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## Mutation in a homolog of yeast Vps53p accounts for the heat and osmotic hypersensitive phenotypes in Arabidopsis hit1-1 mutant.

Lee CF, Pu HY, Wang LC, Sayler RJ, Yeh CH, Wu SJ

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New Finding

### Comments

**This is the first study to show that the tethering complex of the vesicle trafficking machinery is crucial for tolerance to physical stresses in eukaryotes.** Starting with a mutant of Arabidopsis thaliana sensitive to heat and water stress, the group of S-J Wu in Taiwan has isolated the gene responsible, HIT1 (Heat InTolerant), by map-based cloning. HIT1 is homologous to yeast VPS53, encoding a component of the tethering complex involved in vesicle trafficking between the Golgi and the endosomal/prevacuolar compartment. The original hit1-1 allele was a point mutation leading to a Ser-to-Tyr amino acid substitution, which probably introduced thermolabile properties to the protein and partial loss of the original function. Attempts to generate a homozygous double knockout mutant failed because of defective male-specific transmission (haploid pollen grains with the mutation were not fertile). Interestingly, the yeast vps53 null mutant, which is viable, is sensitive to heat stress and thermotolerance can be restored by expression of the Arabidopsis HIT1 gene. These results indicate that the very complicated mechanism for vesicle trafficking in eukaryotic cells constitutes an Achilles heel for heat stress.

**Competing interests:** None declared
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**Planta 224 (2006) 330-338**

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### Faculty of 1000 Biology　生科吳少傑論文獲選

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發表於 2007-03-05 08:56:05

公告單位: 秘書室新聞組

中央大學生命科學系吳少傑助理教授，2006年發表於Planta期刊之論文，最近獲得國際生物學類論文評選組織Faculty of 1000 Biology 推薦爲必讀之優良文章。吳老師以研究植物的耐熱機制爲主。在地球溫室效應逐漸擴大之際，試圖找出農作物耐熱、耐旱的機制，這方面的研究獲得國際間重視。

Faculty of 1000 Biology，爲一個新的研究工具，由世界一千位左右的首席生物學家分別挑選出期刊優良文章作推薦，便於外界更快速掌握生命科學的研究趨勢。評選的標準不是以期刊的排名論高下，而是完全根據文章的科學價值，獲選的都是極具價值之文章。

吳少傑助理教授等人合力撰寫之「Mutation in a homolog of yeast Vps53p accounts for the heat and osmotic hypersensitive phenotypes in Arabidopsis hit1-1 mutant.」在世界諸多文章中被推薦爲must read article。

吳少傑說，當前因世界可耕地面積的減少，了解植物抗高溫逆境的機制，找出提高農作物耐熱能力的方法，顯得迫切需要。他於2002年在生科系成立「植物逆境遺傳學實驗室」，從植物的栽種、突變、雜交、轉殖，小心呵護每一株植物，到之後的分子生物及遺傳工程實驗，歷經長達三年半的努力，展現出研究成果。

該研究是透過功能性遺傳學及基因定位的方法，來株選出與植物耐受高溫逆境有直接關連的基因。當植物內部負責抵禦高溫逆境的基因，因突變而喪失功能，帶有此突變基因的植物，就會對高溫逆境過度敏感，也就是失去對高溫逆境的耐受能力。藉由對高溫逆境敏感型突變植物的篩選，吳少傑的實驗室成功的從阿拉伯芥篩選出一個對高溫逆境過度敏感的突變種植物，並找出造成該突變性狀的遺傳因子。研究過程更進一步發現，該遺傳因子也會影響植物利用環境水分的能力。

他說，植物因無法自由行動，因此需要許多機制來適應環境的變遷。植物看似不動地生長，卻潛藏著很旺盛的生命力。而從該研究所找出的基因，「幸運地得到了新的線索」，指出植物耐受逆境的許多機制之中，包含了過去未被驗證過的囊泡繫鏈因子(vesicle tethering factor)。這個線索本身即是一個重要的新發現，也爲植物與環境逆境這方面的研究，提供了新的出發點。

「研究的樂趣之一在了解生命的運作，及其演化的精神。」吳少傑說，植物和動物一樣必須在複雜多變的環境中求生存。有趣的是，我們常說，「先天不良、後天失調。」不僅人類如此，植物也等同此道理。好的基因，如果後天培育不良，也會導致不好的結果。後天的栽培，著實也是一個關鍵因素。

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ORIGINAL ARTICLE

Chai-Fong Lee · Hsin-Yi Pu · Lian-Chin Wang
Ronald J. Sayler · Ching-Hui Yeh · Shaw-Jye Wu

# Mutation in a homolog of yeast Vps53p accounts for the heat and osmotic hypersensitive phenotypes in Arabidopsis *hit1-1* mutant

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**Abstract** Previously, the growth of Arabidopsis *hit1-1* (heat-intolerant) mutant was found to be inhibited by both heat and water stress (Wu et al. in J Plant Physiol 157:543–547, 2000). In order to determine the genetic mutation underlying the *hit1-1* phenotype, map-based cloning of *HIT1* gene was conducted. Transformation of the *hit1-1* mutant with a *HIT1* cDNA clone reverts the mutant to the heat tolerance phenotype, confirming the identity of *HIT1*. Sequence analysis revealed the *HIT1* gene encodes a protein of 829 amino acid residues and is homologous to yeast (*Saccharomyces cerevisiae*) Vps53p protein. The yeast Vps53p protein has been shown to be a tethering factor that associates with Vps52p and Vps54p in a complex formation involved in the retrograde trafficking of vesicles to the late Golgi. An Arabidopsis homolog of yeast Vps52p has previously been identified and mutation of either the homolog or *HIT1* by T-DNA insertion resulted in a male-specific transmission defect. The growth of yeast *vps53Δ* null mutant also shows reduced thermotolerance, and expression of *HIT1* in this mutant can partially complement the defect, supporting the possibility of a conserved biological function for Vps53p and HIT1. Collectively, the *hit1-1* is the first mutant in higher plant linking a homolog of the vesicle tethering factor to both heat and osmotic stress tolerance.

