Isolation and characterization of the Arabidopsis *heat-intolerant 2* (*hit2*) mutant reveal the essential role of the nuclear export receptor EXPORTIN1A (XPO1A) in plant heat tolerance

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Summary

- The Arabidopsis *heat-intolerant 2* (*hit2*) mutant was isolated on the basis of its impaired ability to withstand moderate heat stress (37°C). Determination of the genetic mutation that underlies the *hit2* thermosensitive phenotype allowed better understanding of the mechanisms by which plants cope with heat stress.
- Genetic analysis revealed that *hit2* is a single recessive mutation. Map-based cloning was used to identify the *hit2* locus. The response of *hit2* to other types of heat stress was also investigated to characterize the protective role of *HIT2*.
- *hit2* was defective in basal but not in acquired thermotolerance. *hit2* was sensitive to methyl viologen-induced oxidative stress, and the survival of *hit2* seedlings in response to heat stress was affected by light conditions. The mutated locus was located at the *EXPORTIN1A* (*XPO1A*) gene, which encodes a nuclear transport receptor. Two T-DNA insertion lines, *xpo1a-1* and *xpo1a-3*, exhibited the same phenotypes as *hit2*.
- The results provide evidence that Arabidopsis XPO1A is dispensable for normal plant growth and development but is essential for thermotolerance, in part by mediating the protection of plants against heat-induced oxidative stress.

Introduction

Heat stress, which results when the ambient temperature is elevated above the normal optimum, can injure a broad spectrum of cellular components, and lead to metabolic malfunction and ultimately cell death. Plants are sessile organisms, and so they frequently encounter such detrimental conditions, which makes heat stress one of the principal factors that limits crop production world-wide (Mittler, 2006).

The heat stress to which plants are exposed can usually be classified as sustained high temperature or heat shock, depending on the intensity, duration, and rate of temperature increase (Sung *et al.*, 2003). In either form, heat stress can affect the function of plant cells directly by inhibiting protein activity and altering membrane integrity. Many biological pathways, in particular, membrane-associated processes such as photosynthesis, can subsequently be disrupted, and this disruption can allow high-energy state electrons to react with molecular oxygen to yield reactive oxygen species (ROS) (Larkindale & Knight, 2002; Suzuki & Mittler, 2006). ROS are toxic molecules that are capable of causing further damage to the cell, which results in so-called secondary stress (Wang *et al.*, 2003). It is well recognized that the induction of heat shock proteins (HSPs) is a primary defense response to heat stress. The role of HSPs in thermotolerance is attributed to their molecular chaperone activities, which enable them to maintain the native configuration and function of cellular proteins, and thus protect these proteins from heat denaturation. Considerable evidence has indicated that acquired thermotolerance, which refers to the ability to withstand normally fatal heat stress after a pre-acclimation period at nonlethal temperatures, is related to the production of HSPs (Sung *et al.*, 2003). To counter the threat of heat-induced oxidative stress, plants employ both enzymatic and nonenzymatic systems to deal with ROS. For example, expression of a

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thermostable isoform of ascorbate peroxidase (APX), one of the plant antioxidant enzymes that detoxify hydrogen peroxide, is induced strongly by heat stress (Panchuk *et al.*, 2002). Other studies, which have included the proteomic analysis of *Agrostis* grass species with different sensitivities to heat stress, have indicated that the amounts of additional ROS scavenging enzymes, such as superoxide dismutase (SOD), and the cellular concentration of antioxidants, such as glutathione (GSH), are also associated with the tolerance of plants to heat (Liu & Huang, 2000; Locato *et al.*, 2008; Xu & Huang, 2008).

Clearly, plants have the genetic potential to alleviate the effects of various forms of heat-related injury. However, to realize this potential, plants must sense stress effectively, which requires appropriate signal transduction pathways and the relocation of a variety of molecules into and out of the nucleus. One of the systems involved is the heat stress factor (HSF) signaling system. HSFs are transcription factors that act as the terminal components of signal transduction pathways by binding to specific heat stress elements (HSEs) in the promoters of genes that encode HSPs to regulate their expression. The promoters of many genes that are involved in oxidative stress signaling and defense also contain HSF-binding motifs (Rizhsky *et al.*, 2004; Davletova *et al.*, 2005), and are induced in response to heat stress in an HSF-dependent manner (Panchuk *et al.*, 2002). Plants carry multiple genes that encode HSFs – for example, > 18 are found in tomato (*Solanum lycopersicum*) and 21 in Arabidopsis (Novel *et al.*, 2001; Baniwal *et al.*, 2004) – and their products comprise a complex regulatory network. Some of these HSFs exist in the cytosol as inactive monomers. Upon receiving a stress signal, these factors are activated through oligomerization and translocation to the nucleus. Indeed, all HSFs contain a nuclear localization sequence (NLS), and, in most cases, a nuclear export sequence (NES), which can be recognized by nuclear transport receptors to allow nucleo-cytoplasmic trafficking (Novel *et al.*, 2001). For example, it has been demonstrated that tomato HsfA2, which is normally localized to the cytosol, becomes a nuclear protein if its NES is defective or absent. The presence of HsfA1 or leptomycin B (LMB), which is an inhibitor of nuclear export, also results in nuclear retention of HsfA2. Furthermore, HsfA2 remains cytoplasmic under conditions of heat stress, even in the presence of LMB (Heerkoltz *et al.*, 2001). These results suggest that the shuttling of molecules between the nucleus and the cytoplasm, which is regulated by the balance of nuclear import and export, is a crucial event during plant responses to heat stress. However, because of the complexity of the nuclear transport receptors and the broad spectrum of substrates whose transport they facilitate, it remains uncertain whether there is a nuclear transport receptor that is specific for plant response to heat stress, and to what extent it affects the heat tolerance of plants.

