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Note

Outcrossing as an Explanation of the Apparent Unconventional Genetic Behavior 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 behavior 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.* (2005) is not consistent with classical Mendelian genetics. Several explanations have been put forward to explain this unexpected reversion of single nucleotide polymorphisms. Lolle *et al.* (2005) 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 mutation accumulation followed by selection (Comai and Cartwright 2005; Henikoff 2005), and the fourth one involves chimerism (Krishnaswamy and Peterson 2007). However, Peng *et al.* (2006) reported that *hth* mutant shows a tendency toward outcrossing and recover a normal genetic behavior 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 (Pennisi 2006; Gawrylewski 2008).

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 hygromycine. In the offspring of wild type, no hygromycine-resistant plant was found among 2980 (MS media, hygromycine 30 mg/liter). Thus Arabidopsis wild-type plants are highly resistant to cross-pollination, at least under our growth conditions. On the contrary, *hth* plants grown in the same context produced 12.1% of hygromycine-resistant plantlets (*hth-4*: 60/377; *hth-8*: 36/224; *hth-10*: 51/611), unambiguously demonstrating the unusual high susceptibility of the *hth* mutant to cross-pollination.

To test if this characteristic of the *hth* mutant could be the cause of its apparent genetic instability, we grew *hth* mutants either in isolation or at various distances from the hygromycine-resistant plants and quantified 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). When grown in complete isolation, no revertant was found in the offspring of the mutants among 9944 plants (Table 1). At 10 and 20 cm, *hth* plants produced 12.4 and 6.9% of revertants, respectively (Table 1). At 50 and 150 cm, this proportion dropped drastically to $<0.1\%$ (Table1). The proportion of revertant is thus dependent on the

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TABLE 1

Phenotypic reversion of *hth* mutants

	$D = 0$, 1 m				$D = 0$, 2 m				$D = 0$, 5 m				$D = 1$, 5 m				Isolation			
	N	Wild type	*hth*	% wild type	*N*	Wild type	*hth*	% wild type	*N*	Wild type	*hth*	% wild type	*N*	Wild type	*hth*	% wild type	*N*	Wild type	*hth*	% wild type
hth-8	10	243	1638	12, 9	9	157	2123	6, 9	10	2	3253	0, 1	9	3	3732	0, 1	35	0	9040	0
hth-4	3	24	255	8, 6	–	ND	ND	ND	3	1	195	0, 5	3	0	632	0	3	0	245	0
hth-10	–	ND	ND	ND	–	ND	ND	ND	–	ND	ND	ND	–	ND	ND	ND	3	0	659	0

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 (wild type) and the *hothead* phenotype (*hth*). The percentages shown are the percentages of wild-type plants among the whole population (wild type + *hth*). *N*, number of mother plants used. ND, not determined.

availability and distance to the *HTH* wild-type pollen grain source. Strikingly, all 430 revertants found were carrying the transgene conferring the resistance to hygromycine 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 two *HTH/HTH* embryos among 141 dissected from 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* plant was found among 230 tested (Lolle *et al.* 2005), thus not confirming the ability of *hth* plants to produce *HTH* ovules. To test this ability again, we used previously isolated "revertants" carrying the transgene conferring the resistance to hygromycine. These plants are the results of a cross between the *hth-8* mutant as female and hygromycine-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. To get rid of contamination or self-pollination 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 F_1 progeny was heterozygous for the *hth* allele. Thus we did not reveal any ability of the *hth* mutant to produce *HTH* pollen grains.

In summary,

i. *hth* plants are highly susceptible to outcrossing.
ii. Reversion is dependent on the presence of a wild-type allele donor plant in the vicinity and its frequency is correlated to the distance between *hth* and donor plants.
iii. A revertant systematically carries genetic information that can be provided only by outcrossing.
iv. As expected under genetic laws (Mendel 1866; Druery and Bateson 1901), 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 outcrossing as the reason for the apparent genetic instability in *hth*.

Arabidopsis plants were cultivated in a greenhouse with a photoperiod of 16 hr/day and 8 hr/night, a temperature of 20°, and humidity at 70%. The *hth-4*, *hth-8*, and *hth-10* mutants were generously provided by R. Pruitt (Purdue University) and are in the Landsberg accession. The hygromycine-resistant plant carried the selection marker *hpt* (Granger and Cyr 2001) at position 1,095,540 bp on chromosome 5. To measure the frequency of reversion in *hth* mutants, we grew a F_2 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 heterozygous 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′-TTCCTAAAACCAAAATC CAG-3′ and 5′-ATCAATTGTAGATCCGGCAAACA-3′) with appropriate controls. Genotyping of the *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′ and 5′-TTGTTTCCAAGTCTCTCCCGAAGAA-3′; ScrF1; *hth-4*, 5′-CGAAGCTGGTGAAGGAGTCGT-3′ and 5′-GT GACCCAATAGCTCCACTAGATAA-3′; Hpy99I; *hth-10*,

5′-ATCAACACCAGGCTTTACTCTCGAA-3′ and 5′-TAGAGATCGGGTAAGCAAGTT-3′; *Mme*I.

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