**Keywords** Heat stress · Osmotic stress · Yeast Vps53p protein · Heat intolerant mutant · Vesicle trafficking · Vesicle tethering factor

**Abbreviations** SNARE: Soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptor · SSLP: Simple sequence length polymorphism · CAPS: Cleaved amplified polymorphic sequence · SNP: Single nucleotide polymorphism · HSP: Heat shock protein · LEA: Late embryogenesis abundant · COR: Cold regulated · VPS: Vesicular protein sorting · RT-PCR: Reverse transcription-polymerase chain reaction

## Introduction

Plants are immobile; hence, their environment constantly affects their growth and development. Extremes in environmental parameters create stressful conditions that can arrest plant growth and even reduce survivability. Understanding how plants respond to stress will help to explain many fundamental questions in plant biology. Because the adverse effects from stress inevitably lead to reduced productivity, elucidating plant responses to environmental stress is a critical step towards increasing stress tolerance in crops through genetic engineering (Mitra 2001; Wang et al. 2003).

High temperatures and water deficit are among the major environmental limitations of plant growth and survival. However, plants have the genetic potential to cope with these stresses. The study of environmental stress-induced gene expression has revealed the activation of a large set of genes that lead to the accumulation of stress-specific proteins in plant cells. Heat shock proteins (Hsps) and late embryogenesis abundant (LEA) proteins are two major types of proteins whose cellular levels are induced by high temperatures and water stresses (Schöffl et al. 1998; Ramanjulu and Bartels 2002; Sun et al. 2002; Sung et al. 2003; Wang

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et al. 2003). Despite evidence for their molecule-protecting function, current strategies for detecting stress-induced gene expression may not be able to identify changes in mRNAs that are expressed at low levels and/or proteins that might play important roles in signal transduction and gene regulation. Besides, because the effects of high temperatures and water stress upon plants are often interconnected (Rizhsky et al. 2004; Wang and Huang 2004; Tester and Bacic 2005), plants may incorporate various responses to cope with these stresses. For example, transpirational cooling can ameliorate heat stress, but it demands water usage. Sufficient water supply is prerequisite for maintaining cotton leaf temperature at a constant level (Upchurch and Mahan 1988; Burke and Upchurch 1989). Accumulation of solutes to maintain cell turgor has also been associated with heat tolerance in cotton (Ashraf et al. 1994). Drought-preconditioned osmotic adjustment can enhance plant heat tolerance as well (Jiang and Huang 2001). Reversely, high-temperature preconditioning can promote osmotic adjustment to increase leaf pressure potential (Morales et al. 2003). Some plants may employ paraheliotropic leaf movement to achieve balance between leaf temperature and transpirational water loss, and changing leaf orientation requires changing the water status in the pulvini (Fu and Ehleringer 1989; Yu and Berg 1994; Wang et al. 2004). Moreover, both high temperatures and water stress can alter the physical properties of the cell membrane and cause loss of function in the embedded proteins. Therefore, it has been suggested that membrane and protein recycling through intracellular vesicular traffic is necessary for healing the injury caused to membranes by environmental stress, and that there are multifunctional sensors and cross-talk among lipid-based signaling pathways that regulate plant responses to high temperatures and water stress (Levine 2002; Jenkins 2003; Meijer and Munnik 2003; Wang et al. 2003; Los and Murata 2004). However, how plants coordinate these various responses to heat and water stress, and the molecular and genetic factors that dictate such responses are unknown.

Previously, we isolated an Arabidopsis heat-intolerant (*hit1-1*) mutant that is hypersensitive to sustained high temperature. Incubation at 37°C for 4 days was lethal for the mutant but not wild-type plants. In addition to its inability to survive at sub-lethal high temperatures, seedling development in *hit1-1* mutants was more sensitive to osmotic stress as well. Furthermore, while the wild-type leaves respond to high temperature by becoming erect, leaves of the *hit1-1* mutant remain horizontal. These data suggest that the function of the mutated locus may be involved in the cross-protection between high temperatures and water stress (Wu et al. 2000). To determine the genetic mutation underlying the *hit1-1* phenotype, map-based cloning was conducted and we report here the identification of the *HIT1* gene.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Colombia (Lehel Seeds, Round Rock, TX, USA) is referred to as the wild type throughout this paper. The *hit1-1* mutant line was isolated from the $F_2$ progeny of plants mutagenized with ethyl methanesulfonate as described (Wu et al. 2000). The T-DNA insertion alleles *hit1-2* and *hit1-3* were obtained from Max-Planck Institute, Cologne, Germany (Rosso et al. 2003) and Arabidopsis Biological Resource Center at Ohio State University, Columbus, Ohio, respectively. Plants were either grown in soil or on agar plates with constant illumination at 23°C.

### Gene mapping of *hit1* locus

Genetic analysis of dominance was performed using the survivability assay by incubating 10-day-old seedlings at 37°C for 4 days. This condition was determined to be lethal for the *hit1-1* but not wild-type plants. For gene mapping, the *hit1-1* mutants were outcrossed with wild-type plants of the Landsberg *erecta* ecotype (Lehel Seeds, Round Rock, TX, USA) by transferring pollen from the mutant to the stigma of emasculated wild-type flowers. Both $F_1$ and $F_2$ 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 and these mutants were scored for cosegregation using genetic markers. Initial mapping was carried out using publicly available simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequences (CAPS) markers (http://www.Arabidopsis.org). For fine-mapping, more than 20 new SSLP and CAPS markers were developed based on the insertions/deletions (INDELs) and single nucleotide polymorphisms (SNPs) between the publicly available Columbia sequences and Landsberg *eracta* sequences. Among them, SSLP markers CER465523 and CER465525, encompassing a 43 kb DNA region at the lower end of the chromosome I, showed minimal recombination with *HIT1* and were detected with primers 5′-GACCTAAAGCTGATGATGATGGT-3′, 5′-CGGAGGGAAGAATGAAGAACAT-3′ and 5′-CAGACAGGGGATTTAACAGTCGT-3′, 5′-CCATCTTCCTGTACTCTGCGTAT-3′, respectively. Further details are available on request.

