophytes of flowering plants are the products of an elaborate developmental programme involving sporophytic and gametophytic gene expression. Although molecular analyses have demonstrated that a large number of genes are expressed specifically or preferentially in pollen (reviewed in Twell 2002), there are few reports demonstrating the functional importance of identified gametophytically expressed genes (Muscietti et al.1994; Xu

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et al. 1995; Sanderfoot et al. 2001; Gupta et al. 2002). Genetic or mutational approaches provide the opportunity to carry out functional screens for gametophytically acting genes important for completion of pollen development. T-DNA insertional mutagenesis, combined with segregation ratio distortion screens, have identified male and female gametophytic mutants in *Arabidopsis thaliana* (Feldmann et al. 1997; Bonhomme et al., 1998; Howden et al. 1998; Procissi et al., 2001). Similarly, transposon insertional mutagenesis has also proven successful in isolating mutants that affect diverse developmental processes in *Arabidopsis* (Sundaresan et al. 1995; Budziszewski et al. 2001), including gametophyte development (Moore et al., 1997)

Although gene-tagging is an effective tool, genomic rearrangements can occur during the transfer process. T-DNA-associated chromosomal rearrangements involving inversion, translocation and deletion have been reported (Nacry et al. 1998; Kaya et al. 2000; Tax and Vernon 2001). Although it has been suggested that deletions may occur in association with Ds in *Arabidopsis* (Budziszewski et al. 2001) substantial DNA rearrangements associated with transposon insertions have not been detailed.

We have screened a Ds enhancer-trap population to identify mutants affecting gametophyte development in *Arabidopsis thaliana*. Here we describe a male-specific gametophytic mutation that is associated with a large chromosomal deletion at the site of transposon insertion. Our data suggest that the deleted region includes one or more genes that have important functions during pollen and seed development.

Materials and methods

Mutant screen and growth conditions

The *Ds* population was generated as described in Sundaressan et al. (1995).

Gametophytic mutants were selected from a transposon-mutagenized population by segregation

ratio distortion as described by Howden et al. (1998) except that 50 µg/ml kanamycin was used

for selection on plates. Histochemical analysis of pollen and genetic transmission analysis were

carried out as described by Park et al. (1998). For developmental analysis more than 1000 spores

were counted from progeny derived from five independent parental lines.

Molecular analysis

Genomic DNA extraction and Southern analysis were carried as described in Howden et

al. (1998). For thermal asymmetric interlaced (TAIL) PCR, each of three degenerate primers

(AD1, AD2, AD3) (Liu et al., 1995) were used in combination with 5' or 3' end-specific DsE

primers as described by Grossniklaus et al. (1998). PCR products were purified using a

QIAquick Gel Extraction Kit (Qiagen, Germany) and sequenced using ABI Big Dye Terminator

Cycle sequencing Kit. Flanking sequences were analysed using BLASTn at the National Centre

for Biotechnology Information (NCBI).

Results and discussion

Isolation of the male-specific gametophytic mutant *halfman*

Progeny seeds from 1,500 DsE enhancer trap lines harbouring a kanamycin resistance

marker (Km[R]) were screened for segregation ratio distortion. Five putative gametophytic mutant

lines showing reproducible segregation ratios approaching 1:1 were identified. Phenotypic

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screening of these lines led to the identification of one line (ET 3696) termed *halfman* (*ham*) that displayed approximately 35% aborted pollen in mature anthers with a collapsed phenotype (Fig. 1A, B). Collapsed pollen grains in *ham* were not viable (Fig. 1C, D). The four other lines (to be described separately) did not show changes in pollen morphology implying their functions during the progamic phase.

Genetic analysis of *ham* showed 1:1 segregation for kanamycin resistance (Table1). Strict cosegregation of the *ham* phenotype and kanamycin resistance was also observed. Self-progeny of heterozygous *ham* plants showed 1:1 segregation for the collapsed pollen phenotype. Moreover, all progeny from segregating sibling plants without collapsed pollen were sensitive to kanamycin (n=1343) but all progeny from *ham* plants showed 1:1 segregation for kanamycin resistance (Km^R: Km^S =865: 924).

The genetic transmission of *ham* was determined in F1 progeny from reciprocal test crosses between wild type and *ham*. The transmission of *ham* was normal through the female, but was strongly reduced through the male to only 6.6% (Table 1). Therefore, *ham* is the result of a gametophytic mutation acting specifically during pollen development. Tetrad analysis in the *quartet* (*qrt*) background further confirmed that *ham* is an incompletely penetrant gametophytic mutation. In particular, +/+;*qrt1*/*qrt1* plants showed only 0.9 % collapsed pollen (n=1088), however +/*ham*;*qrt1*/*qrt1* plants showed 33.2 % aberrant pollen, with 42.6 % of tetrads showing two aberrant spores and 40.9 % a single aberrant spore (n=1408).

