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High irradiance sensitive phenotype of *Arabidopsis hit2/xpo1a* mutant is caused in part by nuclear confinement of AtHsfA4a

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Abstract

In *Arabidopsis*, EXPORTIN1A (HIT2/XPO1A) and EXPORTIN1B (XPO1B) mediate the translocation of nuclear export sequence (NES)-bearing proteins from nucleus to cytoplasm. However, a mutation in *HIT2/XPO1A* but not in *XPO1B* induces sensitivity to high irradiance (HI). *Arabidopsis thaliana* heat stress elements A4a and A5 (AtHsfA4a and AtHsfA5) are involved in plant responses to HI and possess NESs; therefore, their nucleo-cytoplasmic partitioning was analyzed. In wild-type and *xpo1b* mutant cells, AtHsfA4a normally remained in the cytoplasm but became concentrated in the nucleus following exposure to HI, whereas AtHsfA5 was constitutively distributed in both cytoplasm and nucleus. However, in *hit2/xpo1a* mutant, AtHsfA4a and AtHsfA5 were always confined to the nucleus, regardless of the irradiance. Although AtHsfA4a can enhance the ability of plants to scavenge H_2O_2, and AtHsfA5 is a repressor of AtHsfA4a, *athsfa5* but not *athsfa4a* mutant plants exhibited HI sensitivity. Additionally, *athsfa4a* plants expressing *AtHsfA4a*Δ*NES* were sensitive to HI, but *athsfa5* plants expressing *AtHsfA5*Δ*NES* were not. Meanwhile, *hit2/athsfa4a* double mutant was more tolerant to HI than *hit2*. These results indicate that both AtHsfA4a and AtHsfA5 were HIT2/XPO1A-specific substrates. Long-term accumulation of AtHsfA4a contributed to the *hit2* HI-sensitive phenotype independent of the scavenging ability of H_2O_2, and the presence of AtHsfA5 could mitigate this adverse effect.

Additional key words: exportin1 (XPO1), heat stress factor A4a, nuclear transport receptor.

Introduction

Insufficient irradiance limits photosynthesis and plant growth, but excessive irradiance also reduces the photosynthesis due to photodamage (Powles 1984, Takahashi and Murata 2008, Takahashi and Badger 2011). In addition, the excess electrons generated by high irradiance react with molecular oxygen to create reactive oxygen species (ROS) which damage to DNA, lipids, and proteins. Plant cells have evolved enzymatic and non-enzymatic defence systems (Niyogi 1999, Apel and Hirt 2004, Hung *et al.* 2005). In addition, plants also frequently encounter other types of adverse environmental conditions. Therefore, plants evolved an interconnected signalling network to trigger dynamic responses allowing them to adapt to the many changes in their environment. For example, heat stress factors (Hsfs) are transcription factors known to be widespread among all organisms. Their primary function is to activate the expression of heat shock proteins (HSPs). HSPs are molecular chaperones, which can protect other proteins in the cell from heat denaturation. However, unlike the yeast and *Drosophila* genomes, which only possess one *Hsf* gene each, the *Arabidopsis* genome contains 21 *Hsf*s. Based on the amino acid sequences, protein structures, and evolutionary analyses, plant *Hsf*s are classified into three classes: A, B, and C. Different *Hsf* genes are induced by different adverse environmental conditions (Nover *et al.* 2001, Miller and Mittler 2006, Von Köskull-Döring *et al.* 2007). Serving as the terminal

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Abbreviations: APX - ascorbate peroxidase; BiFC - bimolecular fluorescent complementation; CAT - catalase; GFP - green fluorescent protein; Hsf - heat stress factor; HSE - heat stress element; HSP - heat shock protein; LMB - leptomycin B; MV - methyl viologen; NES - nuclear export sequence; NLS - nuclear localization sequence; ROS - reactive oxygen species; SOD - superoxide dismutase; WT - wild-type; XPO1 - exportin1.

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regulator of a signal transduction pathway, Hsfs enter the nucleus and function at promoters containing heat stress elements (HSEs), thereby regulating gene expression. The products of these genes also include many enzymes involved in countering high irradiance (HI) stress. Therefore, all Hsfs contain a nuclear localization sequence (NLS), and some also contain a nuclear export sequence (NES) (Von Koskull-Döring *et al.* 2007, Scharf *et al.* 2012). This allows increasing Hsf signalling diversity through the dynamic equilibrium of nucleo-cytoplasmic transport (Scharf *et al.* 1998, Heerklotz *et al.* 2001, Merkle 2003, 2011).

Among the class A Hsfs, HsfA4 has been found in many different types of plants and participates in many stress reactions, including the response to HI (Yamanouchi *et al*. 2002, Shim *et al.* 2009, Pérez-Salamó *et al*. 2014, Personat *et al*. 2014). In *Arabidopsis*, expression of the *AtHsfA4a* gene is induced by HI and H_2O_2, thereby stimulating expression of the downstream *APX1* gene (Miller *et al*. 2008, Scarpeci *et al*. 2008a,b, Qu *et al*. 2013). Mutations in *APX1* lead to the accumulation of large amounts of H_2O_2 in cells, and increased *AtHsfA4a* gene expression. In addition, expression of a dominant negative mutation of *athsfa4a* (which leads to the retention of the DNA binding domain but removal of the gene activation domain) in wild-type (WT) plants causes the loss of *APX1* gene expression, but increases normal *AtHsfA4a* gene expression. Therefore, AtHsfA4a is considered to have a role as an H_2O_2 sensor (Davletova *et al*. 2005a,b, Pérez-Salamó *et al*. 2014). In rice, *Spl7* (*OsHsfA4d*) mutations result in sensitivity to HI causing reddish-brown lesions on leaves (Yamanouchi *et al.* 2002). However, when *AtHsfA4a* was over-expressed in *Arabidopsis*, growth was inhibited and the size of rosette leaves was reduced by 20 - 30 % compared with control plants (Pérez-Salamó *et al.* 2014). Overexpression of wheat *TaHsfA4a* in rice also resulted in shorter plants under control conditions (Shim *et al*. 2009). In addition, AtHsfA5 was found to be a repressor of AtHsfA4a, which can antagonize the gene activation function of *AtHsfA4a* (Baniwal *et al*. 2007). Therefore, although HSFA4a can help plants tolerate many different stresses, especially HI-induced H_2O_2 stress, its functions have negative effects on plant growth under normal conditions. Therefore, these functions must be precisely regulated.