### Cloning of *HIT1* cDNA and plant transformation

The 43 kb DNA region from *hit1-1* flanked by the CER465523 and CER465525 markers was sequenced and compared to that of wild-type plant to identify the *HIT1* gene. For cloning, DNAse-treated RNA isolated from rosette leaves was used as template to retrotranscribe first-strand cDNA with oligo(dT) primers. The

*HIT1* cDNA was then amplified with gene-specific primers 5′-AGTCATGAATAAGTCGAGTGCTTTA-GAGTA-3′ and 5′-CCCACCTTTGTTTCCTTC-CCCGGG-3′ (*Bsp*HI and *Sma*I sites are underlined). The amplified products were resolved by electrophoresis, gel purified, and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). After digestion with *Bsp*HI and *Spe*I, the *HIT1* cDNA was subcloned into pCAMBIA1305 (CAMBIA, Canberra, Australia) between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator. *Agrobacterium tumefaciens* strain GV3101 was used to deliver this construct into *hit1* plants by vacuum infiltration (Clough and Bent 1998). Transgenic plants were selected on Murashige and Skoog (MS) agar medium containing 25 μg/ml Hygromycin B for 14 days. Resistant T1 seedlings were transferred to soil and grown to maturity. Homozygous T2 plants were selected by examination of their T3 generations through Hygromycin B. T3 seeds derived from homozygous T2 plants were used for subsequent complementation test.

## Heat and osmotic sensitivity assay

For heat tolerance assay, 10-day-old medium grown seedlings were transferred to 37°C for 4 days and then returned to normal growing temperature (23°C). This treatment has been determined to be lethal for *hit1-1* mutants but not wild-type seedlings (Wu et al. 2000). For osmotic sensitivity assay, seeds were surface sterilized and sown on MS agar plates supplemented with various concentrations of mannitol as the osmoticum. Seeds were allowed to germinate at 23°C for 14 days to examine the ability of seedlings showing green, expanded cotyledons.

## Genotyping of T-DNA insertion *hit1* mutants

According to supplier's data, T-DNA was inserted at the ninth exon of *HIT1* in *hit1-2* mutant line. PCR was hence performed on DNA from *hit1-2* to detect the insertion using *HIT1*-complemented forward primer (5′-ATTATTCCCGTGCCAAGTAGG-3′) and T-DNA left border complemented reverse primer (5′-CCC-ATTTGGACGTGAATGTAGACAC-3′) to amplify a 601 bp DNA fragment corresponding to the junction sequence. Control PCR was performed as well using the same forward primer with *HIT1*-complemented reverse primer (5′-GCTGGAACGGATTTTTATTTCTGG-3′) to amplify a 909 bp undisruptive *HIT1* sequence. Amplified fragments were separated by agarose gel electrophoresis and visualized following ethidium bromide staining to determine the genotype of the plants. Similar strategy was also applied to examine the genotype of the *hit1-3* lines with *HIT1*-complemented forward primer (5′-CCAAACCAGCTCATTGTCATT-TTG-3′), *HIT1*-complemented reverse primer (5′-GC-CTATACGGCACATGCCAAG-3′), and T-DNA left border complemented reverse primer (GCGTGGAC-CGCTTGCTGCAACT-3′).

## Gene expression analysis

For tissue-specific gene expression analysis, cDNA was synthesized from total RNA extracted from soil-grown plant tissues with Superscript RT (Life technologies, Carlsbad, CA, USA) according to manufacturer's protocol. For stress-mediated gene expression analysis, seeds were surface sterilized and sown on MS agar (1.2%) plates, whose agar surface was covered with a layer of nylon mesh, and placed vertically to allow the roots to grow on the surface. After 10 days incubation at 22°C, entire plates were transferred into 37°C for 24 h or the mesh was transferred to new plates containing mannitol as the osmoticum for 1 day. After stress treatments, seedlings were immediately frozen in liquid nitrogen and ground to powder for RNA extraction and cDNA synthesis as describe above. For RT-PCR, cDNAs were amplified using the *HIT1* specific primers 5′-CAAATTACAAGTCATGAATAAGTCGAGTGC-3′ and 5′-GATCTTCTTTAGCTTTAGTTCCCGA-3′ with 23 cycles of PCR. In the meantime, the poly-ubiquitin gene (*UBQ10*) was amplified using primers 5′-AGAAGTTCAATGTTTCGTTTCATGTAA-3′ and 5′-GAACGGAAACATAGTAGAACACTTATTCA-3′ as an internal control. Genes encoding a small HSP (*At-HSP17.6A*) and a group II LEA protein (*COR47*) were also amplified for positive controls using primers and conditions described before (Sun et al. 2001; Leonhardt et al. 2004).

## Yeast complementation and growth condition

The *Saccharomyces cerevisiae* wild-type strain in the BY4741 background (*MATa*, *lue2*, *ura3*, *his3*, *met15*, EUROSCARF, Frankfurt, Germany) and corresponding *vps53Δ* knockout strains (Invitrogen Corp., Carlsbad, CA, USA) are regularly maintained in YPG medium (1% yeast extract, 2% peptone, and 2% glycerol). Arabidopsis *HIT1* cDNA was subcloned into the *Bam*H1/*Xba*I sites, flanked by ADH1 promoter and terminator, of the yeast expression vector pRS313 containing HIS selection marker (Sikorski and Hieter 1989). This plasmid was then transformed into *vps53Δ* mutant strain by electroporation. Transformed cells were grown overnight in selective medium (0.67% yeast nitrogen base without amino acids, 2% glucose) with the required supplements to maintain selective pressure on cells carrying vector pRS313 and then maintained in YPG. Growth assays were performed by spotting 5 μl from tenfold dilutions of cultures at $OD_{600} = 0.8$ on YPG plates. For the heat tolerance assay, plates were incubated at 37°C as heat treatment and at 27°C as the control temperature. For osmotic inhibition assay, YPG plates were supplemented with various concentrations of glycerol (2, 4, 6, 8, 10%) or mannitol (0.25, 0.5, 0.75,

1 M) and incubated at 30°C. Growth was monitored after 2–5 days.

## Results

### The chromosome location of the *hit1-1* locus

The *hit1-1* locus was identified by molecular mapping of recombination events and chromosome walking. Initial mapping indicated that *hit1-1* locus was located on the lower arm of chromosome I about 1.1 cM apart from the molecular marker *nga280* (Wu et al. 2000). In this study, about 4,000 $F_2$ individuals from a cross between *hit1-1* (Col) and wild-type (Ler) plants were examined using newly developed SSLP and CAPS markers, and *hit1-1* was further fine mapped to an interval of about 43 kb between CER465523 and CER465525 as shown in Fig. 1. This interval lies within a bacterial artificial chromosome (BAC) F11F12 which was sequenced by the TIGR group of the Arabidopsis Genome Initiative. The entire region of the 43 kb DNA from *hit1-1* plants was PCR sequenced and compared to that from wild-type plants. Result showed a single nucleotide transition from C to A within the gene At1g50500. To determine the genomic structure of the *HIT1* gene, reverse transcription (RT)-PCR was performed to amplify *HIT1* cDNA fragments from total RNA of rosettes leaves using primers corresponding to the putative 5′ and 3′ untranslated region of the gene. Sequence of the amplified cDNA fragments revealed a 2.5-kb open reading frame. When comparing with the genomic sequence, it showed that the *HIT1* gene contains 24 exons, and the *hit1-1* mutation was in the 13th exon, leading to a Ser-to-Tyr amino acid substitution (Electronic supplementary material).