To identify genetic determinants that are essential for plant heat tolerance, we have employed a forward genetic approach to screen for heat-intolerant (*hit*) mutants of Arabidopsis after ethyl methanesulfonate (EMS) mutagenesis (Wu *et al.*, 2000; Lee *et al.*, 2006; Wang *et al.*, 2008). One of these mutants, *hit2*, was isolated because its growth was inhibited under conditions of sustained high temperature. Incubation at 37°C for 4 d was lethal for the mutant but not for wild-type plants. The basal thermotolerance of *hit2* to heat shock treatment was also impaired. Furthermore, the growth and development of *hit2* seedlings were more sensitive to inhibition with methyl viologen (MV) than those of wild-type seedlings. The mutated locus was mapped to the *EXPORTIN1A* (*XPO1A*) gene, which encodes a nuclear export receptor (Haasen *et al.*, 1999). In Arabidopsis, two *XPO1* genes (*XPO1A*, At5g17020 and *XPO1B*, At3g03110) have been identified and are thought to be functionally redundant (Haasen *et al.*, 1999; Merkle, 2004; Blanvillain *et al.*, 2008). However, the *hit2* mutant indicates that XPO1A has its own specific function, which is not necessary for normal growth and development, but is crucial for plant survival under conditions of sustained high temperature or sudden heat shock.

Materials and Methods

Plant material and growth conditions

Wild-type and EMS-mutagenized M_2 seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were purchased from Lehle Seeds (Round Rock, TX, USA). For seed propagation, plants were grown in soil under a 16 h : 8 h light : dark cycle at 23°C. For stress treatments, seeds were first sown on agar plates that contained Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) and 2% (w/v) sucrose, buffered to pH 5.7, with continuous illumination at 23°C. Plates that were subjected to treatment at 37°C were sealed with one layer of cling film followed by a layer of parafilm to prevent dehydration. The light intensities for all growth conditions were *c.* 100 $\mu mol\ m^{-2}\ s^{-1}$. T-DNA insertion alleles *xpo1a-1* (SALK_028886), *xpo1a-3* (SALK_078639C), and *xpo1b-1* (SALK_088267C) were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University, Columbus, OH, USA.

Isolation of mutants

EMS-mutagenized M_2 seeds were sown on MS agar plates and allowed to grow at 23°C to the stage at which the first leaf pair had just emerged. The plates were then moved to a growth chamber set at 37°C and incubated for 1.5 d. Seedlings that showed retarded growth, as determined by slow rates of leaf expansion, or whose leaves became brownish in color, were rescued and moved onto new agar plates, and

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kept at 23°C for another 10 d. Seedlings that could recover from previous heat treatment (putative mutants) were transferred subsequently to soil and grown to maturity. The heat sensitivity of the M_3 seedlings was retested in a heat survivability assay (described in the Genetic analysis and gene mapping section below), and verified true mutants were studied further.

Genetic analysis and gene mapping of the *hit2* locus

The ability of the seedlings to survive heat treatment was tested by incubating 10-d-old seedlings at 37°C for 4 d. This assay was used for genetic analysis (Lee *et al.*, 2006). This treatment was shown to be lethal for the *hit2* mutant but not for wild-type seedlings. An F_2 mapping population was prepared by crossing *hit2* in the Col-0 background with Arabidopsis ecotype Landsberg *erecta* (L*er*-0). F_2 plants were self-pollinated to produce the F_3 generation, which was subjected to the survivability assay. DNA was isolated from individual mutant plants of the F_2 generation for PCR-based gene mapping. Initially, 50 *hit2* mutant F_2 plants were selected for rough mapping using published genetic markers (Konieczny & Ausubel, 1993; Bell & Ecker, 1994). This allowed the *hit2* mutation to be located *c.* 2 cM downstream of the simple sequence length polymorphism (SSLP) marker *nga151* on the upper arm of chromosome 5. For fine mapping, publicly available SSLP and cleaved amplified polymorphic sequence (CAPS) markers in the vicinity of *hit2* were used (http://www.arabidopsis.org). In addition, potential SSLP and CAPS markers that were based on insertions/deletions (INDELs) and single nucleotide polymorphisms (SNPs) were identified by comparing the genomes of Col-0 and Cereon L*er*-0 (Jander *et al.*, 2002). The markers that showed minimal recombination with *HIT2* were the CAPS markers CER483173 and CER483157, which were identified in the present study. CER483173 was amplified with the primers 5′-TAACGCCTCCCTACGACATTCT-3′ and 5′-ACCTTCAGAGGAAAGGAACGCT-3′ and the L*er*-0 amplicon was cleaved by *Cla*I. CER483157 was amplified with the primers 5′-ATGCCATCGATCATCACCAAGT-3′ and 5′-CATCGACGCTATTCAGAATCTAG-3′ and the Col-0 amplicon was cleaved by *Pst*I. Further details are provided in Supporting Information Fig. S1, Table S1. The 60-kb region that was flanked by the CER483173 and CER483157 markers was sequenced and compared with that of wild-type plants to identify the *HIT2* gene.

MV and heat shock treatments of Arabidopsis seedlings

To measure sensitivity to MV, seeds were sown on MS agar plates supplemented with various concentrations of MV and incubated at 23°C with continuous light. The germination rate was calculated from the number of seeds with visible protruding radicals, and the seedling maturation rate was calculated from the number of seedlings with green, expanded cotyledons. For heat shock tests, seeds were sown on pre-sterilized plastic Petri plates (90 mm diameter × 20 mm deep; Viogene, Sijhih, Taiwan). Freshly autoclaved medium (33 ml) was poured into each dish and allowed to solidify on a level bench, to assure uniformity of temperature during the heat shock treatment. Plates were incubated at 23°C with constant light for 7 d. For the heat shock treatment, the plates were sealed tightly with parafilm and then submerged entirely in a water bath set at 44°C for 20 min. After the treatment, the plates were returned to 23°C for another 7 d for observation.

Genotyping and gene expression analysis

To genotype the T-DNA insertion mutants, DNA from *xpo1a-1*, *xpo1a-3* or *xpo1b-1* was amplified by PCR using specific primers to detect the insertion, as described previously (Blanvillain *et al.*, 2008). To analyze tissue-specific gene expression, total RNA was extracted from plant tissues grown in soil using a GeneMark Plant Total RNA Miniprep Purification Kit (Hopegen Biotechnology, Taichung, Taiwan). Reverse transcription was performed using Moloney murine leukaemia virus (MMLV) HP Reverse Transcriptase (Epicentre, Madison, WI, USA). To analyze gene expression mediated by heat stress, seeds were sown on MS agar plates. After incubation for 10 d at 23°C, entire plates were transferred to 37°C for various times. After stress treatment, seedlings were frozen immediately in liquid nitrogen and ground to powder for RNA extraction and cDNA synthesis as described for tissue-specific gene expression analysis above. For reverse transcription PCR (RT-PCR), cDNAs were amplified using the *HIT2*-specific primers 5′-GGGCAGCTGCGGATAATTCTCCCACCG-3′ and 5′-GACGCCGCTGCATGACTTGAGAACC-3′. The poly-ubiquitin gene (*UBQ10*) and a small HSP gene (*At-HSP17.6A*) were also amplified with gene-specific primers, described previously, as internal and positive controls, respectively (Lee *et al.*, 2006).