The *Arabidopsis heat-intolerance 2* (*hit2*) mutant was identified due to its heat-sensitive phenotype (Wu *et al*. 2010). In addition to being defective in basal thermotolerance, *hit2* plants are also sensitive to the oxidative stress inducer methyl viologen (MV). Heat treatment in the dark can also significantly increase the survival of *hit2* mutant plants. Accordingly, HIT2 is believed to participate in the mechanism of tolerance against light-dependent heat-induced oxidative stress. *HIT2* encodes the nuclear transport receptor EXPORTIN1A (XPO1A), whose function is to transport proteins with a NES from the nucleus to the cytoplasm (Haasen *et al*. 1999). In addition to the *XPO1A* gene (At5G17020), *Arabidopsis* carries another homolog of *EXPORTIN1* called *XPO1B* (At3G03110). The amino acid sequences of the proteins encoded by these two genes share 86 % identity. Early reports indicate that mutation of either *XPO1A* or *XPO1B* does not result in any distinguishable traits. However, when *XPO1A* and *XPO1B* were simultaneously mutated, the development of the female gametophyte and embryo was affected, and offspring homozygous for both mutations could not be generated. Accordingly, HIT2/XPO1A (hereafter referred to as HIT2) and XPO1B are considered to be functionally redundant (Blanvillain *et al.* 2008). However, later studies showed that *xpo1b* mutant plants do not have the same sensitivity to heat as *hit2* mutant plants. This indicated that the physiological reactions affected by HIT2 and XPO1B are partially independent. It also implies that the cargo substrates of HIT2 and XPO1B are partially specific (Wu *et al.* 2010). However, it remains unknown which HIT2-specific substrates are trapped in the nucleus, causing *hit2* mutant plants to become sensitive to adverse conditions, such as HI, high temperature, and oxidative stress.

The heat sensitivity trait of *hit2* is dependent on irradiance, *AtHsfA4a* gene activation is induced by light, AtHsfA5 is a repressor of AtHsfA4a, and both AtHsfA4a and AtHsfA5 have a NES. Therefore, a series of experiments was conducted to determine the correlation among AtHsfA4a, AtHsfA5, and HIT2 with the aim to answer following questions: *1*) are AtHsfA4a and AtHsfA5 specific substrates of HIT2; *2*) are the nuclear-cytoplasmic distributions of AtHsfA4a and AtHsfA5 regulated by irradiance; *3*) what are the effects of retaining either AtHsfA4a or AtHsfA5 alone in the nucleus on the HI sensitivity of plants? The results from these experiments allowed us to clarify whether HI sensitivity in *hit2* mutant is related to the AtHsfA4a/AtHsfA5 regulatory system.

Materials and methods

Plants and growth conditions: The WT *Arabidopsis thaliana* L. (ecotype Col-0) seeds were obtained from *Lehle Seeds Company* (Round Rock, TX, USA). The *hit2* mutant line was as previously described (Wu *et al*. 2010) and *xpo1b* (SALK_088267C) and *athsfa5* (SALK_004385) mutant lines were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA) (Alonso *et al*. 2003). The *athsfa4a* (GK-181H12) was obtained from the Genomanalyse im Biologischen System Pflanze

(Bielefeld University, Bielefeld, Germany) (Kleinboelting *et al.* 2012). The *hit2/athsfa4a* (*hit2/a4a*) double mutant was obtained by crossing *hit2* and *athsfa4a*. The *athsfa4a/athsfa5* (*a4a/a5*) double mutant was obtained by crossing *athsfa4a* and *athsfa5*. To grow plants *in vitro*, seeds were surface sterilized, kept at 4 °C for 2 d for stratification, sown in agar plates containing 1/2 strength Murashige and Skoog (1962; MS) medium with 2 % (m/v) sucrose (pH adjusted to 5.7), and grown in an incubator at a temperature of 23 °C and a continuous irradiance of 100 μmol m^{-2} s^{-1} provided by cool, white fluorescent tubes (Wu *et al*. 2010). For the HI stress treatment, 4-d-old seedlings were transferred to an incubator with a irradiance of 500 μmol m^{-2} s^{-1} and grown for an additional 12 d. For some experiments, plants were also cultivated in soil at 23 °C, an irradiance of 100 μmol m^{-2} s^{-1}, and a 16-h photoperiod.

Genotyping of T-DNA insertion lines and double mutants: PCR using three primers was performed for genotyping the *athsfa4a* mutant. The primers p-*AtHsfA4a*-F and p-*AtHsfA4a*-R yielded an 894-bp wild-type amplicon, whereas the primers p-*AtHsfA4a*-F and p-*AtHsfA4a*-T-DNA-R yielded a 560-bp amplicon containing a sequence at the junction of plant *AtHsfA4a* and the T-DNA insertion. Similarly, when genotyping the *athsfa5* mutant, the primers p-004385-F and p-004385-R yielded a 599-bp amplicon containing the wild-type *AtHsfA5* sequence, whereas the primers p-004385-R and p-Lba1 yielded a 560-bp amplicon including the DNA sequence at the junction of plant *AtHsfA5* and the T-DNA insertion. When genotyping the *hit2/a4a* double mutants, the *hit2* genotype was identified by derived cleaved amplified polymorphic sequence. The primers p-*hit2*-*Eco*RI-F and p-*hit2*-*Eco*RI-R were used for PCR and yielded a 284-bp amplicon. The amplicon obtained using the wild type as the template could be digested into two fragments of 257- and 27-bp using the restriction enzyme *Eco*RI, whereas amplicons obtained with *hit2* as the template could not. The *athsfa4a* genotype was identified as described above. All primer sequences used are shown in Table 1 Suppl.

Gene expression analysis: To determine whether the T-DNA insertions in *athsfa4a* and *athsfa5* knocked out the transcription of the two genes, total RNA was extracted from seedlings grown for 10 d in culture medium using the *GeneMark Plant Total RNA Miniprep* purification kit (*Hopegen Biotechnology*, Taichung, Taiwan). *SuperScript*^{III} reverse transcriptase (*Invitrogen*, Carlsbad, CA, USA) was then used for reverse transcription into cDNA (Wu *et al.* 2017). The primers p-*AtHsfA4a*-511-F and p-*AtHsfA4aΔNES*-R were used to detect *AtHsfA4a* cDNA. The primers p-*AtHsfA5*-600-F and p-*AtHsfA5ΔNES*-R were used to detect *AtHsfA5* cDNA. The primers p-*UBC28*q-F and p-*UBC28*q-R were used to detect the expression of the internal control gene *UBC28*. All primer sequences used are shown in Table 1 Suppl.