### *HIT1* is conserved among eukaryotes

According to the cDNA sequence, the predicted HIT1 protein contains 829 amino acids with molecular mass of 89 kDa. BLAST analysis revealed the HIT1 protein is most homologous to yeast Vps53p protein, sharing 19% identity and 38% similarity. Predicted homologous proteins are also found in other eukaryotic organisms like *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens*, with 23.9–24.6% identity and 47–56% similarity (Fig. 2). The yeast Vps53p protein has been shown to involve in the retrograde trafficking of vesicles to the late Golgi (Conibear and Stevens 2000; Siniossoglou and Pelham 2001; Conibear et al. 2003).

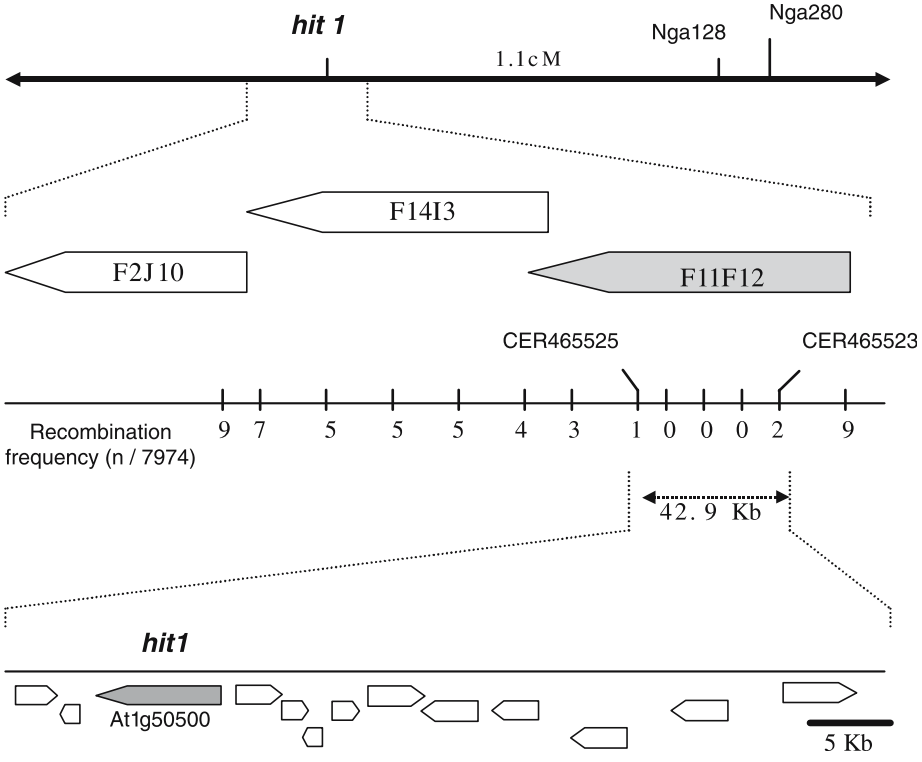


**Fig. 1** Fine mapping of the *HIT1* locus. The *top line* shows a segment of the lower arm of chromosome I with simple sequence length polymorphism markers nga280 and nga128 from which *HIT1* locus is adjacent. The *second line* shows an expansion of the region encompassing the *HIT1* locus. Corresponding bacterial artificial chromosomes (The Institute for Genomic Research, TIGR; http://www.tigr.org) are presented as *arrowheads* above the line. The positions of molecular markers used for mapping and their recombination frequencies are indicated. The chromosome region between markers CER465525 and CER465523 is blown up and shown in a higher resolution at the *bottom line*. This entire region of DNA was sequenced and compared to that of wild type. Gene shaded in *gray* represents the gene At1g50500 in which a single base substitution was found in the *hit1-1* mutant plant

**Fig. 2** Amino acid sequence comparison of HIT1 protein with their putative orthologs in *C. cerevisiae* (Vps53p, accession number P47061), *C. elegans* (C.e., accession number CAA81595), *D. melanogaster* (D.m., accession number AAF51022), and *H. sapiens* (H.s., accession number AAS20944). Sequence alignment was performed with Vector NTI software (Invitrogen, Carlsbad, CA, USA). Amino acids which share identity (*black shaded*) and similarity (*gray shaded*) are indicated

Since all eukaryotic cells require vesicle transport machinery to move lipids and proteins between various cellular compartments, conservation of protein sequence among eukaryotic species is to be expected. However, no specific structure, signal peptide, or particular motifs are found in the HIT1 sequence.

## Mutation of *HIT1* accounts for the heat and osmotic hypersensitivity

To confirm that the heat intolerant and osmotic stress hypersensitive phenotypes in *hit1-1* mutant were resulted from the mutation in the gene of At1g50500 (*HIT1*), *hit1-1* mutant plants were transformed with pCAMBIA 1305 vector with *HIT1* cDNA driven by a cauliflower mosaic virus (CaMV) 35S promoter. Twelve hygromycin-resistant T1 plants were obtained and their corresponding T2 homozygous progeny were isolated independently. Seeds from the wild-type, *hit1-1* mutant and homozygous transgenic plants were then subjected to heat and osmotic stress analysis. Results showed that while *hit1-1* seedlings were totally bleached after 4 days exposure at 37°C, transgenic seedlings remained green in color like those seen in wild-type seedlings (Fig. 3a). On the other hand, when germinated on mannitol-containing agar plates (300 mM), none of the *hit1-1* seeds were able to develop into mature seedlings as categorized by green, expended cotyledon. In contrast, seeds from transgenic plants were able to develop into mature seedlings as did seeds from wild-type plants (Fig. 3b). Similar analysis was performed on *hit1-1* plants transformed with pCAMBIA 1305 vector alone and their heat and osmotic stress hypersensitivity remained unchanged (data not shown), demonstrating the corresponding *hit1-1* phenotypes were indeed rescued by wild-type *HIT1* gene.