Results

Heat intolerance of *hit2* is attributable to a single recessive mutation

The *hit2* mutant was isolated by screening EMS-mutagenized M_2 seeds by examining seedling development under permissive high temperature. The phenotype of hypersensitivity to heat was confirmed in the M_3 and subsequent generations with a survivability assay; that is, when 10-d-old agar-grown plants were exposed to 37°C for 4 d, *hit2* mutant plants became bleached, whereas wild-type seedlings

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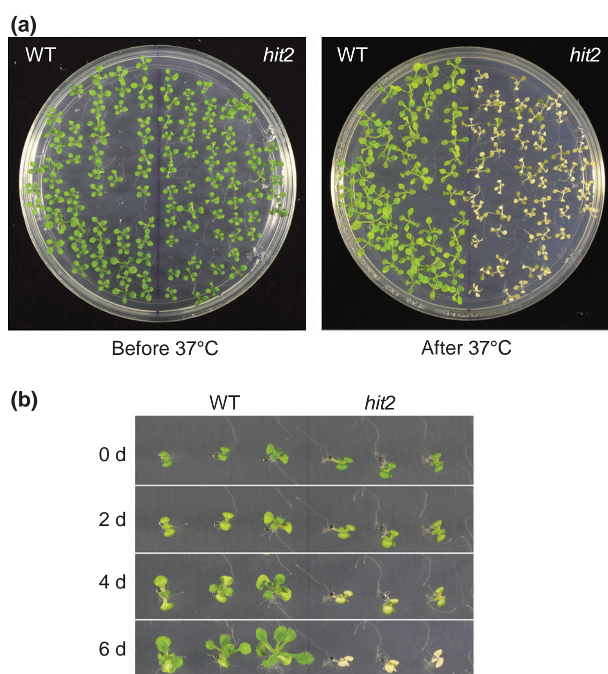

Fig. 1 Sensitivity of Arabidopsis *heat-intolerant 2* (*hit2*) mutant to sustained high temperature and sudden heat shock. (a) Incubation at 37°C for 4 d was lethal for *hit2* but not wild-type (WT) plants. Photographs were taken before and after treatment. (b) Progression of phenotypes of wild-type and *hit2* seedlings after heat shock treatment. Seven-day-old plants grown on medium, with opened cotyledons and an emerging first pair of leaves, were subjected to heat shock at 44°C for 20 min. After treatment, plants were returned to room temperature for recovery and photography. Wild-type plants continued to grow, whereas mutant plants were bleached completely after 6 d.

remained green in color. Subsequent transfer of plants from this stress treatment to 23°C allowed the growth of wild-type but not *hit2* plants, which revealed that such treatment was lethal only for the *hit2* mutant (Fig. 1a). This assay was used for genetic analysis, which indicated that F_1 plants from a cross between *hit2* and the wild type could all tolerate heat. Furthermore, analysis of the self-pollinated F_2 plants showed a 3 : 1 segregation ratio of wild-type to *hit2* plants (Table 1). Hence, the heat intolerance phenotype was caused by a single recessive nuclear mutation.

The *hit2* mutant is defective in basal thermotolerance

As part of our effort to characterize the responses of *hit2* to various forms of heat stress, we tested the thermotolerance of *hit2* to sudden heat shock. This test was performed by heating the seedlings directly to 44°C for a short period of time. Fig. 1(b) shows the development of the seedlings after they were exposed to heat shock for 20 min. No difference in appearance between the mutant and wild-type seedlings was observed immediately after treatment. However, after 6 d at 23°C, the leaves of wild-type seedlings remained green in color and exhibited noticeable growth and expansion, whereas the leaves of the *hit2* seedlings were bleached completely and showed no sign of growth. Therefore, the ability of the *hit2* mutant to tolerate sudden heat shock stress was impaired, in addition to its ability to tolerate sustained high temperature stress. However, pretreatment of the seedlings at 37°C for 60 min allowed the *hit2* mutant to withstand severe heat shock to the same extent as the wild type (Fig. 2). These results imply that the *hit2* mutant was defective only in basal and not in acquired thermotolerance.

The *HIT2* locus maps to Arabidopsis *XPO1A*

To identify the genetic determinant responsible for the heat intolerance phenotype of *hit2*, the location of the *hit2* locus was determined by map-based cloning. Initially, rough mapping located the mutation on the upper arm of chromosome 5 near the molecular marker *nga151*. Fine mapping with *c.* 2700 F_2 individuals from a cross between *hit2* (Col-0) and wild-type (L*er*-0) plants localized the *hit2* locus further to a *c.* 60-kb region that was delimited by the genetic markers CER483173 and CER483157 (Fig. 3a–d). This interval, which was covered by the bacterial artificial chromosome (BAC) F2K13, consisted of genes with Arabidopsis Genome Initiative (AGI) annotation numbers from At5g16910 to At5g17070. Genomic DNA from *hit2* homozygous plants was sequenced in this region by PCR sequencing and the sequence compared with that from wild-type plants. This revealed a nucleotide transition of G to A in the 13th exon of At5g17020. This gene encodes the nuclear export receptor XPO1A (Haasen *et al.*, 1999;

Table 1 Genetic analysis of the Arabidopsis *heat-intolerant 2* (*hit2*) and *exportin1b-1* (*xpo1b-1*) mutants

Strains or crosses (♂ × ♀)	Generation	Total	Tolerant[a]	Sensitive[a]	χ^{2b}
Wild-type Col-0		143	143	0	
hit2/hit2		140	0	140	
Wild-type × *hit2/hit2*	F_1	57	57	0	
	F_2	440	327	113 (34.6%)	0.109[b]
xpo1b-1/xpo1b-1		45	45	0	

[a]Tolerance or sensitivity was determined in the survivability assay as described in the Materials and Methods section.
[b]The calculated χ^2 value was based on the expected ratio of 3 : 1 for tolerant to sensitive individuals, assuming that *hit2* was a single recessive mutation ($P > 0.05$).

