Mutation in Arabidopsis *HIT1* locus causing heat and osmotic hypersensitivity

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Summary

A mutant strain of *Arabidopsis thaliana* var. Columbia whose seedling development was 2 to 3 °C more sensitive to high temperature inhibition than wild type was isolated. This mutant (*hit1*, for *h*eat-*i*ntolerant) did not show any phenotypic change when grown at 22 °C. However, incubation at 37 °C for 4 days was determined to be lethal for the mutant but not for wild-type plants. Leaves of wild type respond to high temperature by becoming erect, but by comparison leaves of the *hit1* mutant remain horizontal and show a slightly wilty appearance. Furthermore, seedling development in *hit1* mutants was more sensitive to osmotic stress imposed by exogenous mannitol (>300 mmol/L). These data imply that *HIT1* may be responsible for water status regulation by which plants survive heat stress. Genetic analysis shows that the *hit1* phenotype is conditioned by a single recessive, nuclear mutation, and the *HIT1* locus was closely linked to the *nga280* microsatellite marker on chromosome 1.

Introduction

The synthesis and accumulation of a variety of heat shock proteins (HSPs) that function as molecular chaperones are undoubtedly significant molecular events leading to the recovery from sublethal heat shocks and leading to the acquisition of tolerance to subsequent lethal heat shock (for review, see Vierling 1991, Schöffl et al. 1998). However, there is additional evidence that other molecular, biochemical, and physiological events beside the synthesis of HSPs also may play a role in tolerance to heat stress (Cherry et al. 1994, Nover 1994), and defining the genetic determinants of both

HSP-mediated and/or alternative pathways has not yet significantly advanced. We have investigated the possibility of defining mutants that are more thermosensitive than wild type, in an attempt to define those genes important to basal thermotolerance in *Arabidopsis thaliana*.

Here we report the isolation of the first such heat intolerant mutant *hit1*. The *hit1* mutant was identified by looking for seedlings that were able to germinate but not able to mature at high temperature. Genetic analysis showed that *hit1* is a single, recessive nuclear mutation. Its cross sensitivity to osmotic stress and its inability to show erect leaves during heat stress suggest that the normal function of the mutated locus may be required to adjust the water status in plant, and this adjustment is crucial for plants to survive during heat stress.

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Materials and Methods

Plant material and mutant isolation

Arabidopsis thaliana (ecotype Colombia) carrying the homozygous genetic marker *gl1* (glabra) is referred to as the wild type throughout this report. Both wild type and ethyl methanesulfonate (EMS)-mutagenized seeds were obtained from Lehle Seeds Inc. (Round Rock, TX). Plant growing conditions were as described unless specified (Wu et al. 1996).

EMS-mutagenized M_2 seed were sown on agar plates and allowed to germinate at 30 °C for 1 week. Seeds that failed to display aerial organs (opened, green cotyledons) were moved onto new agar plates and placed at 22 °C for another 10 days. Seeds that could recover from previous heat stress and become fully-developed seedlings (putative mutants) were subsequently transferred to soil and grown to maturity. Heat sensitivity of the obtained M_3 seeds was retested again by germination at both low (22 °C) and high (30 °C) temperature for one week. Those plants whose progeny demonstrated a germination rate equal to wild type at 22 °C but lower than wild type at 30 °C were considered true mutants, and backcrossed to wild type at least once before further study.

Measurement of temperature and osmotic sensitives

The temperature sensitivity of seedling development was determined by incubating Petri plates containing seeds at various temperature (22–37 °C). The seed germination rate was determined as the percentage of seeds showing visible protruding radicles, and the seedling maturation rate was determined as the percentage of seeds showing fully opened, green cotyledons after one week. Survival of matured seedlings upon heat stress was tested by transferring entire plates containing 10-day-old seedlings to higher temperature conditions (30–37 °C). A critical temperature and duration that could unambiguously discriminate mutant from wild type individuals was determined to be 37 °C for four days. These conditions were used in subsequent genetic analysis.

For osmotic sensitivity measurement, seedling maturation rate was determined by sowing seeds on agar plates supplemented with various mannitol levels. The number of seedlings showing expanded cotyledons after 2 weeks incubation at 22 °C was determined. For the root elongation assay, seeds were sown on mannitol-free plates which were placed vertically to allow the roots to grow on the agar surface. After 4 to 5 days incubation at 22 °C, seedlings with roots 1 to 1.5 cm in length were carefully transferred to plates containing various mannitol levels. Plates were again placed vertically to allow roots to continuously grow. The increase of root length was measured daily for up to 7 days. At least two replicated plates were used for each temperature and mannitol treatment.