## *HIT1* may be involved in male-specific transmission

To learn more about the possible role of *HIT1*, we attempted to isolate *hit1* null mutant by screening the T-DNA insertion libraries from the Arabidopsis knockout facilities in Max-Planck Institute and The Salk Institute Genomic Analysis Laboratory. The *hit1-2* allele with a T-DNA inserted in the ninth exon and the *hit1-3* allele with a T-DNA inserted in the seventh intron of the *HIT1* gene were identified. For *hit1-2* allele, the isolation of homozygous mutant plants was unsuccessful. Antibiotic screening of the progeny from a heterozygous parental plant showed 1:1 ratio of segregation for sulfatidine resistance to sensitive instead of 3:1 ratio, as expected. Further analysis was conducted using PCR with *HIT1*- and T-DNA complemented primers to amplify the junction sequence of *HIT1* and T-DNA for genotyping the presence of the inserted T-DNA. The result revealed a 1:1 ratio of heterozygous to no-insert progeny (T/−, −/−) instead of the expected 1:2:1 ratio

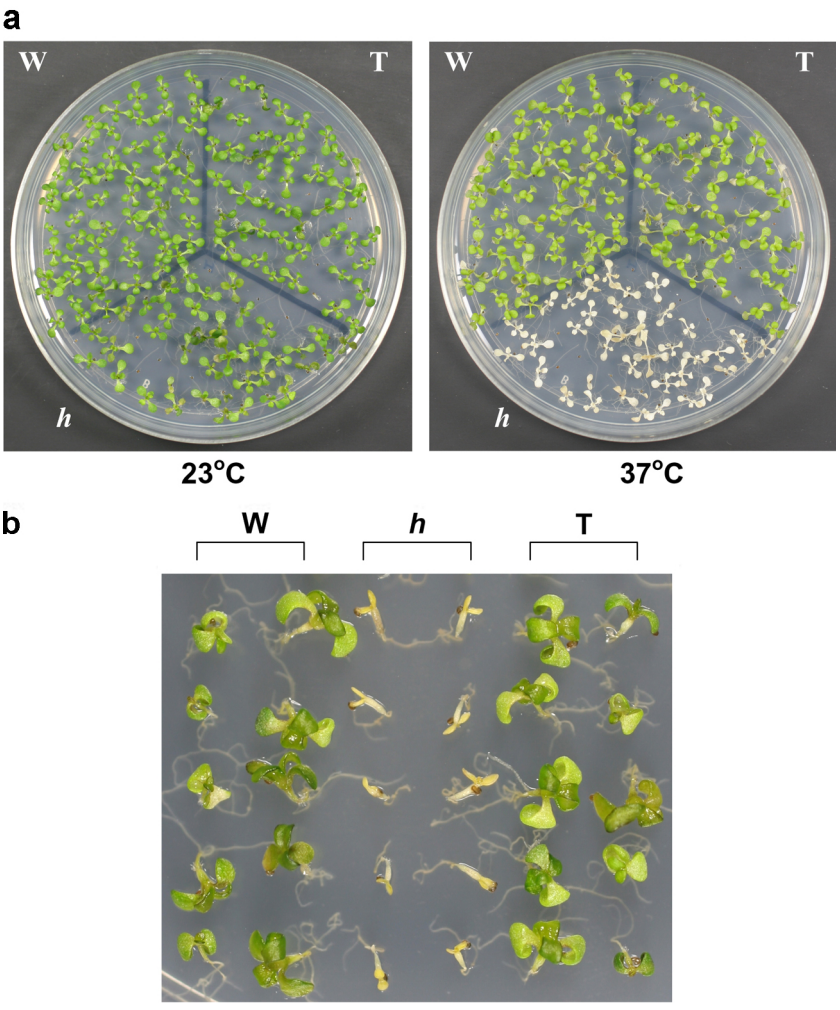


**Fig. 3** Phenotypic restoration of *hit1-1* mutant by transformation with a *HIT1* cDNA clone. **a** Ten-day-old, 23°C grown seedlings were transferred to 37°C and incubated for 4 days. Picture was taken before and after the heat treatment. **b** Seeds were sown on MS agar plate containing 0.3 M mannitol as exogenous osmoticum. Plate was incubated at 23°C for 14 days before picture was taken. Treatment labels include the following: *W* untransformed wild type, *h hit1-1* mutant, *T hit1-1* transformed with *HIT1* cDNA

of homozygous to heterozygous to no-insert progeny (T/T, T/−, −/−). Heterozygous plants were phenotypically indistinguishable from wild type consisting in the expected dominance of the *HIT1* gene. Similar results were also found while attempting to screen *hit1-3* homozygous plants. Since the siliques produced from the heterozygous parental plants were fully filled with viable seeds (data not shown), it is suggested that the T-DNA insertion in the *HIT1* gene may result in the male-specific transmission defect and can only be isolated as a hemizygous line.

## *HIT1* expressed at a constant level regardless of stress treatments

To study the expression of the *HIT1* gene, semi-quantitative RT-PCR was performed to amplify the 3′ region of the *HIT1* cDNA. Result showed that *HIT1* is ubiquitously expressed in root, rosette leaves, silique, and stem (Fig. 4a). This result is consistent with a previous finding reported by Lobstein et al. (2004). RT-PCR was also performed on 10-day-old seedlings, which had been incubated at 37°C for 0, 6, 12, and 24 h, respectively, to examine the *HIT1* expression pattern upon heat stress (Fig. 4b). Result showed that *HIT1* expressed at a relatively constant level regardless of the heat stress treatments. Furthermore, RT-PCR was performed on 10-day-old seedlings, which have been treated with 0, 0.1, 0.2, and 0.3 M mannitol, to examine the effect of osmotic stress on *HIT1* expression (Fig. 4c). Again, no significant alternation in the expression level was found.