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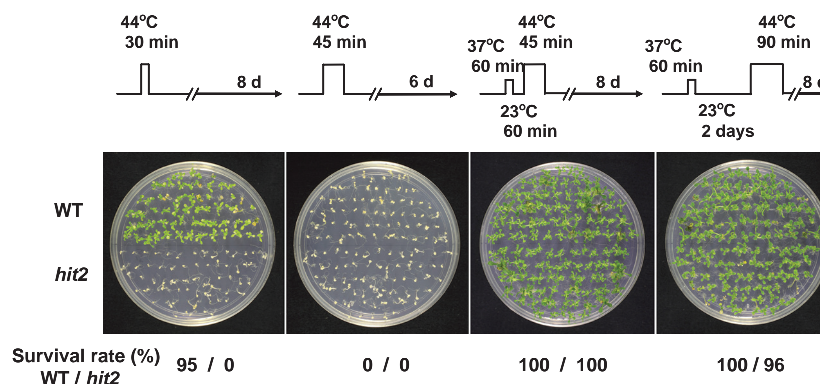

Fig. 2 Arabidopsis *heat-intolerant 2* (*hit2*) seedlings were defective in basal but not acquired thermotolerance. Seven-day-old seedlings grown on medium were subjected to the different heating regimens indicated above each photograph. Survival rates were calculated from the number of seedlings that showed continuous growth of green leaves after 6 d. Apart from heat treatment, plants were incubated at 23°C. Plates were photographed at 6–8 d after the final heat treatment. After 1 h of pre-acclimation at 37°C, *hit2* plants were able to survive heat shock that was otherwise lethal, as were wild-type (WT) plants.

Blanvillain *et al.*, 2008), and the *hit2* mutation changed the codon for tryptophan 364 (TGG) to a premature TGA stop codon (Fig. 3e).

Mutation of *XPO1A* accounts for the *hit2* phenotype

To verify the causal relationship between the *XPO1A* gene and the observed *hit2* phenotype, two additional *XPO1A* alleles, *xpo1a-1* and *xpo1a-3*, were obtained from a collection of T-DNA insertion mutants (Alonso *et al.*, 2003) for evaluation. These two alleles were chosen because *xpo1a-1* has been shown to cause a deletion in the *XPO1A* transcript and *xpo1a-3* has been shown to be a null allele. In the heat survivability assay or after heat shock treatment, *xpo1a-1* and *xpo1a-3* homozygous seedlings exhibited the same thermosensitive phenotype as that of *hit2* (Fig. 3f,g). Moreover, analysis of progeny from heterozygous parental plants (e.g. *xpo1a-1/+*) indicated that wild-type (+/+) and heterozygous (T/+) individuals were heat tolerant, and only homozygous (T/T) individuals displayed the heat-sensitive phenotype (data not shown). These results indicated that the observed *hit2* phenotype could be accounted for by mutation of the *XPO1A* gene. We also analyzed an *xpo1b* T-DNA insertion mutant, *xpo1b-1*, and found that *xpo1b-1* seedlings were heat tolerant in the heat survivability assay (Table 1). This suggests that *XPO1A* and *XPO1B* have different roles in plants under conditions of heat stress.

hit2 is more sensitive than the wild-type to MV-induced oxidative stress

Conditions of high temperature can induce oxidative stress, and plants that have lost their antioxidant ability are often defective specifically in basal thermotolerance (Larkindale & Knight, 2002; Larkindale *et al.*, 2005). It has been demonstrated that proteins that are involved in plant tolerance to oxidative stress may be subject to nucleo-cytoplasmic partitioning that is modulated by XPO1 (Blanvillain & Ow, 2004; Tsou *et al.*, 2007; Blanvillain *et al.*, 2008). The *hit2* mutant contains a mutation in *XPO1A* and shows a specific defect in basal thermotolerance. Therefore, it is plausible to assume that the mutant is also sensitive to oxidative stress. To investigate this possibility, we tested seed germination and seedling development of *hit2* on medium that contained various concentrations of MV and was exposed to light. The rate of germination was determined from the number of seeds that showed visible protruding radicals, and the rate of seedling development from the number of seeds with green, opened cotyledons. As shown in Fig. 4(a), wild-type and *hit2* mutant seeds exhibited similar germination rates at various concentrations of MV. However, the development of *hit2* seedlings was more sensitive to inhibition by treatment with MV than that of wild-type seedlings (Fig. 4b). Furthermore, although some of the *hit2* seeds were able to reach the seedling stage, their cotyledons soon became pale and their growth ceased (Fig. 4c,d). Similar results were obtained when *xpo1a-1* and *xpo1a-3* seedlings were grown in the presence of MV (data not shown). These results indicate that the sensitivity of *hit2* to inhibition by MV is more severe after the photosynthetic organs have developed, which is consistent with the known role of MV in inducing light-catalyzed oxidative stress.

The sensitivity of *hit2* to heat stress is influenced by light conditions

Heat-induced oxidative damage is known to be exacerbated by light, and the light conditions to which plants are exposed have been shown to influence plant survival after heat stress (Larkindale & Knight, 2002; Larkindale *et al.*, 2005). *hit2* is sensitive to MV-induced photo-oxidative stress, and therefore it is likely that light conditions will