Subcellular localization of *AtHsfA4a* and *AtHsfA5*: To analyze the nucleo-cytoplasmic distribution of *AtHsfA4a*, RNA was first isolated from wild-type plants using the *GeneMark Plant Total RNA Miniprep* purification kit (*Hopegen Biotechnology*). Next, *SuperScript*^{III} reverse transcriptase (*Invitrogen*) was used to synthesize cDNA. Using this cDNA as a template, the primers p-*AtHsfA4a*-*Bam*HI-F and p-*AtHsfA4a*-*Pst*I-R were used to amplify a 1 203-bp DNA fragment containing the *AtHsfA4a* cDNA sequence. After digesting this amplicon with the restriction enzymes *Bam*HI and *Pst*I, it was inserted into the previously described pLOLA-*GFP* vector to create pLOLA-*GFP*-*AtHsfA4a* (Wang *et al.* 2013). Conversely, using genomic DNA as a template, the primer pair p-p*AtHsfA4a*-*Xho*I-F and p-p*AtHsfA4a*-*Sma*I-R amplified a 3 722-bp DNA fragment containing the *AtHsfA4a* promoter. After digestion with the restriction enzymes *Xho*I and *Sma*I, this amplicon was inserted into the pLOLA-*GFP*-*AtHsfA4a* vector to create the pLOLA-p*AthHsfA4a*-*GFP*-*AtHsfA4a* construct. To analyze the subcellular localization of AtHsfA5, the primer pair p-p*AtHsfA5*-*Xho*I-F and p-p*AtHsfA5*-*Sma*I-R was first used with wild-type genomic DNA as a template to amplify an 870-bp DNA fragment containing the *AtHsfA5* promoter sequence. After digestion with the restriction enzymes *Xho*I and *Sma*I, this amplicon was inserted into the pLOLA-*mCherry* vector to make the pLOLA-p*AtHsfA5*-*mCherry* construct (Wang *et al.* 2011). With cDNA as the template, the primer pair p-*AtHsfA5*-*Bam*HI-F and p-*AtHsfA5*-*Pst*I-R amplified a 1 401-bp DNA fragment containing the *AtHsfA5* cDNA. After digestion with the restriction enzymes *Bam*HI and *Pst*I, this amplicon was inserted into pLOLA-p*AtHsfA5*-*mCherry* to generate the pLOLA-p*AtHsfA5*-*mCherry*-*AtHsfA5* construct. All primer sequences used are shown in Table 1 Suppl. Protoplasts were prepared from rosette leaves of 4-week-old plants (Yoo *et al.* 2007). Polyethylene glycol (PEG)-mediated transfection was performed by mixing 0.2 cm^3 of freshly prepared protoplasts (4×10^4 protoplasts), 0.02 cm^3 of plasmid DNA (10 μg) and 0.22 cm^3 of PEG transfection solution (40 % PEG_{4000} in 0.2 M mannitol and 0.1 M $CaCl_2$) (Wang *et al*. 2015). The transfection mixtures were incubated at 22 °C for 10 min. The transfected protoplasts were then washed and suspended in W5 solution as described by Yoo *et al.* (2007). Fluorescent proteins were observed and imaged using an *Olympus IX71* fluorescence microscope (Center Valley, PA, USA). To determine the nucleo-cytoplasmic distributions of AtHsfA4a and AtHsfA5, transformed protoplasts were exposed to irradiance of 500 μmol m^{-2} s^{-1} for 5 h before observation and imaging.

Transgenic plants for stress analysis: To determine the

effect of AtHsfA4a or AtHsfA5 accumulation in cell nuclei on plant tolerance to high irradianmce, transgenic plants producing either AtHsfA4a or AtHsfA5 protein lacking the NES were generated (Nover *et al.* 2001). First, the primers p-*AtHsfA4a-Bam*HI-F and p-*AtHsfA4aΔNES-Pst*I-R were used with cDNA prepared from wild-type plants as the template to amplify a 1 164-bp DNA fragment encoding the AtHsfA4aΔNES protein. After this DNA fragment had been digested with the restriction enzymes *Bam*HI and *Pst*I, it was inserted into the pLOLA-*GFP* vector (Wang *et al*. 2013) to generate the pLOLA-*GFP-AtHsfA4aΔNES* construct. Conversely, the *AtHsfA4a* promoter fragment was prepared as described previously by amplification with the primers p-p*HsfA4a-Pst*I-F and p-p*HsfA4a-Sma*I-R. After digestion with the restriction enzymes *Pst*I and *Sma*I, the promoter was inserted into the binary vector pCAMBIA2301 (*CAMBIA*, Canberra, ACT, Australia) to generate the pCAMBIA2301-p*AtHsfA4a* construct. Next, the *GFP-AtHsfA4aΔNES* was excised from the pLOLA-*GFP-AtHsfA4aΔNES* using *Sma*I and *Sac*I, and then inserted into pCAMBIA2301-p*AtHsfA4a* to generate pCAMBIA2301-p*AtHsfA4a-GFP-AtHsfA4aΔNES*. The same strategy was used for generation of the pCAMBIA1300-p*AtHsfA5-mCherry-AtHsfA5ΔNES* construct. First, the primers p-*AtHsfA5-Bam*HI-F and p-*AtHsfA5ΔNES-Pst*I-R were used to amplify a DNA fragment (1383 bp) encoding the AtHsfA5ΔNES polypeptide. After digestion with the restriction enzymes *Bam*HI and *Pst*I, this amplicon was inserted into pLOLA-*mCherry* to generate pLOLA-*mCherry-AtHsfA5ΔNES*. The *AtHsfA5* promoter was amplified using the primers p-p*AtHsfA5-Sal*I-F and p-p*AtHsfA5-Sma*I-R. After digestion with the restriction enzymes *Sal*I and *Sma*I, this amplicon was inserted into pCAMBIA1300 (*CAMBIA*) to generate pCAMBIA1300-p*AtHsfA5*. Next, *mCherry-AtHsfA5ΔNES* was excised from pLOLA-*mCherry-AtHsfA5ΔNES* using *Sma*I and *Sac*I, and inserted into pCAMBIA1300-p*AtHsfA5* to generate pCAMBIA1300-p*AtHsfA5-mCherry-AtHsfA5ΔNES*. All primer sequences used are shown in Table 1 Suppl. These two vectors were then delivered to *athsfa4a* and *athsfa5* mutants, respectively, using the *Agrobacterium*-mediated floral dipping method (Clough and Bent 1998). Positive transformants were selected on agar medium containing 50 μg cm^{-3} kanamycin (for pCAMBIA2301) or 25 μg cm^{-3} hygromycin (for pCAMBIA1300). Resistant T1 seedlings were transferred to soil for continued growth to collect T_2 seeds. Homozygous T_2 plants were distinguished by the antibiotic resistance of T_3 seeds in culture medium containing antibiotics. Homozygous T_3 seeds served as the experimental material for use in high irradiance experiments.