## HIT1 may have a biological function similar to that of yeast Vps53p

Previous study has shown that the growth of the yeast *vps53Δ* mutant is inhibited at 37°C compared to that of wild-type cells (Conibear and Stevens 2000). This high temperature mediated growth-inhibiting phenomenon is similar to the heat hypersensitive phenotype of *hit1-1* mutant and prompted us to hypothesize that HIT1 has the same biological function as that of yeast Vps53p. To test this hypothesis, complementation tests were conducted. The *HIT1* cDNA was cloned into a yeast expression vector pRS313 to generate pRS313-*HIT1*, wherein the *HIT1* cDNA is driven by a yeast constitutive ADH1 promoter. The yeast wild-type and mutant strain lacking *VPS53* were transformed with the empty vector pRS313

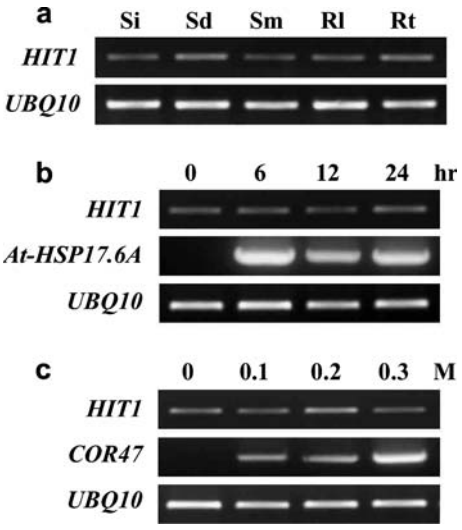


**Fig. 4** Reverse transcription (RT)-PCR analysis of normal or stress-mediated transcript levels of *HIT1*. **a** RT-PCR was performed on first strand cDNA made from seedlings or different plant tissues grown at 23°C. Treatment designations are as follows: *Sd* seedlings, *Si* silique, *Sm* stem, *Rl* rosette leaf, *Rt* root, *St* shoot. **b** Ten-day-old seedlings were transferred from 23 to 37°C and incubated for 0, 6, 12, or 24 h before total RNA was extracted from tissues for RT-PCR analysis. **c** Ten-day-old seedlings were transferred onto plates containing 0, 0.1, 0.2, and 0.3 M mannitol and incubated for 1 day before total RNA was extracted from seedlings for RT-PCR analysis. *At-HSP17.6A* and *COR47* RNAs served as positive controls and *UBQ10* RNA as internal control for the RT-PCR

or pRS313-*HIT1*. Results showed that the growth rate of both wild-type and mutant strains was not affected by the vector pRS313 at either 27 or 37°C. However, while pRS313-*HIT1* had no effect on the growth rate of wild-type strain, it could partially complement the retarded growth at 37°C in the mutant strain lacking *VPS53* (Fig. 5), suggesting a conserved biological function between Vps53p and HIT1 proteins. On the other hand, when using glycerol or mannitol as exogenous osmoticum, the growth of wild-type and *vps53Δ* mutant cells was equivalent. Transformation of wild-type and mutant cells with either pRS313 or pRS313-*HIT1* revealed no significant differences in growth under conditions of osmotic stress (data not shown), suggesting that the HIT1-mediated mechanism for plant osmotic stress tolerance may not exist in the yeast.

## Discussion

Plants have the ability to employ osmotic adjustment and enhanced water usage to acclimate to sustained high temperature stress (Neuner et al. 1999; Jiang and Huang 2001; Sung et al. 2003; Wang et al. 2004). Additionally, high temperature stress may be accompanied by water deficit under field conditions, forcing plants to resist both stresses simultaneously (Rizhsky et al. 2004; Wang and Huang 2004; Tester and Bacic 2005). Therefore, it has been suggested that plants have genetic determents and signaling pathways to regulate various protecting mechanisms upon heat stress, water stress, or their combination. Unfortunately, traditional experiments aimed at analyzing the response of plants to either of these stresses individually under controlled conditions have provided little information to this end (Mittler and Berkowitz 2001). Recent approaches using genomic analysis have revealed interrelated patterns of gene expression mediated by heat and/or water stress (Rizhsky et al. 2002, 2004). Nevertheless, functional analysis is required to complement expression profiling (Cushman and Bohnert 2000), and the identification of the *HIT1* gene provides new insight to this end.

The deduced HIT1 protein sequence has the most homology to yeast Vps53p. The yeast *VPS53*, along with the *VPS52* and *VPS54*, was identified through the screening for new genes that are involved in vesicle trafficking between the Golgi and the prevacuolar/endosomal compartment (Conibear and Stevens 2000). Vesicle trafficking is a complex system existing in plants and other eukaryotes for moving lipids, membrane-enclosed proteins, and other substances among the various subcellular compartments. The specificity of the docking and fusion process between a vesicle and its special target is partly mediated by a group of membrane-an-

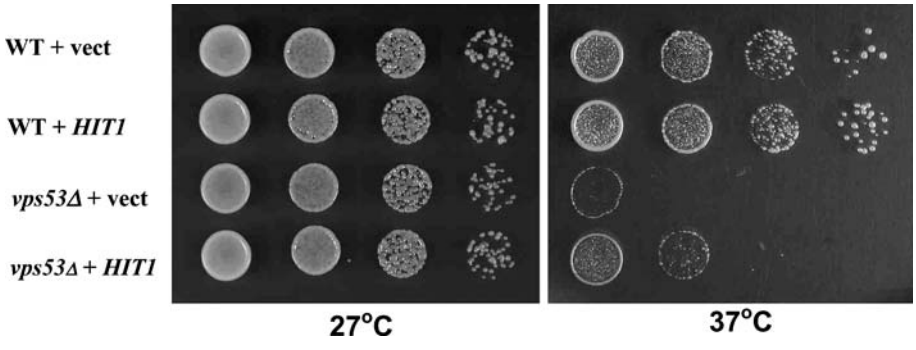


**Fig. 5** Complementation assay of yeast *vps53Δ* mutant cells by Arabidopsis *HIT1*. Tenfold dilutions of yeast cells were spotted on YPG plates and incubated at either 27 or 37°C for 2 or 3 days, respectively, before pictures were taken. Treatment designations were as follows: *WT + vect* wild type transformed with vector only, *WT + HIT1* wild type transformed with vector containing *HIT1* cDNA clone, *vps53Δ + vect* mutant transformed with vector only, *vps53Δ + HIT1* mutant transformed with vector containing *HIT1* cDNA clone