Genetic analysis

Crosses between mutant and wild type were accomplished by transferring pollen from the mutant to the stigma of emasculated wild type flowers. The F_1 and F_2 plants were tested for their heat sensitivity by planting seeds on agar plates at 22 °C for 10 days followed by treatment at 37 °C for 4 days.

For mapping of the mutated locus, mutants in the Columbia *gl-1* background were crossed to plants of the Landsberg *erecta* background. F_2 plants were self-pollinated to produce the F_3 generation for the survivability assay described above. DNA of individual mutant plants from the F_2 generation was isolated for gene-mapping using microsatellite markers (Bell and Ecker 1994) or using cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel 1993).

Results and Discussion

Upon imbibition, the development of an *Arabidopsis* seedling begins with the protrusion of the radicle (seed germination) followed by the expansion and greening of cotyledons (seedling maturation). Elevated temperatures interrupt this ongoing developmental process. On agar plates no reduction was found in either germination or maturation rates for wild-type *Arabidopsis* seeds at temperatures from 25 °C up to 30 °C. However, while most seeds were still able to germinate, seedling maturation was completely arrested at 35 °C. Subsequent transfer of 35 °C-germinated seeds back to 25 °C permitted their normal development. Observing for seedlings unable to complete maturation at a high but normally permissive temperature, e.g. 30 °C, allows temperature sensitive mutants to be screened.

About 20,000 EMS-mutagenized M_2 seeds were screened for heat hypersensitive mutants by examining their seedling development at 30 °C for 7 days. One selected mutant, *hit1*, was later confirmed by retesting seedling development in the M_3 generation. The heat sensitivity of *hit1* mutant seedlings during development is shown in Figure 1. Germination of the *hit1* mutant was completely inhibited at 35 °C, while wild type germination was fully inhibited only at 37 °C (Fig. 1 A). For seedling maturation, the temperatures at which complete inhibition occurred was 32 °C for *hit1* and 35 °C for wild type (Fig. 1 B). Thus, the *hit1* mutant was 2 to 3 °C more sensitive to heat inhibition than wild type during seed germination and seedling maturation.

Because heat sensitivity may depend on the stage of seedling development, we determined whether *hit1* was also hypersensitive to heat at the subsequent stages of seedling development. For 10-day-old plants, i.e. with one visible leaf pair, the *hit1* mutant was totally bleached after 4 days exposure to 37 °C while wild-type seedlings remained green in color (Fig. 2). Subsequent transfer of plants from this stress treatment to room temperature allowed the growth of wild type, but not *hit1* (data not shown). This treatment was subsequently employed for genetic analysis. Results indicated that *hit1* is a single, recessive, nuclear mutation (Table 1) and the *HIT1* locus was mapped to chromosome 1 closely linked (1.1% recombination frequency) to *nga280* marker (Table 2).

When mature plants, with 6–8 rosette leaves, were exposed to 37 °C, leaves of wild-type plants lifted upwards to an erect position whereas *hit1* leaves failed to do so (Fig. 3). Maximum erectness of wild-type leaves was reached within 24 hours after the initiation of treatment and was maintained
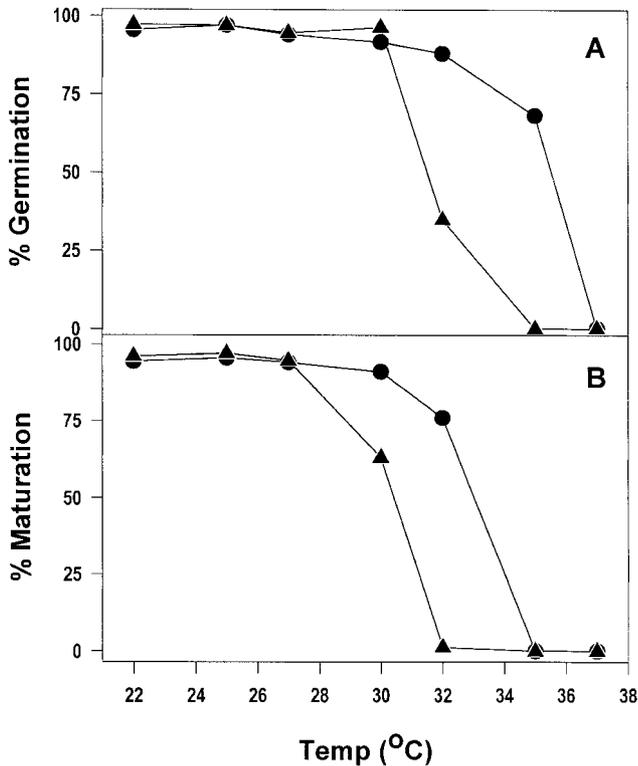
Figure 1. Seedling development of the *hit1* mutant is 2~3 °C more sensitive to heat inhibition than wild-type seedlings. Seeds were sown on agar plates and incubated at various temperatures. Percentage of germination (**A**) was determined as the percentage of seed that showed radical emergence, and percentage of maturation (**B**) was determined as the percentage of seedlings that showed opened cotyledons within one week. Data are obtained from a sample size of ~100 seeds at each temperature tested. ●, wild type; ▲, *hit1*.