Measurement of chlorophyll content: To measure chlorophyll content after HI treatment, the shoots (~25 mg) were collected, immersed in liquid nitrogen, ground into a powder, thoroughly mixed with 2 cm^3 of 80 % (v/v) acetone, and placed at 4 °C in the dark for 30 min. Following centrifugation at 15 000 *g* for 10 min, a spectrophotometer (*UV-VIS SP-8001*; *Metertech*, Taipei, Taiwan) was used to measure the absorbance of the supernatant at 663 and 645 nm (chlorophyll *a* and *b*), and chlorophyll content was calculated using a formula described by Misyura *et al.* (2013).

Determination of H_2O_2 content: To assess the ability of the plants to scavenge H_2O_2, seeds were placed in agar plates with or without 1.5 mM H_2O_2 and allowed to germinate and grow under normal temperature and irradiance. After 2 weeks, shoots (50 mg) were placed in liquid nitrogen, ground into a powder, dissolved in 0.3 cm^3 of 0.1 % (m/v) trichloroacetic acid, and centrifuged at 13 000 *g* and 4 °C for 20 min. After centrifugation, 0.25 cm^3 of potassium phosphate buffer (10 mM, pH 7.0) and 0.5 cm^3 of 1 M KI solution were added to 0.25 cm^3 of supernatant and mixed thoroughly. After 10 min, absorbance at 390 nm was measured. An H_2O_2 standard curve was constructed and H_2O_2 content was calculated as described by Zsigmond *et al*. (2012).

Values are reported as means ± standard deviations of three biological replicates.

Results and discussion

Previous results have shown that dark conditions can increase the survival rate of *hit2* mutants under heat stress, and that *hit2* mutants are sensitive to oxidative stress inducer MV (Wu *et al.* 2010). Although these results showed that HIT2 plays an important role in plant resistance to HI, it remains unknown whether HIT2 directly protects plants from photodamage. To this end, 4-d-old wild-type, *hit2*, and *xpo1b* seedlings grown on medium were transferred from a normal irradiance of 100 μmol m^{-2} s^{1} to HI of 500 μmol m^{-2} s^{-1} and allowed to grow for an additional 12 d; following this, the seedlings were observed and compared. This treatment eliminated influence of other factors, such as high temperature, and can be adopted to determine the direct relationship between HIT2 and plant tolerance to HI. The results showed that all plants continued to develop and grow new leaves. Although wild-type and *xpo1b* mutant leaves maintained their green colour, *hit2* mutant leaves became yellow (Fig. 1*A*). Analysis of chlorophyll content indicated that, after the HI treatment, the chlorophyll content in *hit2* mutant leaves was lower than in wild type and *xpo1b* mutant leaves (Fig. 1*B*). These results confirm

that *hit2* mutants are inherently sensitive to HI.

AtHsfA4a is known to participate in the regulation of oxidative stress induced by HI, and AtHsfA5 is a repressor of AtHsfA4a. Both AtHsfA4a and AtHsfA5 carry NESs (Davletova *et al*. 2005a, Pérez-Salamó *et al*. 2014). In addition, transient expression assays in tobacco (*Nicotiana benthamiana*) protoplasts have shown that AtHsfA4a exists simultaneously in the nucleus and the cytoplasm under normal conditions. AtHsfA5 is primarily located in the nucleus, with little being found in the cytoplasm. However, after treatment with the XPO1 inhibitor leptomycin B (LMB), both AtHsfA4a and AtHsfA5 accumulate only in the nucleus. This indicates that AtHsfA4a and AtHsfA5 are both substrates of tobacco XPO1 (Kotak *et al.* 2004). However, unlike the tobacco genome, which possesses only one *XPO1* gene, the *Arabidopsis* genome contains two copies of *XPO1* genes, *HIT2/XPO1A* and *XPO1B*. To elucidate whether AtHsfA4a and AtHsfA5 are specific substrates of HIT2, reporter constructs were generated, which showed that the *AtHsfA4a* promoter drives the expression of the C-terminus of green fluorescence protein (GFP) linked to the N terminus of *AtHsfA4a*, and the *AtHsfA5* promoter drives the expression of the C terminus of mCherry

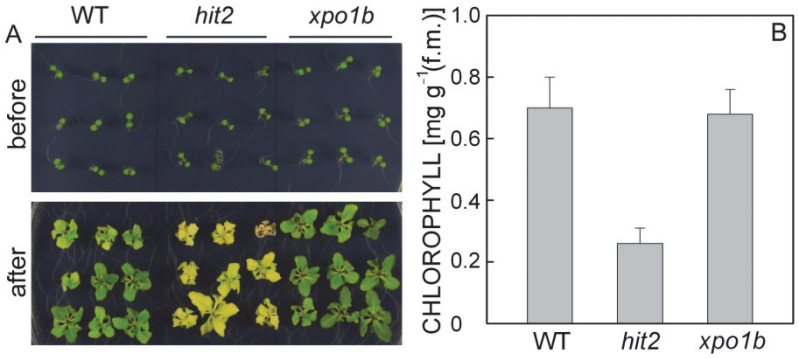

Fig. 1. Development of *Arabidopsis* mutants sensitive to high irradiance (HI): *A* - 4-d-old seedlings grown under irradiance of 100 μmol m^{-2} s^{-1} (before) were transferred to HI of 500 μmol m^{-2} s^{-1} and grown for 12 d (after). Wild-type (WT), *hit2*, and *xpo1b* plants could grow under HI, however, unlike WT and *xpo1b* plants, which maintained green leaves, *hit2* plants exhibited yellow leaves. *B* - Chlorophyll content after HI treatment was similar in *xpo1b* and WT but lower in *hit2*. Means ± SDs, $n = 3$.