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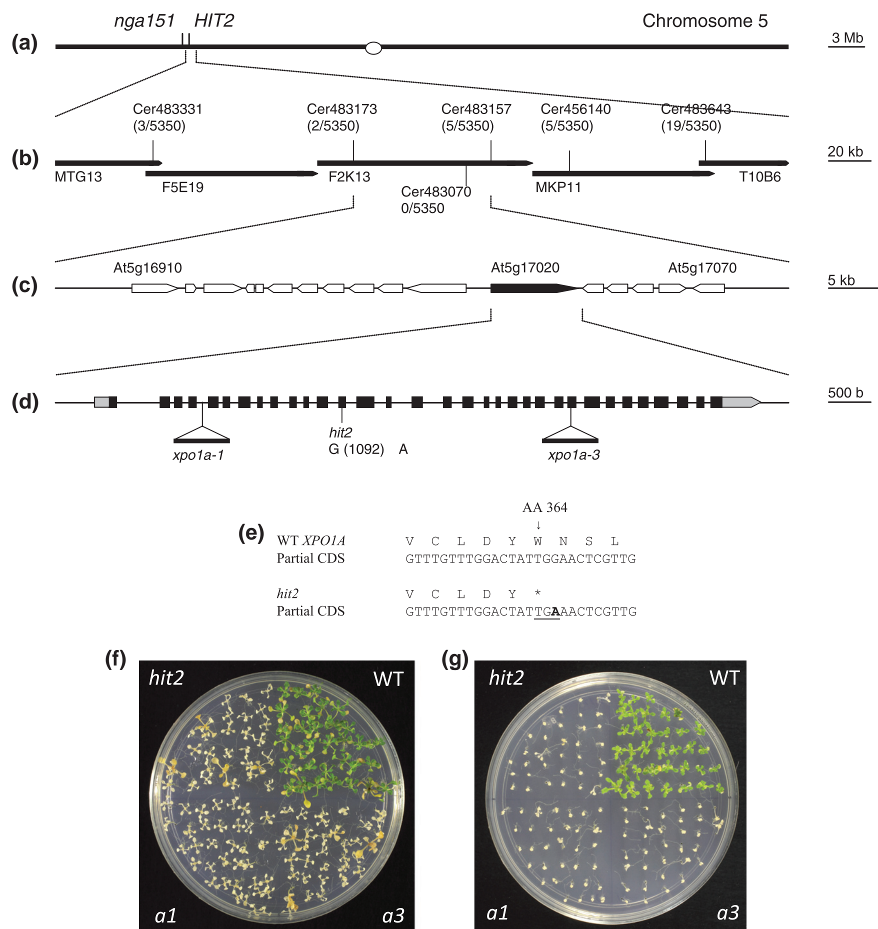

Fig. 3 Recombination mapping of the *HEAT-INTOLERANT 2* (*HIT2*) locus. (a) Arabidopsis chromosome 5 with simple sequence length polymorphism (SSLP) marker *nga151*, which was adjacent to the *HIT2* locus. (b) Expansion of the region that encompassed the *HIT2* locus. Five overlapping bacterial artificial chromosomes within the region are shown. The relative positions of the molecular markers (vertical lines) are indicated. The number of recombinants versus the total number of chromosomes tested for a given marker is shown in parentheses. (c) The chromosomal region between markers CER483173 and CER483157 was sequenced and compared with that of the wild type. The gene shown in black represents At5g17020, in which a single base substitution was found in the *hit2* mutant plants. (d) Exon–intron structure of the *HIT2* gene. The *hit2* mutation was a G-to-A mutation within the 13th exon. Additional T-DNA insertion alleles of At5g17020, *exportin1a-1* (*xpo1a-1*) and *xpo1a-3*, are also indicated. (e) Amino acid and nucleotide changes found in *hit2* corresponded to a nonsense mutation that truncated the HIT2 protein at amino acid 364. (f) Phenotypes of 10-d-old wild-type, *hit2*, *xpo1a-1*, and *xpo1a-3* plants after incubation at 37°C for 4 d. (g) Phenotypes of wild-type, *hit2*, *xpo1a-1*, and *xpo1a-3* Arabidopsis seedlings after heat shock at 44°C for 20 min. Photographs were taken at 8 d after challenge. WT, wild type; *a1*, *xpo1a-1*; *a3*, *xpo1a-3*.

affect the sensitivity of *hit2* to heat. To test this hypothesis, *hit2* seedlings were subjected to the heat survivability assay as before, but the plates were wrapped with aluminum foil to avoid exposure to light. Fig. 5 shows that the viability of *hit2* seedlings was increased markedly, from zero survival in the light to near total survival in the dark. In the case of heat shock stress, *hit2* seedlings were treated at 44°C for 20 min as before, but were allowed to recover in the dark. Again, the survival of *hit2* seedlings was increased, but to a much lesser extent; only 10% of the *hit2* seedlings were able to recover from the stress treatment. This phenomenon might have been caused by other forms of damage, which were more severe than heat-induced oxidative injury and which occurred rapidly in heat-shocked *hit2* seedlings, and masked the influence of light during later recovery. Collectively, these results indicate that the *hit2* phenotype is attributable, at least in part, to the lack of a sufficient response to oxidative injury, which strongly affects *hit2* viability under sustained high temperature, but is less significant for protection against heat shock.

Expression of *XPO1A* is not affected by heat stress treatment

To evaluate expression of the *XPO1A* gene, the 3′ region of the *XPO1A* cDNA was amplified by semiquantitative RT-PCR. The *XPO1A* transcript was detected in the root, rosette leaves, cauline leaves, stem, and flowers (Fig. 6), which

