Outcrossing as an explanation of the apparent unconventional genetic behaviour of

*Arabidopsis thaliana hth* mutants.

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Abstract

The reappearance of HTH alleles in the offspring of homozygous Arabidopsis hth mutants is not consistent with classical Mendelian genetics. It has been suggested that stored RNA may be used to restore genetic information. However, Peng et al. reported that hth mutants tend to display outcrossing and suggested that outcrossing might provide an alternative explanation for the apparent genetic instability. We have confirmed and extended these results, corroborating that the apparent non-Mendelian behaviour of hth mutants can be explained by their susceptibility to outcrossing.
In *Arabidopsis*, the reappearance of HTH (HOTHEAD) alleles from the grandparents in the offspring of homozygous *hth* mutants reported by Lolle *et al.* is not consistent with classical Mendelian genetics (Lolle *et al.* 2005). Several explanations have been put forward to explain this unexpected reversion of single nucleotide polymorphisms. Lolle *et al.* suggested that RNAs synthesized by the parent may be stored in plants of subsequent generations that do not carry the corresponding genomic information, and that these RNAs may be used as a template to restore the genomic information carried by the previous generation. Four alternative hypotheses have been proposed to account for this non-Mendelian behavior: two of them are also based on template-directed gene conversion (Chaudhury 2005; Ray 2005), the third one appeals to a process of mutations accumulation followed by selection (Comai and Cartwright 2005; Henikoff 2005) and the fourth one involves chimerism (Krishnaswamy and Peterson 2007). However, Peng *et al.* (Peng *et al.* 2006) reported that *hth* mutant shows a tendency to outcrossing and recover a normal genetic behaviour when grown in isolation. The authors suggested that this propensity to outcross may provide an alternative explanation for the apparent genetic instability of *hothead* mutants. Nevertheless, Lolle *et al.* (2006) argued that outcrossing, while possible, could not be the sole source of "reversion" (Lolle *et al.* 2006) leaving open the debate whether *hothead* is a true genetic outlaw (Gawrylewski 2008; Pennisi 2006).

In order to measure the level of outcrossing in *hth* mutant compared to wild type, we grew each of them next to (10 cm) a plant homozygote for a transgene conferring resistance to hygromycin. In the offspring of wild type no hygromycin resistant plant was found among 2980 (MS media, hygromycin 30mg/L). Thus *Arabidopsis* wild type plants are highly resistant to cross-pollinisation, at least in our growth conditions. On the contrary, *hth* plants
grown in the same context produced 12.1% of hygromycin-resistant plantlets (hth-4: 60/377; hth-8: 36/224; hth-10: 51/611), unambiguously demonstrating the unusual high susceptibility of hth mutant to cross-pollinisation.

To test if this characteristic of hth mutant could be the cause of its apparent genetic instability we grew hth mutants either in isolation or at various distances from the hygromycin-resistant plants and quantify the appearance in the hth progeny of “revertant” plants (i.e. showing a wild type phenotype and not the typical abnormal flower of hth mutants) (see Materials and methods). When grown in complete isolation no revertant was found in the offspring of the mutants among 9944 plants (table 1). At 10cm and 20cm, hth plants produced 12.4% and 6.9% of revertants, respectively (table 1). At 50cm and 150cm this proportion dropped drastically to less than 0.1% (table1). The proportion of revertant is thus dependant on the availability and distance to HTH wild type pollen grain source. Strikingly, all the 430 revertants found were carrying the transgene conferring the resistance to hygromycin that can be originated only from the pollen grain donor, demonstrating that all these "reversion" events are caused by outcrossing.

Lolle et al (2005) detected 2 HTH/HTH embryos among 141 dissected out of fruits developing on selfed hth plants, suggesting that hth plants can produce HTH ovules, which is not consistent with the outcrossing explanation. However, among 92 plantlets obtained from the same self cross, none were HTH/HTH. Moreover, when hth plants were crossed as female with wild type pollen, no HTH/HTH plants was found among 230 tested (LOLLE et al. 2005), thus not confirming the ability of hth plants to produce HTH ovules. In order to test again this ability, we used previously isolated "revertants" carrying the transgene conferring the resistance to hygromycin. These plants are the results of a cross between hth-8 mutant as female and hygromycin-resistant plants (HTH/HTH) as male. The genotype of these plants, determined by PCR-based assay (LOLLE et al. 2005), was systematically HTH/hth (337/337).
and never $HTH/HTH$. Thus the two crossing experiments using $hth$ plants as female did not reveal any ability of $hth$ mutant to produce $HTH$ ovule (0/230 and 0/337).