Plants homozygous for *ham* appear to be embryo lethal, since 3.4 % of seeds present in mature green siliques of *ham* heterozygotes were smaller, white and eventually aborted seeds (n=782, not shown). This is similar to the predicted frequency (3.3%) of *ham* homozygote embryos.

No significant morphological differences between *ham* and wild-type pollen were observed in microspores or in pollen at the early bicellular stage. The first appearance of aberrant pollen occurred during mid-bicellular stage after detachment of the generative cell from the pollen wall (Fig. 2B). At this stage ~35 % of pollen grains from +/*ham* plants showed evidence of cytoplasmic degeneration including membrane separation from the pollen wall, and at tricellular stage, extensive vacuolation (Fig. 2C middle). The collapsed pollen phenotype was first observed at tricellular stage and was associated with the complete loss of cellular contents (Fig. 2C right).

Since the collapsed pollen phenotype was only 35 % in *ham* heterozygotes, a further 15% of the pollen population are predicted to harbour the mutant allele, but show wild-type morphology. If all of these 15 % are functional, the male transmission efficiency of *ham* is predicted to be 30 %. However, the observed male transmission efficiency was 6.6 %. This implies a further role for *HAM* during the progamic phase.

Molecular analysis reveals a single copy of DsE element in *ham*.

Southern analyses using GUS or NPTII probes showed that a single copy of DsE was present in *ham* heterozygotes, but was absent from wild-type siblings (data not shown). Genomic sequences flanking the DsE element were rescued using TAIL PCR. Sequence analysis of the PCR products showed that the 5' end of DsE element was inserted adjacent to the second exon of the *RLK5* gene (At4g28490) on BAC F20O9. However the 3' end of DsE was inserted into a putative gene (At4g28830) on F16A16, located two BACs south of *RLK5* (Fig.3A). This suggested that ~150Kb of genomic DNA was deleted immediately adjacent to the 3' end of DsE. Southern analysis with GUS and NPTII probes confirmed the predicted sizes of 5' DsE- and 3'

DsE-junction fragments, based on the sequenced ends of the single DsE insertion (data not shown and Fig.3A).

Sequence analysis revealed an additional eight nucleotides (GTTTAGAC) at the 5' junction between RLK5 and the 5' end of DsE (Fig. 3B), however, no extra nucleotides were present at the 3' junction between DsE and native genomic sequence. Furthermore, three nucleotides of the Ds inverted repeat were deleted at the 5' end of Ds in *ham*. In this regard, it was reported that only 44% of transposon insertions in Arabidopsis contained authentic inverted repeat elements and a target site duplication (Ito et al., 2002). Moreover, the existence of a large DNA deletion combined with Ds insertion has recently been suggested based on the rescue of Ds plant flanking sequences of a Ds enhancer trap line (ET4401) (Budziszewski et al., 2001).

The deleted region in *ham* is predicted to contain 38 genes according to TIGR/MIPS annotation (Fig. 3C) suggesting that the *ham* phenotypes are caused by the lack of one or more of these genes. Moreover, the deleted region does not contain non-redundant genes important for megagametogenesis. Studies of the consequences of loss of function mutations will be required to establish whether one or more of these deleted genes contribute to the developmental defects observed during pollen and seed development in *ham*.

Acknowledgements

Figure Legends

Fig. 1 Mature pollen phenotype of wild type (Ler) and *ham*. Upper panels show brightfield images and lower panels show the same pollen grains visualised using DAPI (A, B) or FDA staining (C, D). Wild type (A, C), *ham* (B, D). Scale bar shown in (A) is 20µm. All images are at the same magnification.

Fig. 2 Morphology of isolated microspores and pollen grains during development in *ham*. Light panels show the morphology and dark panels DAPI staining at early bicellular (A), late bicellular (B), and tricellular (C) stages. Scale bar shown in (A) is 10µm. All images are at the same magnification.

Fig. 3 Map of the enhancer trap element (DsE) and the region deleted in *ham*. Neomycin phosphotransferase (*NPTII*) and a modified GUS marker gene in the DsE are indicated (Sundaresan et al, 1995). The ~150kb deleted region is indicated by a dashed line (A). Sequence of the original Ds element (Up) and the insertion site of *ham* (Bottom) are shown (B). The plant flanking sequences are shown in lowercase, three deleted nucleotides in the inverted repeat as dots, and eight extra nucleotides underlined. (C) List of predicted genes in the deleted region of *ham*.