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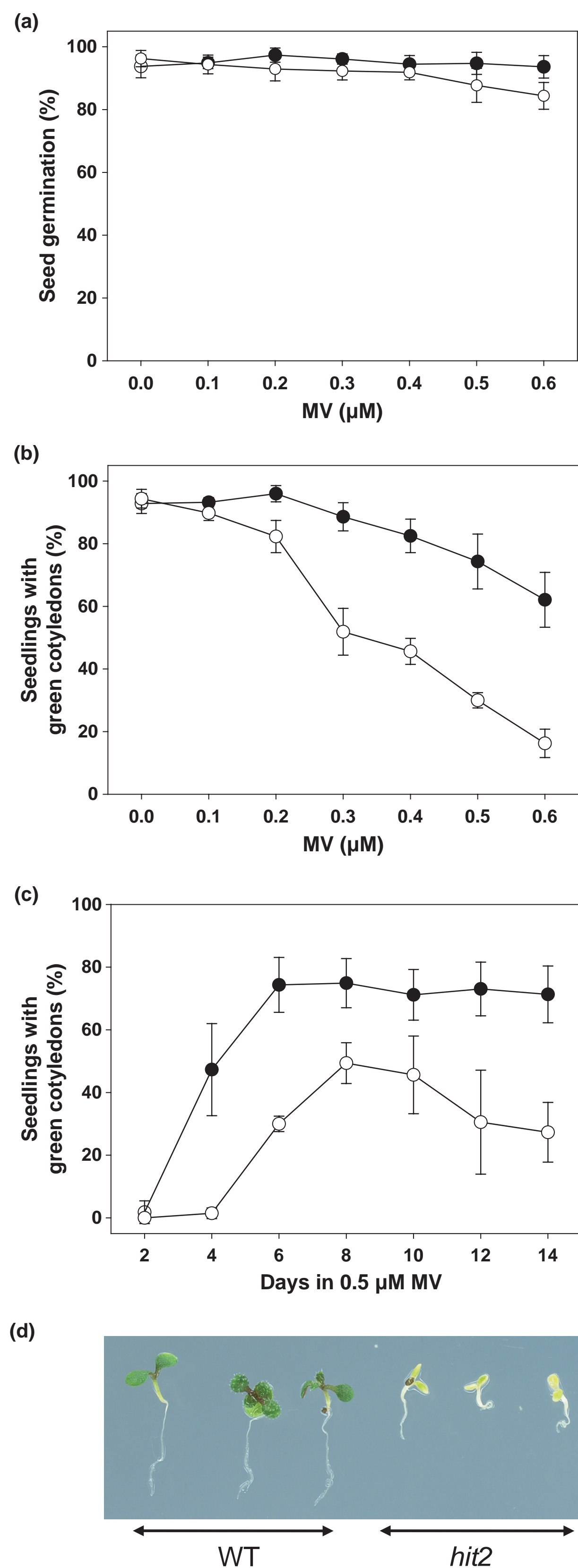

Fig. 4 Development of Arabidopsis *heat-intolerant 2* (*hit2*) seedlings (open circles) was more sensitive to methyl viologen (MV)-induced oxidative stress than that of wild-type (WT) seedlings (closed circles). Seeds were sown on agar plates that contained various concentrations of MV and allowed to germinate at 23°C with continuous illumination. (a) The percentage of seeds that germinated was calculated by counting the seeds with visible protruding radicals after 10 d. Seed germination of the *hit2* mutant was relatively unaffected by MV inhibition. (b) The percentage of seedlings that matured was calculated from the number of seeds that showed green opened cotyledons after 14 d. (c) Progression of seedling maturation of wild-type and *hit2* seeds sown on agar supplemented with 0.5 μM MV. The decline in maturation rate of *hit2* seedlings after day 7 might have been caused by fading of the green color of the cotyledons of *hit2* seedlings that had developed. Data for plots were obtained from a sample size of *c*. 60 seeds on each plate, and at least three replicated plates were used for each treatment. Error bars represent SD for all experiments. (d) Phenotypes of representative wild-type and mutant seedlings grown at 0.5 μg MV for 14 d. Seedlings from the same plate were removed and reorganized for the photograph.

indicated that *XPO1A* was expressed ubiquitously in sporophytic tissues. We investigated the effect of heat stress on *XPO1A* expression by performing RT-PCR on RNA from 10-d-old seedlings that had been incubated at 37°C for 0, 6, 12, 24, 36, or 48 h, respectively. Although expression of the heat-responsive small HSP gene *AtHSP17.6A* was induced within 6 h, *XPO1A* was expressed at a relatively constant level throughout the treatment.

Discussion

Plant cells, like those of other eukaryotic organisms, contain a bilayered nuclear membrane that separates the nucleoplasm from the cytoplasm. Translocation of molecules across this membrane barrier occurs through the nuclear pore complex (NPC), which is a structure made of nucleoporin proteins (Alber *et al*., 2007). Small molecules can pass through the NPC by passive diffusion. However, the efficient and directed translocation of large molecules requires the mediation of nuclear transport receptors. These receptors recognize unique domains within their specific cargo substrates and interact with nucleoporins to facilitate the passage of diverse macromolecules through the NPC. Many studies have shown that plants can convey signals from and trigger appropriate responses to environmental stimuli by changing the nucleo-cytoplasmic partitioning of certain regulatory proteins. This implies that nucleo-cytoplasmic trafficking mediated by nuclear transport receptors is vital for plant tolerance to stress (Merkle, 2004; Meier & Brkljacic, 2009). However, because of the fact that a receptor may have a broad range of cargo substrates, or one species of macromolecule may use several different receptors for nuclear translocation, the existence of a nuclear transport receptor with a specific role in plant stress tolerance had not

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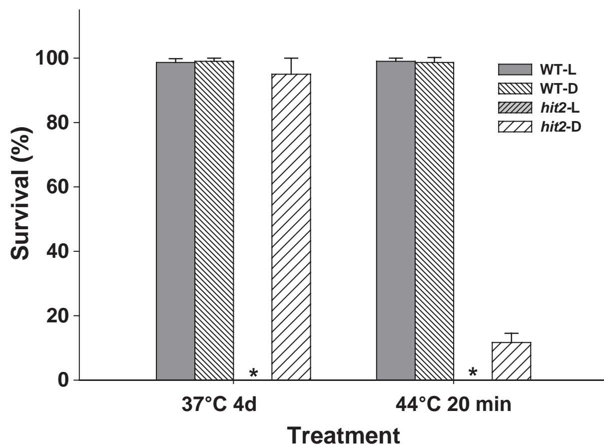

Fig. 5 Survival of Arabidopsis *heat-intolerant 2* (*hit2*) seedlings after heat stress was affected by light conditions. Ten-day-old plants grown on medium were heated to 37°C in the light or dark for 4 d. Seven-day-old seedlings were heated to 44°C for 20 min and allowed to recover at 23°C in the light or dark for 6 d. The numbers of plants that were alive before and after treatment were counted to determine the survival rate. Data shown are the averages of six replicates; error bars represent SD. WT, wild type; L, light; D, dark. *Zero survival rates.