chored proteins called SNAREs (SNARE stands for *s*oluble *N*-ethylmaleimide-sensitive factor *a*daptor protein *r*eceptor, for review see Blatt et al. 1999), and additional factors such as Rab GTPases and multisubunit tethering complexes (Whyte and Munro 2002). It was shown that Vps52p, Vps53p, and Vps54p form such a tethering complex in a 1:1:1 ratio that, while regulated by a fourth component Vps51p, can associate with the t-SNARE Tlg1p and the Rab/Ypt GATase Ypt6p to mediate retrograde vesicle trafficking from the endosome back to the *trans*-Golgi network (Conibear and Stevens 2000; Siniossoglou and Pelham 2001; Conibear et al. 2003). Arabidopsis genes *POK* and *AtVps54* (At1g71270 and At4g19490, respectively) were recently identified to encode potential homologs of yeast Vps52p and Vps54p (Lobstein et al. 2004). The molecular interaction and cytological function of these Arabidopsis homologs are not clear. However, a POK::GFP fusion protein was shown to localize to Golgi in plant cells. It has also been shown that *POK* is required for effective pollen tube growth, which involves enhanced trafficking of Golgi-derived vesicles (Cheung et al. 2002; Lobstein et al. 2004). Furthermore, disruption of many individual Arabidopsis SNARE proteins is lethal in the male gametophyte (Sanderfoot et al. 2000). These data, together with the results that T-DNA insertional *hit1-2* and *hit1-3* mutants showed defect in the male-specific transmission, support the likely involvement of HIT1 in vesicle trafficking.

The normal appearance of *hit1-1* plants at the regular growth temperature of 22°C indicates that the mutated locus is not essential for normal plant growth and development but is important for plant survival upon heat stress. However, because of the lack of homozygous T-DNA *hit1-2* mutant, it was not possible to assume that *HIT1* gene directly participated in plant heat tolerance. One reason for this is that changes in the thermal stability of a protein involved in basic cellular functions could potentially generate a temperature sensitive phenotype. Nevertheless, since *hit1-1* plants are more sensitive to osmotic stress inhibition during seedling development than wild type, it is unlikely that the heat hypersensitive phenotype of *hit1-1* mutation is derived from a simple alternation in the thermal stability of a gene product. Most likely, the *hit1-1* mutation results in changes in the temperature-dependent regulation of plant–water relations. In contrast, deletion of *VPS53* gene does not abolish yeast viability but shows retarded growth under high temperature conditions comparing to that of wild-type cells (Conibear and Stevens 2000). In addition, expression of HIT1 in yeast *vps53Δ* mutant can partially complement this heat dependant growth retardation. These results indicate that both HIT1 and Vps53p may have a direct role in heat tolerance. On the other hand, the responses from yeast *vps53Δ* mutant and wild-type cells to osmotic stress were indistinguishable, and expression of HIT1 did not alter their sensitivity to osmotic stress as well (Wang and Wu, unpublished data); suggesting the HIT1-mediated mechanism for plant osmotic stress tolerance may not exist in or be required by yeast cells.

Vesicle trafficking has been proposed to participate in plant stress responses in various aspects. Because there are many environmental stresses that can damage cell membranes, and subsequently, the function of their embedded proteins (Sung et al. 2003; Chaves and Oliveira 2004; Los and Murata 2004), the action of vesicle trafficking in membrane recycling is important for recovering from injury due to environmental stress. This protecting role has been demonstrated in Arabidopsis by overexpression of a gene encoding the vesicle-associated membrane protein AtVAMP7C, which was shown to function in trafficking between late endosomes and lysosomes/vacuoles and has no antioxidant activity, to rescue cells from the attack of reactive oxygen species (Advani et al. 1999; Levine et al. 2001). Vesicle trafficking has also been linked to stress-related signaling pathways. For example, using a functional screening for abscisic acid (ABA)-related signaling has revealed a tobacco SNARE gene, *Nt-SYR1*, whose encoded protein is required for ABA-mediated ionic flux in guard cells (Leyman et al. 1999). An Arabidopsis SNARE protein OSM1/SYP61 was found to have a similar role in the ABA regulated stomatal responses and was important for osmotic stress tolerance (Zhu et al. 2002). In addition, many lipid-based molecules, such as those derived from the phosphatidylinositol metabolic pathway, can regulate membrane trafficking, and have been shown to mediate the signaling processes that lead to ABA responses (Meijer and Munnik 2003; Lin et al. 2004). More generally, stress stimuli change the global pattern of gene expression in plants; it predictably requires the coordination of vesicle trafficking systems involved in the removal of existing proteins and lipid molecules from damaged membranes and the delivery of the freshly synthesized ones to their target sites (Levine 2002). Despite circumstantial evidence supporting membrane rejuvenation through vesicle trafficking, little empirical data has come to light to elucidate the protective mechanisms by which vesicle trafficking improves the plants tolerance to heat and drought stress. Additionally, current understanding of vesicle trafficking in plant stress responses is restricted to the study of the SNARE proteins. The role and importance of the tethering complex in plant stress responses have not yet been defined. To our knowledge, *HIT1* is the first gene homologous to tethering factors linked to the tolerance of multiple stresses in plants. The study of the *hit1* mutant, as well as further characterization and analyses of the function of HIT1, should provide new insights into the importance of vesicle trafficking in plant stress responses.

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ERRATUM

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Ronald J. Sayler · Ching-Hui Yeh · Shaw-Jye Wu

# Mutation in a homolog of yeast Vps53p accounts for the heat and osmotic hypersensitive phenotypes in Arabidopsis *hit1-1* mutant

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Unfortunately, Fig. 2 was published with errors. The correct figure is given here:

The online version of the original article can be found at http://dx.doi.org/10.1007/s00425-005-0216-6