plant responses to cold, salt, as well as heavy metals (Hugly et al. 1990, Howden and Cobbett 1992, Wu et al. 1996, Liu and Zhu 1997, Liu and Zhu 1998). Nonetheless, using such a genetic approach to study plant responses to heat stress has been limited. One reason for this is that mutants resulting in changes in the thermal stability of any protein involved in basic cellular functions such as metabolism could potentially generate a temperature sensitive phenotype. However, the *hit1* mutant presented here does not support this assumption since the heat-hypersensitivity always cosegregates with the osmotic hypersensitive phenotype which requires no involvement of temperature. The heat hypersensitivity of the *hit1* mutant is also unlikely to be in defect of transpirational cooling because seed germination and seedling development are also inhibited by high temperature. Rather, the defect of the *hit1* mutant most likely lies in the mechanism for regulating the water status, especially the aerial portion of the plant, that is essential for plant survival and development during heat and osmotic stresses. Results from a recent study in which enhanced thermotolerance in both seed germination and seed-

as long as the plants remained at the high temperature. Maintaining erect leaves in herbaceous plants may rely on the buildup of turgor pressure, particularly in cells of leaf bases. This implies the establishment of a new water status within the aerial portions of the wild-type plants, and prompts us to further examine the water-regulating capability of the *hit1* mutant upon osmotic stress.

As shown in Figure 4 A, none of the *hit1* seeds were able to develop into mature seedlings when the concentration of mannitol was above 300 mmol/L. For wild-type seeds, a mannitol concentration for complete inhibition of seedling development occurred at greater than 400 mmol/L. Although *hit1* seeds failed to develop into mature seedlings on high mannitol media (300–400 mmol/L), they were still able to germinate. Figure 4 B shows that there is little difference in root growth between wild type and *hit1* at high mannitol concentrations. This result suggests that the osmotic hypersensitivity of the *hit1* mutant may involve water relation only in the aerial portion but not in the root of the plant.

Functional genetics involving isolation and characterization of hypersensitive mutants, has provided new insight into

Table 1. Genetic Analysis of Arabidopsis *hit1* mutant.

Strains or Crosses ($♀ × ♂$)	Generation	Total	Tolerant[a]	Sensitive[a]	$\chi^{2\,b}$
Wild-type Columbia		551	551	0	
hit 1/hit 1		520	0	520	
Wild-type × *hit 1/hit 1*	F_1	37	37	0	
	F_2	530	412	118 (22.3%)	2.097[b]

[a] Tolerant or sensitive was determined by viability of 10-day-old seedlings at 37 °C for 5 days.
[b] The calculated χ^2 value was based on the expected ratio of 3:1 for T:S individuals, assuming that *hit 1* was a single recessive mutation ($P > 0.05$).

Table 2. Genetic Mapping of *hit 1*.

Marker	Chromosome	n^a	Recombination Frequency[b]
nga 63	I	40	50.0±5.6
nga 280	I	44	1.1±1.1
GPA 1	II	35	37.1±5.8
nga 168	II	35	40.0±5.9
nga 126	III	44	59.0±5.2
nga 8	IV	44	39.8±5.2
AG 4	IV	44	40.9±5.2
nga 76	V	34	51.5±6.1
nga 225	V	30	50.0±6.5