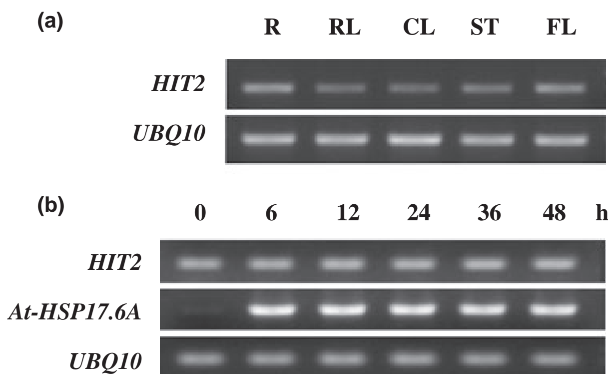

Fig. 6 Expression analysis of *HEAT-INTOLERANT 2* (*HIT2*). (a) Reverse transcription–polymerase chain reaction (RT-PCR) was performed on first-strand cDNA generated from different Arabidopsis plant tissues grown at 23°C. Tissue designations were as follows: R, root; RL, rosette leaf; CL, cauline leaf; ST, stem; FL, flower. Poly-ubiquitin (*UBQ10*) RNA served as an internal control for RT-PCR. (b) Ten-day-old seedlings were transferred to 37°C and incubated for 0–48 h before total RNA was extracted for RT-PCR analysis. Heat shock protein 17.6A (*At-HSP17.6A*) RNA served as a positive control.

been demonstrated. The isolation and characterization of the *hit2* mutant, in which the mutation clearly affected a nuclear transport receptor that was required for heat tolerance but dispensable for normal plant growth and development, have shed light on this issue.

XPO1 belongs to the large family of importin β-like nuclear transport receptors. It was identified first in the fission yeast *Schizosaccharomyces pombe* as chromosome region maintenance 1 (CRM1) and later renamed XPO1 (Adachi & Yanagida, 1989; Nishi *et al.*, 1994; Stade *et al.*, 1997). XPO1 interacts with leucine-rich NESs, which are found in a large variety of proteins that are exported from the nucleus. In addition, XPO1 mediates the transport of many RNA species across the nuclear envelope (Hutten & Kehlenbach, 2007; Merkle, 2009). Furthermore, as its original name implies, XPO1 is involved in functions other than nucleo-cytoplasmic trafficking. In mammalian cells, XPO1 has been shown to recruit partner proteins at the centrosome and kinetochores. These mitotic structures are crucial for microtubule organization and for the anchoring of chromatin to the spindle. Defects in these functions result in the over-duplication of centrosomes, disrupt chromosome segregation, and ultimately prevent progression of the cell cycle (Arnaoutov *et al.*, 2005; Wang *et al.*, 2005). The fact that Arabidopsis contains two copies of the *XPO1* genes immediately raises the question of whether these two paralogs are redundant functionally. Previously, it was shown that transcripts of both Arabidopsis *XPO1A* and *XPO1B* are detectable in seedlings and reproductive tissues. Using a reverse genetics approach, it has also been demonstrated that plants that contain a mutation in either one of the *XPO1* genes appear normal (Blanvillain *et al.*, 2008). However, it has not been possible to obtain a double-mutant homozygous plant. Furthermore, analysis of gametophyte and zygote development has indicated that the main reason the mutant alleles cannot be co-transmitted is that a viable embryo cannot be established from double-mutant female gametophytes (Blanvillain *et al.*, 2008). These results have suggested that: each of the *XPO1* paralogous genes can cover the loss of function of the other under nonstress conditions; XPO1 is not strictly required for haploid mitotic divisions; it is likely that the lack of XPO1-dependent nuclear transport machinery compromises embryo development (Blanvillain *et al.*, 2008). Nevertheless, we showed that the relationship between *XPO1A* and *XPO1B* was more complicated than described previously (Blanvillain *et al.*, 2008). The fact that the phenotypes of wild-type and *xpo1a* mutant plants can be distinguished under stress conditions suggests that *XPO1A* has a different function from that of *XPO1B*, at least with respect to stress tolerance in the vegetative body. Alternatively, the expression of *XPO1A* and *XPO1B* might be subject to regulation that is as yet unknown, for example the expression of one of the *XPO1* paralogous genes might become specific to a certain tissue type, growth stage, environmental condition, or phase of ploidy. It is also possible that different substrates undergo nucleo-cytoplasmic redistribution under stress and nonstress conditions and these substrates might be recognized by only one of the XPO1 receptors. Meanwhile, given that heat stress affects seedlings at the whole-organism level rather than just in the relatively confined meristem cells, it is likely that the heat intolerance phenotype of the *hit2* mutant results from the absence of XPO1A-mediated transport.

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In the study described herein, the *hit2* mutant could not tolerate heat shock that was nonlethal to the wild type, but it did retain the ability to withstand otherwise lethal heat shock after pre-acclimation at a moderately high temperature. This suggests that *HIT2* is crucial for the basal thermotolerance of plants. Heat stress often has a complex effect on the functions of plant cells. High temperatures directly cause protein denaturation and membrane destabilization. In turn, these effects perturb metabolic equilibration, alter redox homeostasis, induce the overproduction of ROS, and result in so-called secondary oxidative stress (Mittler, 2002; Wang *et al.*, 2003). Given the diverse effects caused by heat, it is reasonable to suggest that different protective mechanisms contribute to the survival of plants during different types of heat stress. In this regard, the results of previous studies have suggested that genes that are involved in the production of antioxidants are more likely to participate in plant basal thermotolerance, and are less crucial for acquired thermotolerance (Larkindale & Knight, 2002; Larkindale *et al.*, 2005). Meanwhile, it has been shown that some proteins related to tolerance to oxidative stress in plants relocate from the cytoplasm to the nucleus in the presence of the nuclear export inhibitor LMB (Blanvillain & Ow, 2004; Tsou *et al.*, 2007; Blanvillain *et al.*, 2008). Together with the evidence that the *hit2* mutant is also sensitive to oxidative stress induced by MV, these findings suggest that XPO1A-mediated heat tolerance is achieved in part by protecting cells against heat-induced oxidative damage.