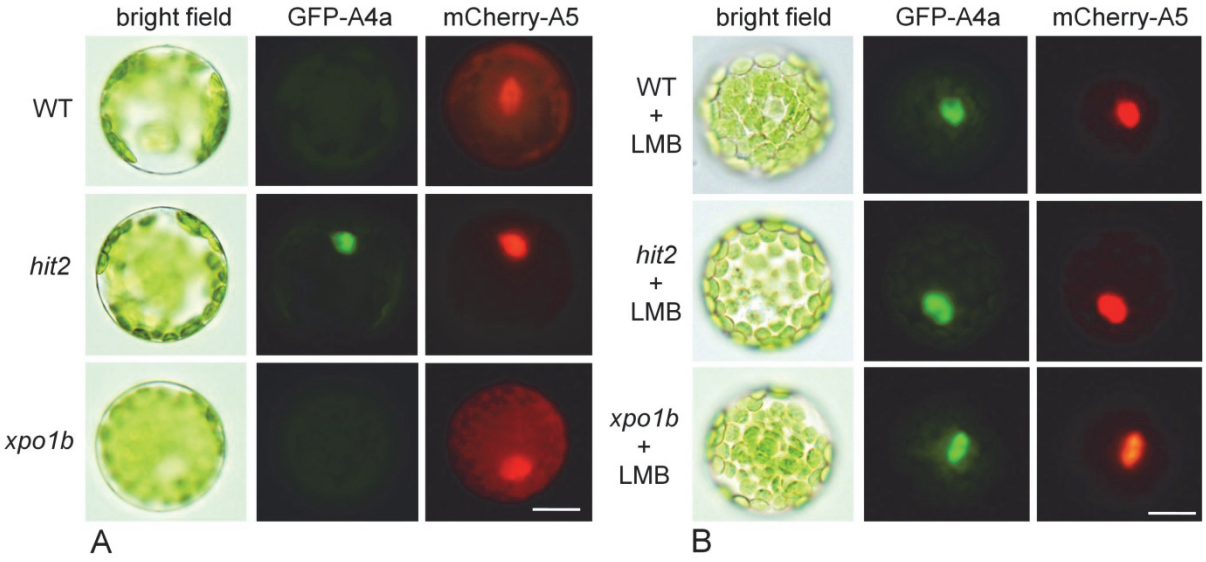

Fig. 2. AtHsfA4a and AtHsfA5 are specific substrates of HIT2: Protoplasts were collected from wild-type (WT), *hit2*, and *xpo1b* leaf tissue, then co-transformed with the AtHsfA4a promoter-driven GFP-AtHsfA4a construct and the AtHsfA5 promoter-driven mCherry-AtHsfA5 construct. Fluorescence microscopy was used to observe GFP and mCherry fluorescence signals; *A* - No GFP-AtHsfA4a fluorescence was observed in WT and *xpo1b* protoplast nuclei. However, GFP-AtHsfA4a could be clearly observed in *hit2* protoplast nucleus. mCherry-AtHsfA5 fluorescence was observed in WT and *xpo1b* nuclei and cytoplasm. However, mCherry was concentrated in the nucleus in *hit2* protoplasts. *B* - The same materials were used as in *A* but 5 ng cm^{-3} leptomycin (LMB) was applied 1 h before observation. Both GFP and mCherry were observed only in the nucleus in WT, *hit2*, and *xpo1b* cells. *Bar* = 10 μm.

fluorescence protein (mCherry) linked to the N terminus of *AtHsfA5*. These were then co-transformed into protoplasts cultured from wild-type, *hit2*, and *xpo1b* plants for transient expression analysis. The results showed that *GFP-AtHsfA4a* was not present in the nuclei of wild-type and *xpo1b* protoplasts. However, in *hit2* protoplasts, *GFP-AtHsfA4a* accumulation was clearly observed in the nuclei (Fig. 2*A* and Fig. 1 Suppl.). The *mCherry-AtHsfA5* was observed in the nuclei of wild-type, *hit2*, and *xpo1b* cells, and the fluorescence was most intense in the *hit2* nuclei (Fig. 2*A*). In addition, mCherry fluorescence was also observed in the cytoplasm of wild-type and *xpo1b* cells, but not in the cytoplasm of *hit2* cells (Fig. 2*A* and Fig. 2 Suppl.).

We aimed to confirm that *GFP-AtHsfA4a* was observed in the *hit2* nuclei but not in the wild-type or *xpo1b* nuclei because the amount of AtHsfA4a was small and diffusely distributed in the cytoplasm and not because GFP-AtHsfA4a was not synthesized. Wild-type and *xpo1b* protoplasts transformed with *GFP-AtHsfA4a* were treated with the XPO1 inhibitor LMB for 1 h before observation. After LMB treatment, GFP fluorescence in WT and *xpo1b* cells could be observed in the nucleus, similar to that observed in *hit2* cells not treated with LMB. Similarly, the distribution of mCherry fluorescence was consistent with that observed in *hit2* cells not treated with LMB, with fluorescence appearing only in the nucleus (Fig. 2*B* and Fig. 2 Suppl.). These results indicate

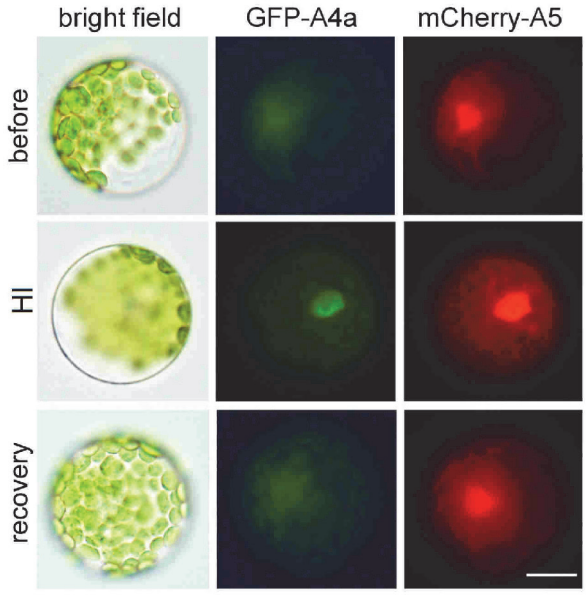

Fig. 3. High irradiance (HI) led to reversible movement of AtHsfA4a in and out of the nucleus. Protoplasts were collected from wild-type leaf tissue, then co-transformed with the AtHsfA4a promoter-driven GFP-AtHsfA4a construct (GFP-A4a) and the AtHsfA5 promoter-driven mCherry-AtHsfA5 construct (mCherry-A5). The image was taken 16 h after polyethylene-glycol-mediated transfection (before) followed by 5 h of HI of 500 μmol m^{-2} s^{1} (HI) and recovery for 2 h under 100 μmol m^{-2} s^{-1} (recovery). *Bar* = 10 μm.

that *AtHsfA4a* and *AtHsfA5* are both expressed under normal conditions, and that homeostasis is maintained between the nucleus and the cytoplasm. AtHsfA4a is primarily located in the cytoplasm and AtHsfA5 is located in both nucleus and cytoplasm. In addition, only HIT2, and not XPO1B, aids exit of AtHsfA4a and AtHsfA5 from the nucleus (Fig. 3 Suppl.).

AtHsfA4a carries both an NLS and an NES (Nover *et al.* 2001), but AtHsfA4a is only distributed in the cytoplasm under normal conditions. Being a positive transcriptional regulator of genes induced by HI, it is reasonable to speculate that AtHsfA4a enters the nucleus under these conditions. To test this hypothesis, wild-type protoplasts transformed with *GFP-AtHsfA4a* were exposed to HI for 5 h, and then the distribution of AtHsfA4a was observed. The results showed that AtHsfA4a indeed accumulated in the nucleus upon stimulation with HI (Fig. 3). After protoplasts treated with HI were transferred back to normal irradiance for 2 h, AtHsfA4a was observed to exit the nucleus. This indicates that the nucleo-cytoplasmic distribution of AtHsfA4a dynamically depended on the irradiance. In contrast, AtHsfA5 was constantly present in both nucleus and cytoplasm, suggesting that its subcellular localization is not affected by irradiance (Fig. 3).