[a] Number of individual F_2 analyzed.
[b] Calculated by the Kosambi function (Koornneef and Stam, 1992).
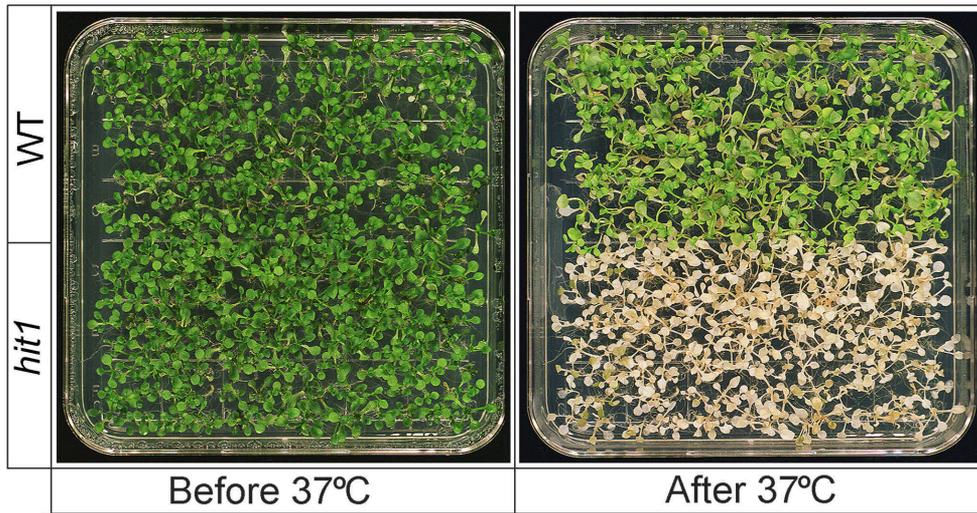
Figure 2. Incubation at 37°C for 4 days was lethal to *hit1* but not wild type. Ten-day-old media-grown plants, with the first pair of leaves, were transferred to 37°C and incubated for four days. WT, wild type.
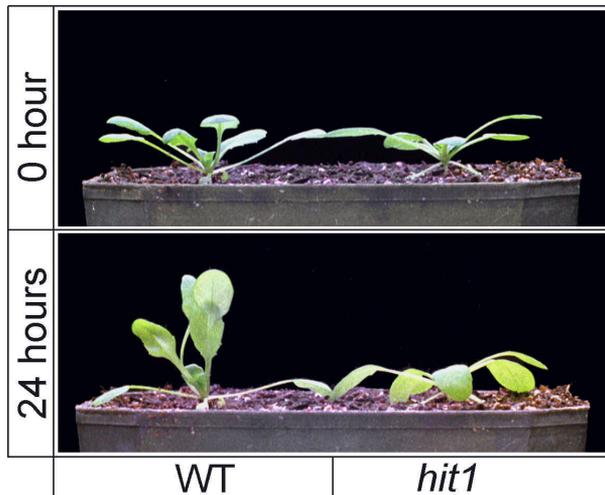
Figure 3. Soil-grown *hit1* plants failed to lift their leaves upwards to an erect position as did wild-type plants. Plants with 6–8 rosette leaves were transferred to 37 °C and incubated for 24 hours.
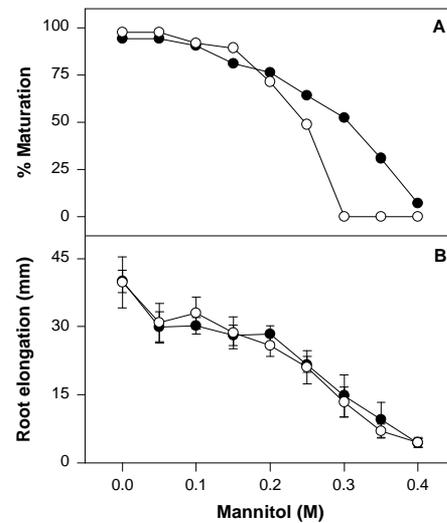
Figure 4. Seedling development of *hit1* was more sensitive to osmotic stress inhibition. (**A**) Seeds were sown on agar plates containing various concentrations of mannitol as an osmoticum. The percentage of seedling maturation was calculated from the number of seedlings that showed green, opened cotyledons within two weeks from the beginning of the experiment. Data are obtained from a sample size of ~100 seeds at each temperature tested. (**B**) Root elongation of *hit1* and wild type seedlings was measured to quantify sensitivity toward osmotic stress. Root elongation data 7 days after transfer to mannitol containing plates is shown. ●, wild type; ○, *hit1*. Each point represents the means ± SE (n=10).

ling maturation was achieved by overproduction of glycine-betaine support such a notion (Alia et al. 1998).

In summary, the isolation of the *hit1* mutant demonstrates the feasibility of a molecular genetic approach to study plant heat stress responses. The *hit1* mutant is valuable for understanding the mechanisms by which plants cope with heat stress at both the molecular and whole plant levels. The cross sensitivity of *hit1* to osmotic stress also provides us with the opportunity to examine the interaction between thermal and osmotic stress responses.

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