It is tempting to speculate about the identity of the molecule whose pattern of nucleo-cytoplasmic partitioning through XPO1A must change in order for plants to survive under heat stress. Although many XPO1-mediated transport substrates have been identified and their roles characterized, most of our understanding has been obtained from vertebrate and yeast cells, and much less is known about this transport in plants. Nevertheless, the results of some studies suggest that HsfA2 is a possible candidate. HsfA2 is one of the key regulators involved in the development of thermotolerance in plants. Its role in regulating the genes required for defense against oxidative stress has also been characterized (Nishizawa *et al.*, 2006; Schramm *et al.*, 2006). In tomato, HsfA2 contains an NLS and NES, and its nuclear export is controlled by XPO1. High temperature can induce conformational changes in HsfA2, which result in a weak or inaccessible NLS and heat-dependent cytoplasmic retention of HsfA2 (Heerkoltz *et al.*, 2001). These findings have demonstrated that XPO1-mediated shuttling of HsfA2 between the nucleus and cytoplasm is a potential regulatory switch that controls the response of plants to heat stress (Merkle, 2004). However, in Arabidopsis, *HsfA2* itself is a heat-induced gene and is considered to be less involved in basal thermotolerance (Schramm *et al.*, 2006). Indeed, functional characterization of an Arabidopsis *hsfA2* knockout line has revealed that there is no significant difference in appearance or survivability between mutant and wild-type plants under prolonged moderate heat stress or after direct heat shock at 44°C (Charng *et al.*, 2007). Plants contain complex families of *Hsf* genes and HSF proteins may form multimeric complexes; therefore, an alternative HSF might account for the observed *hit2* phenotype. In addition, heat-induced nucleocytoplasmic redistribution of HSPs has been reported (Sun *et al.*, 2004; Tsukahara & Maru, 2004; Tkach & Glover, 2008). In the *hit2* mutant, the lack of XPO1A-mediated export might cause accumulation of negative regulators in the nucleus that would normally be excluded during stress conditions. Further study is necessary to answer this intriguing question, and should provide new insights into the importance of regulatory control at the level of nucleo-cytoplasmic trafficking for plant stress responses.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Relative positions of the molecular markers used for determination of the location of the *heat-intolerant 2* (*hit2*) locus.

Table S1 Oligonucleotides used for determination of the location of the *heat-intolerant 2* (*hit2*) locus.

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Supporting Information for

Isolation and characterization of the Arabidopsis *hit2* mutant reveals the essential role of the nuclear export receptor XPO1A in plant heat tolerance

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Supporting Information Table S1

Supporting Information Figure S1

Supporting Information Table

Table S1. Oligonucleotides used for determination of the *hit2* locus

Molecular marker	Type	Corresponding BAC clone	Primer sequences		Size of product	
					Col (restriction)	L*er* (restriction)
CER456140	SSLP	MKP11	5'-cccatgctttaagaaaagcctga-3'	5'-tggcgatggaaagactgatgat-3'	139	128
CER456148	SSLP	MKP11	5'-tgttgcagcctttatcaggtgat-3'	5'-caggacacatatttggagtgga-3'	119	109
CER483157	CAPS	F2K13	5'-atgccatcgatcatcaccaagt-3'	5'-catcgacgctattcagaatctag-3'	539/373 (*Pst*I)	912
CER483173	CAPS	F2K13	5'-taacgcctccctacgacattct-3'	5'-accttcagaggaaaggaacgct-3'	998	372/626 (*Cla*I)
CER456830	SSLP	MQK4	5'-gctcaactactgtttggtagact-3'	5'-gccagtcacaaaactcaattcct-3'	172	162
nga151	SSLP	T15N1	5'-gttttgggaagttttgctgg-3'	5'-cagtctaaaagcgagagtatgatg-3'	150	122
CER456932	SSLP	MRG7	5'-gggaggtccttcgtcataattacc-3'	5'-tctgaggagcagagagcgataga-3'	167	149
CER457188	SSLP	MTG13	5'-ggccacaactgttacattac-3'	5'-caaccaaaacatcgaaaaagc-3'	112	102
CER483311	SSLP	F5E19	5'-taacatgttccagcctgcaagg-3'	5'-ctagatcgtaatgccaaaactc-3'	201	183
CER483461	SSLP	K3M16	5'-tagcgaatgaatacacaagcta-3'	5'-cgagaaagtaacttgggtaaca-3'	143	135
CER457401	SSLP	MVA3	5'-agagaacaccattgttttcgac-3'	5'-gttattagcctaatgggctggc-3'	153	143
CER456657	SSLP	MPI7	5'-cactcaggaaatccgagcatgc-3'	5'-cagcatccatgtcgatgatgga-3'	254	156
CER483643	SSLP	T10B6	5'-caagtagctagaagcctagaag-3'	5'-catcacccatgacttggtaact-3'	152	136

Supporting Information Figure

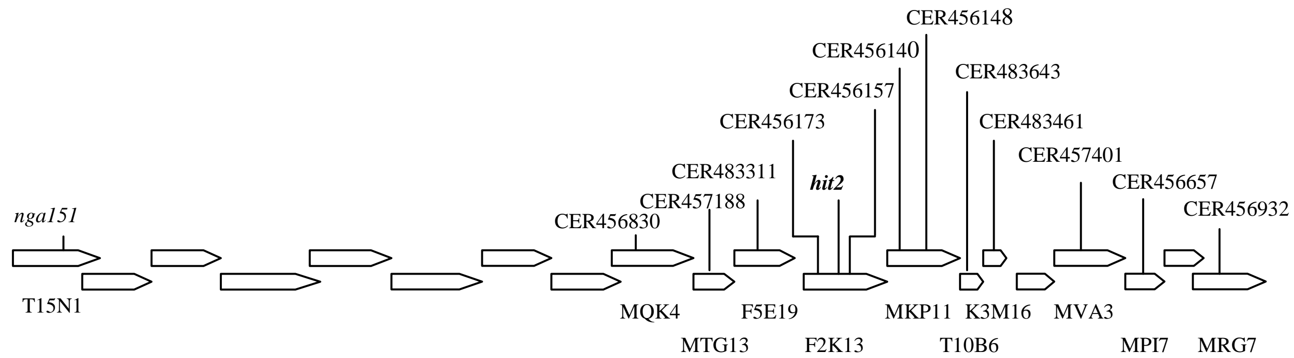

Figure S1. Relative positions of the molecular markers used for determination of the *hit2* locus.

Region of the upper arm of chromosome 5 with 20 overlapping bacterial artificial chromosomes (horizontal arrow bars) that encompassed the *hit2* locus. The relative positions of the molecular markers (vertical lines) used for determination of the *hit2* locus are indicted. The *hit2* mutation was mapped within BAC F2K13.