The transmission through pollen grains of a wild-type $HTH$ allele from a homozygous mutant ($hth/hth$) described previously (Lolle et al. 2005) is also not consistent with the outcrossing explanation. We tested this transmission through pollen grains by crossing $hth$ mutants as male with $HTH$ plants as female. In order to get rid of contamination or self pollinisation of the female plant, crosses were done in isolation and a male sterile plant ($bm3$) (Gaillard et al. 1998) was used as female. The genotype of the progeny was determined by PCR-based assay (Lolle et al. 2005). Under these conditions, 100% ($hth-4$: 168/168; $hth-8$: 92/92; $hth-10$: 92/92) of the F1 progeny was heterozygote for $hth$ allele. Thus we did not reveal any ability of $hth$ mutant to produce $HTH$ pollen grains.

In summary: (i) $hth$ plants are highly susceptible to outcrossing. (ii) Reversion is dependant on the presence of wild type allele donor plant in the vicinity and its frequency is correlated to distance between $hth$ and donor plants. (iii) Revertant systematically carries genetic information that can be provided only by outcrossing. (iii) As expected under genetic laws (Mendel 1866), we did not confirmed that $hth$ plants can be a source of pollen or ovule bearing an $HTH$ allele. Altogether these results strongly argue for the outcrossing as the reason for the apparent genetic instability in $hth$.

Materials and methods

Arabidopsis plants were cultivated in a greenhouse with a photoperiod of 16 h/day and 8 h/night, a temperature of 20°C and humidity at 70%. The $hth-4$, $hth-8$ and $hth-10$ mutants were generously provided by Dr Pruitt (Purdue University) and are in the Landsberg accession. The hygromycine resistant plant carried the selection marker $hpt$, (Granger and Cyr 2001) at position 1095540 bp on chromosome 5. To measure the frequency of reversion
in *hth* mutants, we grew F2 population derived by selfing of *hth* heterozygous plants and selected *hth* mutants according to the shape of the first flowers. The first inflorescence was then cut to eliminate any possible prior pollen contamination, notably from heterozygote or wild type sister plants. *hth* mutants were then placed either in isolation in a Plexiglas cabinet or at various distances from a line of hygromycine resistant plants. The offspring of these plants were scored for the wild type phenotype ("revertant"). The presence of the hygromycine transgene in the "revertant" plants was tested by PCR (5'-TTCCTAAAAACCAAAATCCAG 3'; 5'- ATCAATTGTAGATCCGGCAAACA 3') with appropriate controls. Genotyping of *HTH* locus was performed with specific primers and digestion to reveal the presence or absence of a polymorphic restriction site; *hth*-8: 5'-TTGGAGAAACTTGCTTACCCGATCT 3'; 5'- TTGTTTCCAAGTCTCTCCCGAAGAA 3'; ScrF1; *hth*-4: 5'-CGAAGCTGGTGAGGAGTCTCGT 3'; 5'-GTGACCCATAAGCTCCACTAGATAA 3'; Hpy99I; *hth*-10: 5'-ATCAACACCAGGCTTTACTCTCGAA 3'; 5'- TAGAGATCGGGTAAGCAAGTT 3'; MmeI.
Bibliography


Table 1. Phenotypic reversion of hth mutants

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<tr>
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<th>D=0.1 M</th>
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<td>10 243 1638 12.9</td>
<td>9 157 2123 6.9</td>
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<td>9 3 3732 0.1</td>
<td>35 0 9040 0</td>
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<td>- nd</td>
<td>3 1 195 0.5</td>
<td>3 0 632 0</td>
<td>3 0 245 0</td>
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<tr>
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<td>- nd</td>
<td>- nd</td>
<td>- nd</td>
<td>3 0 659 0</td>
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Homozygous hth plants were grown in isolation or at various distances from HTH allele donor plants. Progeny from these populations were scored for plants with the wild-type phenotype [wt] and the hothead phenotype [hth]. The percentages shown are the percentages of [wt] plants among the whole population ([wt]+[hth]). N: Number of mother plants used. nd: not determined.