After confirming that AtHsfA4a and AtHsfA5 are specific substrates of HIT2 (Fig. 2 and Fig. 4 Suppl.), and that the nuclear and cytoplasmic distribution of AtHsfA4a is regulated by irradiance, *athsfa4a* (GK-181H12) and *athsfa5* (SALK_004385) mutant lines were obtained from the GABI-KAT and ABRC T-DNA insertion mutation libraries, respectively. Their phenotypes were compared with that of the *hit2* mutant for further assessment of the relationship among AtHsfA4a, AtHsfA5, and HIT2 in HI responses in plants (Fig. 4*A*). Reverse transcription (RT)-PCR was performed to confirm that both *athsfa4a* and *athsfa5* are null mutants without normal *AtHsfA4a* and *AtHsfA5* transcription products, respectively (Fig. 4*B*). In addition, under normal temperature and irradiance, the growth rate and appearance of *athsfa4a* and *athsfa5* were consistent with those of *hit2*, without any difference from wild-type plants (data not shown). However, following treatment of 4-d-old seedlings with HI for 12 d, *athsfa5* displayed the same chlorosis phenotype as *hit2*, whereas *athsfa4a* exhibited the same green colour as the wild type (Fig. 4*C*). Analysis of the chlorophyll content also showed that the chlorophyll content of *athsfa5* and *hit2* plants was significantly lower than that of wild-type and *athsfa4a* plants under HI (Fig. 4*D*). These results indicate that the lack of functional AtHsfA5, but not AtHsfA4a, causes plants to be sensitive to HI stress, similar to the *hit2* mutation.

The AtHsfA4a and AtHsfA5 are specific substrates of HIT2; therefore, both should be simultaneously restricted to the nucleus in *hit2* mutants. We aimed to further elucidate the effect of retaining either AtHsfA4a or AtHsfA5 alone in the nucleus on the HI sensitivity of

plants. Transgenic *athsfa4a* plants expressing *AtHsfA4a* without NES (*A4aΔNES*) and transgenic *athsfa5* plants expressing *AtHsfA5* without NES (*A5ΔNES*) were generated, and the growth and development of seedlings were observed under HI conditions. The *A4aΔNES* was similar to *hit2* in terms of sensitivity to HI, with the symptom of chlorosis. However, *A5ΔNES* was similar to the wild type, with leaves retaining their green colour (Fig. 5). At the same time, *athsfa4a* and *athsfa5* were crossed to generate an *athsfa4a/athsfa5* (*a4a/a5*) double mutant to analyze the ability of plants to tolerate HI when both AtHsfA4a and AtHsfA5 were absent from the nucleus. The growth and development of *a4a/a5* plants under HI were similar to those of wild type plants (Fig. 5). It can be inferred from these results that continuous accumulation of AtHsfA4a in the nucleus (*A4aΔNES*) or lack of AtHsfA5 in the nucleus (*athsfa5*) results in HI sensitivity similar to that observed in *hit2*. Plants are still able to maintain wild-type tolerance to HI with the long-term accumulation of AtHsfA5 (*A5ΔNES*), or lack of AtHsfA4a in the nucleus (*athsfa4a* and *a4a/a5*). AtHsfA5 negatively regulates AtHsfA4a activity (Baniwal *et al.,* 2007); therefore, these results indicate that cells become sensitive HI when the nucleus contains high content of AtHsfA4a that is not regulated by AtHsfA5.

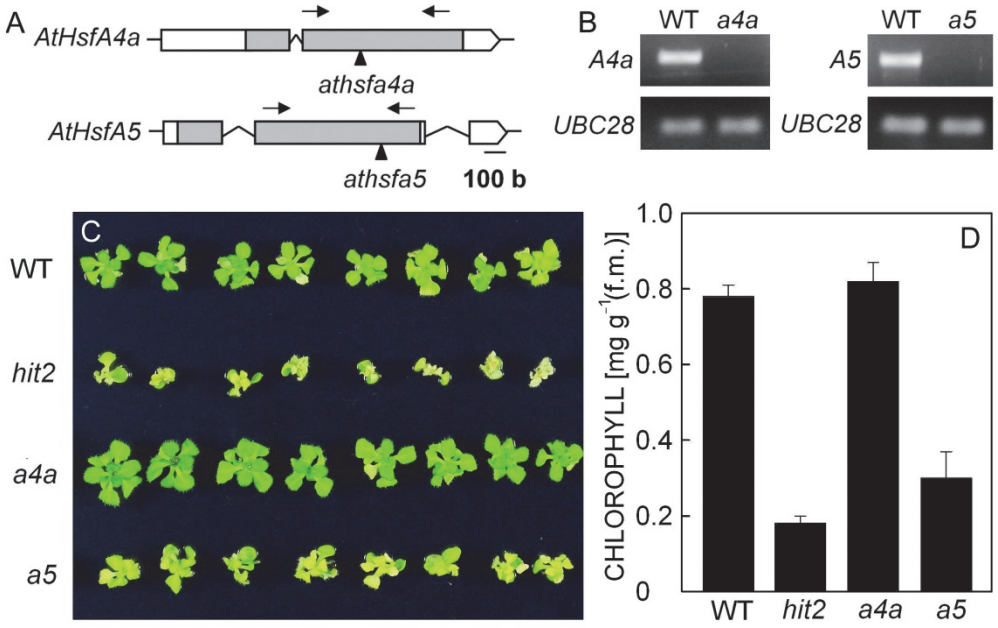

Fig. 4. *Athsfa5* mutants, but not *athsfa4a* T-DNA insertion mutants (*A*) were sensitive to high irradiance (HI). *Arrows* indicate the locations of reverse transcription (RT)-PCR primers used to determine the presence or absence of gene expression. *B* - RT-PCR showed that *athsfa4a* (*A4a*) and *athsfa5* (*A5*) were both null mutations. The *UBC28* gene was used as a loading control. *C* - 4-d-old seedlings grown under irradiance of 100 μmol m^{-2} s^{-1} were transferred to HI of 500 μmol m^{-2} s^{-1}, grown for 12 d, and their shoots were imaged. *D* - Chlorophyll content after HI stress. Means ± SDs, $n = 3$.