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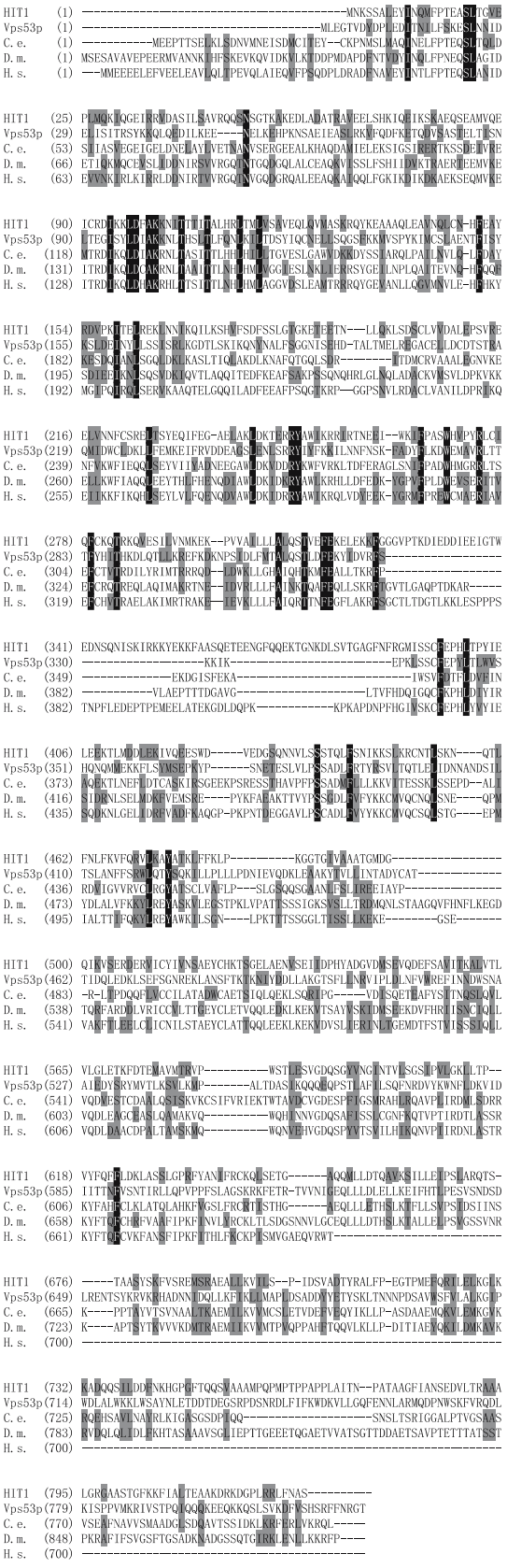

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**Fig. 2** Amino acid sequence comparison of HIT1 protein with their putative orthologs in *C. cerevisiae* (Vps53p, accession number P47061), *C. elegans* (C.e., accession number CAA81595), *D. melanogaster* (D.m., accession number AAF51022) and *H. sapiens* (H.s., accession number AAS20944). Sequence alignment was performed with Vector NTI software (Invitrogen, Carlsbad, CA, USA). Amino acids which share identity (*black-shaded*) and similarity (*gray-shaded*) are indicated

*Planta -- Electronic Supplementary Material*

http://www.springerlink.com/content/7162tw8628337751/MediaObjects/425_2005_216_MOESM1_ESM.pdf

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 F  K  L  P  K  G  G  T  G  I  V  A  A  A  T  G  M  D  G  Q  I  K  V  S  E  R  D  E  R  V  510
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 I  C  Y  I  V  N  S  A  E  Y  C  H  K  T  S  G  E  L  A  E  N  V  S  E  I  I  D  P  H  Y  540
GCTGATGGTGTAGACATGTCAGAGGTCCAGGATGAATTTTCAGCCGTCATAACGAAAGCATTGGTAACGCTGGTCCTTGGACTTGAGACT 1710
 A  D  G  V  D  M  S  E  V  Q  D  E  F  S  A  V  I  T  K  A  L  V  T  L  V  L  G  L  E  T  570
AAATTTGACACAGAAATGGCAGTGATGACACGTGTTCCATGGAGCACACTTGAGAGTGTTGGTGACCAGTCTGGGTACGTGAATGGAATA 1800
 K  F  D  T  E  M  A  V  M  T  R  V  P  W  S  T  L  E  S  V  G  D  Q  S  G  Y  V  N  G  I  600
AATACAGTCCTTAGCGGCAGCATACCTGTCCTCGGGAAACTTCTAACACCAGTTTACTTCCAGTTTTTCCTTGACAAGCTGGCATCATCC 1890
 N  T  V  L  S  G  S  I  P  V  L  G  K  L  L  T  P  V  Y  F  Q  F  F  L  D  K  L  A  S  S  630
CTAGGACCACGGTTCTATGCTAATATTTTCAGATGCAAGCAACTATCCGAAACTGGAGCTCAACAGATGTTACTAGATACGCAAGCTGTG 1980
 L  G  P  R  F  Y  A  N  I  F  R  C  K  Q  L  S  E  T  G  A  Q  Q  M  L  L  D  T  Q  A  V  660
AAGTCGATTCTACTAGAAATTCCTTCCCTTGCCAGACAGACCTCAACTGCTGCCAGCTATTCCAAATTTGTGAGCCGTGAAATGAGCAGA 2070
 K  S  I  L  L  E  I  P  S  L  A  R  Q  T  S  T  A  A  S  Y  S  K  F  V  S  R  E  M  S  R  690
GCTGAAGCACTCCTTAAGGTCATACTATCCCCAATCGACTCAGTGGCAGACACTTATCGTGCACTGTTTCCGGAGGGAACGCCCATGGAG 2160
 A  E  A  L  L  K  V  I  L  S  P  I  D  S  V  A  D  T  Y  R  A  L  F  P  E  G  T  P  M  E  720
TTTCAACGCATCTTAGAACTTAAGGGTCTTAAGAAAGCTGATCAGCAGAGTATACTAGATGATTTCAACAAGCATGGTCCAGGATTCACA 2250
 F  Q  R  I  L  E  L  K  G  L  K  K  A  D  Q  Q  S  I  L  D  D  F  N  K  H  G  P  G  F  T  750
CAGCAGTCGGTTGCAGCAGCAATGCCACAACCAATGCCAACCCCACCTGCACCACCATTGGCAATCACTAATCCGGCTACAGCTGCAGGG 2340
 Q  Q  S  V  A  A  A  M  P  Q  P  M  P  T  P  P  A  P  P  L  A  I  T  N  P  A  T  A  A  G  780
TTCATAGCAAACAGCGAGGATGTTTTAACCAGAGCAGCTGCTCTTGGCAGAGGAGCTGCAAGCACTGGCTTCAAGAAGTTCATTGCTCTT 2430
 F  I  A  N  S  E  D  V  L  T  R  A  A  A  L  G  R  G  A  A  S  T  G  F  K  K  F  I  A  L  810
ACCGAAGCTGCCAAAGACCGCAAAGATGGGCCTTTAAGGAGACTCTTCAACGCATGA
 T  E  A  A  K  D  R  K  D  G  P  L  R  R  L  F  N  A  S
TTTGTTCCTTTACTACCTCATCTCAACTTTTGATTTATGCAATTCCCACCTTTGTTTCCT
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Arabidopsis *HIT1* cDNA and its deduced protein sequence. The gene has 2487 nucleotides encoding an 829 amino acid protein. The position for *hit1-1* mutation is indicated by an asterisk above the codon.