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Table 1. Genetic transmission analysis of *ham*

	Crosses	Km^R	Km^S	TE
Self	+/ *ham* x +/ *ham*	312	298	-
Female	+/ *ham* x +/+	250	254	98.4
Male	+/+ x +/ *ham*	17	258	6.6

Km^R: Number of plants resistant to kanamycin

Km^S: Number of plants sensitive to kanamycin

TE: Transmission efficiency represents the percentage of *ham* alleles successfully transmitted through male or female gametes. TE= Km^R/ Km^S x100
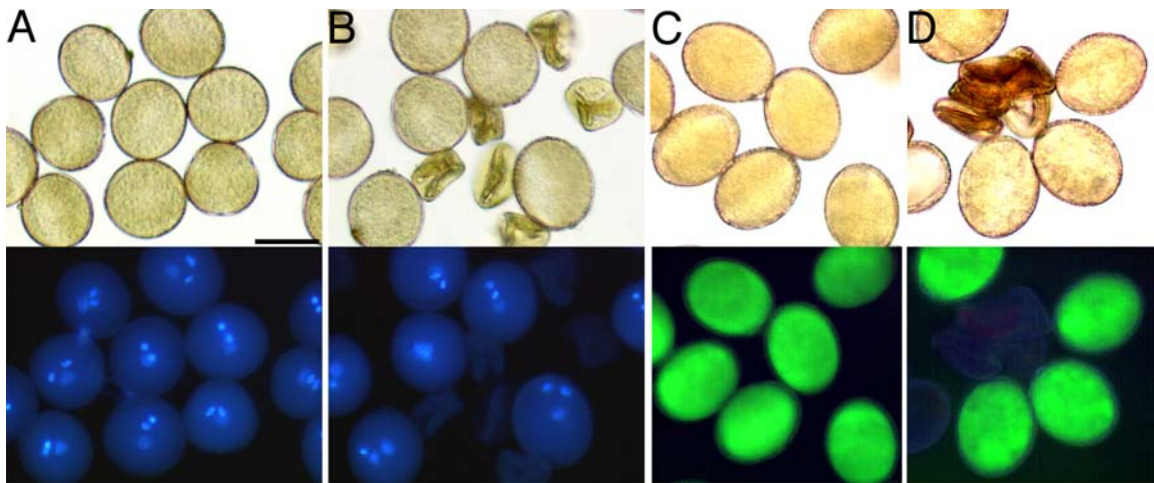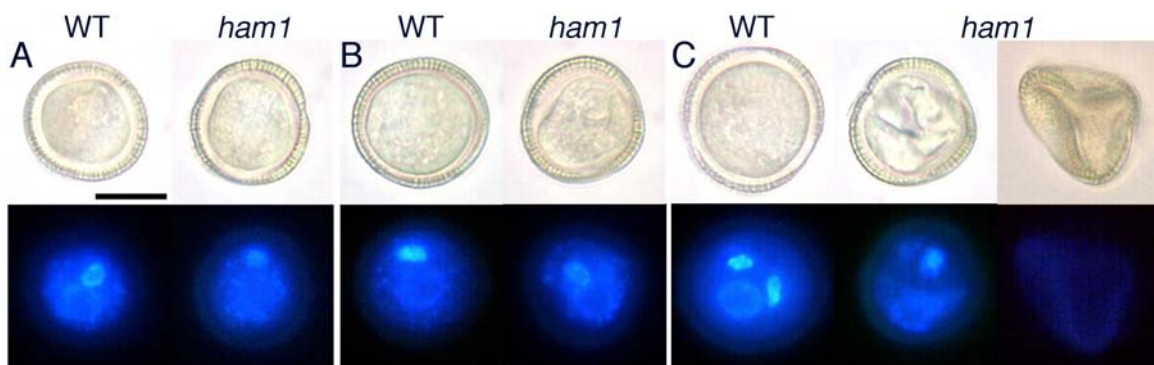
Figure 1

Figure 2

Figure 3

(A)

(B)


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              5' CAGGGATGAAAGTAG (DS) CCGTTTTCATCCCTA 3'
ctctaaaGTTTAGAC - - - GGATGAAAGTAG (DS) CCGTTTTCATCCCTAgtggtac
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(C)

At4g28490	RLK5	At4g28680	AA decarboxylase-like
At4g28500	Predicted protein	At4g28690	Hypothetical protein
At4g28510	Prohibitin-like	At4g28700	Ammonium transporter-like
At4g28520	12S seed storage	At4g28703	Unknown protein
At4g28530	NAM/CUC2-like protein	At4g28706	Unknown protein
At4g28540	ADK1-like	At4g28710	Myosin heavy chain-like
At4g28550	Putative protein	At4g28720	Putative protein
At4g28560	Putative protein	At4g28730	Putative protein
At4g28570	Predicted GPI anchor	At4g28740	Putative protein
At4g28580	Putative protein	At4g28750	PSI subunit PSI-E
At4g28590	Hypothetical protein	At4g28760	Unknown protein
At4g28600	Putative protein	At4g28770	Unknown protein
At4g28610	Putative protein	At4g28780	Proline rich APG-like
At4g28620	ABC transporter-like	At4g28790	Putative transcription factor
At4g28630	ABC transporter ATM1	At4g28800	Putative transcription factor
At4g28640	Auxin-inducible protein	At4g28811	Putative transcription factor
At4g28650	Receptor kinase-like	At4g28815	AtbHLH127
At4g28660	PS II protein W-like	At4g28820	Putative protein
At4g28670	Ser/Thr kinase-like	At4g28830	Putative protein