AtHsfA4a is known to induce *APX1* expression, whose product can convert excess harmful H_2O_2 in the cells into water, and AtHsfA5 is known to repress AtHsfA4a (Baniwal *et al.* 2007). Theoretically, the long-term accumulation of AtHsfA4a in the nucleus (*A4aΔNES*) or loss of AtHsfA5 (*athsfa5*) should improve the antioxidant capacity of cells and increase their tolerance to stresses which induce H_2O_2 such as HI. However, we showed that continuous accumulation of AtHsfA4a in the nucleus (*A4aΔNES*), or AtHsfA4a lacking regulation by AtHsfA5 (*athsfa5*), causes sensitivity to HI. To elucidate the relationship between AtHsfA4a/AtHsfA5-mediated antioxidant capacity and HI sensitivity, we assessed the ability of the afore-mentioned plants to scavenge H_2O_2. H_2O_2 content in *hit2* and *hsfa4a* seedlings grown in MS culture medium supplemented with 1.5 mM H_2O_2 was more than 400 % higher than that of seedlings grown in medium without H_2O_2. In contrast, the H_2O_2 content of wild-type seedlings grown in medium containing 1.5 mM H_2O_2 was less than 300 % higher than the H_2O_2 content of seedlings grown in H_2O_2-free medium (Fig. 6). This indicates that *hit2* and *athsfa4a* mutants have lower H_2O_2-scavenging ability than the wild type. In contrast, *A4aΔNES* transgenic plants showed better H_2O_2-scavenging ability than the wild type. The H_2O_2-scavenging abilities of *athsfa5* and *A5ΔNES* were similar to that of the wild type (Fig. 6). These results show that increased AtHsfA4a activity in the nucleus can indeed increase the H_2O_2-scavenging capacity of the cell. Reduction of AtHsfA4a activity in the nucleus thus decreases the H_2O_2-scavenging capacity of the cell. Nevertheless, it was the *A4aΔNES* with

enhanced H_2O_2-scavenging capacity that displayed a *hit2*-like HI sensitive phenotype, whereas the *athsfa4a* mutant with reduced-H_2O_2 scavenging ability retained tolerance to HI to an extent similar as the wild type (Figs. 5 and 6). In addition, there was little difference in the H_2O_2-scavenging ability among *athsfa5*, *A5ΔNES*, and wild-type plants. Nevertheless, *athsfa5* is sensitive to HI. Collectively, these results indicate that the HI sensitivity induced by the unbalanced coordination of AtHsfA4a/AtHsfA5 is not directly related to a decrease in the H_2O_2-scavenging capacity and suggest that other factors may compensate for the ultimate influence of AtHsfA5 on the H_2O_2 scavenging ability.

One of the original goals of this study was to determine whether HI sensitivity in *hit2* mutants is related to the AtHsfA4a/AtHsfA5 regulatory system. AtHsfA4a and AtHsfA5 are specific substrates of HIT2, and long-term over-accumulation of AtHsfA4a, but not AtHsfA5, in the nucleus leads to HI sensitivity. Therefore, it was reasonable to propose that *hit2/athsfa4a* (*hit2/a4a*) double-mutant plants would have better tolerance to HI than do *hit2*. To test this hypothesis, a *hit2/a4a* double mutant was generated by crossing *hit2* with *athsfa4a* plants, and the results showed that *hit2/a4a* was indeed more tolerant to HI than *hit2*. However, the degree of tolerance lower than that observed in the wild type, or in *athsfa4a* when plant leaf colour was visually assessed and overall chlorophyll content was compared (Fig. 7). This indicates that the retention of AtHsfA4a in the nucleus is not the only cause of HI sensitivity in *hit2*. It is probable that other currently unknown HIT2-specific substrates are retained in the nucleus, which further increase the HI sensitivity of *hit2* mutants.

The *Arabidopsis* genome contains two *XPO1* genes,

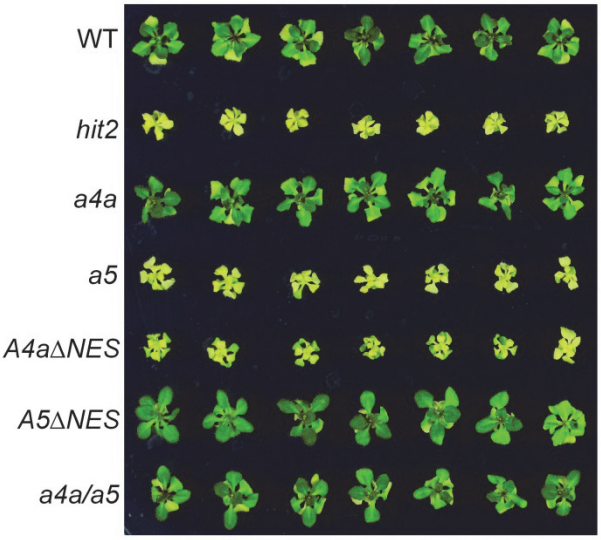

Fig. 5. The seedlings (4-d-old) grown under irradiance of 100 μmol m^{-2} s^{-1} were transferred to high irradiance of 500 μmol m^{-2} s^{1}, grown for 12 d, and their shoots were removed and arranged for photographs. The *hit2*, *athsfa5* (*a5*), and *A4aΔNES* show higher HI sensitivity than other plants.

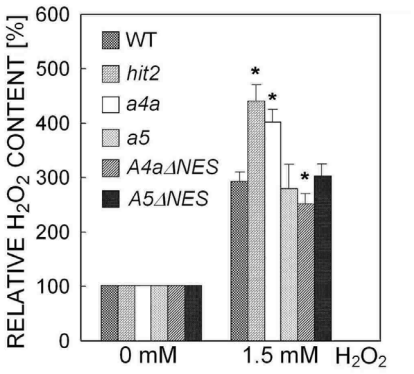

Fig. 6. *Arabidopsis* seeds were directly sown on agar with 0 or 1.5 mM H_2O_2, and allowed to grow under irradiance of 100 μmol m^{-2} s^{-1} for 8 d. Relative H_2O_2 content was calculated by setting the H_2O_2 content of each plant line grown in H_2O_2-free medium as 100 %. Means ± SDs, n = 3, * indicates significant differences at $P < 0.05$ compared with wild-type (WT). (*a4a* - *athsfa4a*, *a5* - *athsfa5*).

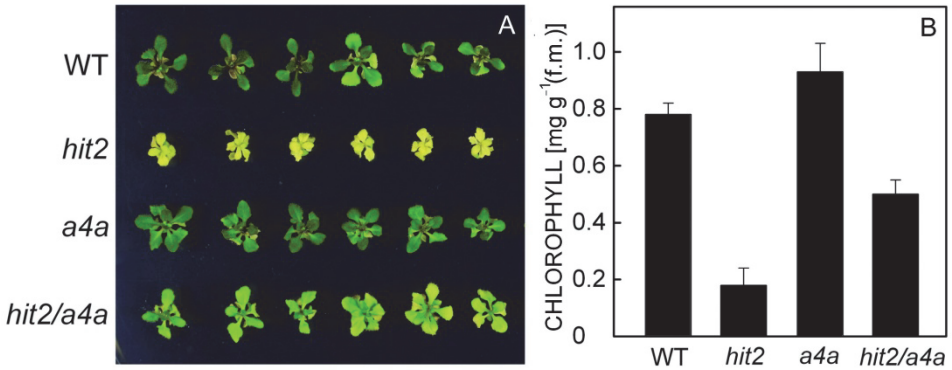

Fig. 7. Null mutation of *athsfa4a* can alleviate the sensitivity of *hit2* to high irradiance (HI). *A* - 4-d-old seedlings grown under irradiance of 100 μmol m^{-2} s^{-1} were transferred to HI of 500 μmol m^{-2} s^{-1} and allowed to grow for an additional 12 d before their shoots were removed and arranged for photographs: *hit2/a4a* double mutant was more tolerant to HI stress than the *hit2* single mutant. *B* - Chlorophyll content after HI treatment. Means ± SDs, n = 3. (WT - wild type, *a4a* - *athsfa4a*, *hit2/a4a* - *hit2/athsfa4a* double mutant).

HIT2 and *XPO1B*. Although their gene products share a high amino acid sequence similarities, *hit2* but not *xpo1b* mutants exhibit many abiotic stress-sensitive phenotypes. This implies that the export of some NES-containing proteins from the nucleus requires HIT2 but not XPO1B. Nevertheless, the identity of these proteins and the proteins confined to the nucleus of *hit2* mutants responsible for the observed *hit2* phenotypes remain unknown. The results of the present study reveal that AtHsfA4a and AtHsfA5 are HIT2-specific substrates, and the confinement of AtHsfA4a but not AtHsfA5 to the nucleus results in HI sensitivity in *hit2* mutants, thus providing answers to the above questions for the first time.

HsfA4a plays important roles in resistance to environmental stresses in plants. For example, overexpression of wheat *TaHsfA4a* in rice can increase tolerance to cadmium, and knockdown of *OsHsfA4a* decreases this tolerance. This is partly explained by the fact that HsfA4a in wheat and rice can positively regulate the expression of the gene encoding cadmium-binding metallothionein protein. However, rice plants over-expressing *TaHsfA4a* are shorter than wild-type plants (Shim *et al.* 2009). Similar results have been found in *Arabidopsis* by Pérez-Salamó *et al.* (2014). This indicates that, despite HsfA4a being able to help plants tolerate many types of environmental stress, it actually has negative effects on plant growth and development under normal conditions. This also implies that the function of HsfA4a needs to be tightly regulated to avoid potential adverse effects to wild-type plants. Indeed, the transcription of *AtHsfA4a* is elevated as long as plants are under salt or osmotic stress; under UV-B or cold stress, it is induced during the first 3 - 6 h, but thereafter it decreases and returns to normal levels 12 - 24 h later (Pérez-Salamó *et al.* 2014). These findings indicate that the expression of AtHsfA4a is regulated in different ways under different stress conditions. A novel finding of this study is that although AtHsfA4a enters the nucleus upon stimulation with HI, trapping of AtHsfA4a in the nucleus (*hit2* and *A4aΔNES*) causes sensitivity to HI, which reveals that the function of AtHsfA4a can be regulated by nuclear-cytoplasmic trafficking.

Photosystem (PS) II is the primary target of photodamage. This occurs when high-energy UV radiation impairs the catalytic Mn_4Ca cluster of water oxidation, and long-wave visible radiation reduces electron acceptors within the reaction center of PS II (Takahashi and Murata 2008). Together, these effects induce the formation of the short-lived, but highly reactive singlet oxygen ($^1O_2$), which attacks the adjacent PS II reaction center protein D1 to which the Mn_4Ca cluster binds (Vass 2012). In order to repair this damage, the affected D1 protein is degraded and replaced by *de novo* synthesized protein, which is then ligation to a Mn_4Ca cluster and other components to restore a functional PS II reaction center (Aro *et al.* 1993, 2005; Nishiyama and Murata 2014). It has been shown that the expression of the *PsbA* gene, which encodes the D1 protein, and the rate of the D1 protein turnover are strictly controlled by irradiance in order to maintain the D1 protein at a steady-state level. Tracking the phenotype during the course of a HI treatment reveals that, initially, *hit2* and wild-type leaves both become yellowish to a similar degree. Later, the leaves of wild-type plants revert to a green colour, whereas the leaves of *hit2* mutants remain yellow (Fig. 5 Suppl.). This suggests that the HI-sensitive phenotype of *hit2* arised from a weakened ability to undergo repair rather than the prevention of photodamage. This may explain the finding that, although AtHsfA4a induced the expression of the gene encoding the H_2O_2-degrading enzyme APX1 and nucleus-trapped AtHsfA4a (*A4aΔNES*) plants do show an increased ability to degrade H_2O_2, such AtHsfA4a-mediated ability does not confer HI tolerance to plants. Instead, considering that AtHsfA4a is potentially unfavorable for plant growth and development under normal growth conditions, the long-term accumulation of AtHsfA4a may have adverse effects on the repair of the damaged PS II, and alleviation of such adverse effects requires AtHsfA5.

Previous studies have indicated that during transient expression assays in tobacco protoplasts, AtHsfA4a localizes in the nucleus and cytoplasm. Similarly, *Helianthus annuus* HaHsfA4a can be observed simultaneously in the cytoplasm and nucleus when expressed in tobacco protoplasts (Tejedor-Cano *et al.* 2014). In contrast to the above-mentioned studies in which both *AtHsfA4a* and *HaHSFA4A* expression is driven by the CaMV35S promoter to maintain high content of AtHsfA4a or HaHSFA4a in tobacco cells, in the present study, *AtHsfA4a* was driven by its native promoter and expressed in *Arabidopsis* cells in which AtHsfA4a was localized only to the cytoplasm under normal conditions. These results indicate that the nucleo-cytoplasmic homeostasis of AtHsfA4a was affected by its inherent content and cellular environment. In particular, *Arabidopsis* contained two XPO1 homologs, which could potentially increase the diversity of the nucleo-cytoplasmic homeostasis of any NES-containing substrates. Additionally, because the *hit2/a4a* double mutant could only partially rescue the *hit2* HI sensitive phenotype (Fig. 7), this indicated that other unknown HIT2-specific substrates accumulated in *hit2* nuclei, which could also result in the HI sensitive phenotype. The identification of these unknown HIT2-specific substrates can improve our understanding of biomolecules dynamically distributed in the nucleus and cytoplasm that affect the tolerance of plants to HI stress. In this context, *hit2* is undoubtedly a valuable material for investigating how plants adjust the nucleo-cytoplasmic distribution of their components to respond to various environmental stresses.

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