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# The newly identified *heat-stress sensitive albino 1* gene affects chloroplast development in rice

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ABSTRACT

High temperature, a major abiotic stress, significantly affects the yield and quality of crops in many parts of the world. Components of the photosynthetic apparatus are highly susceptible to thermal damage. Although the responses to acute heat stress have been studied intensively, the mechanisms that regulate chloroplast development under heat stress remain obscure, especially in crop plants. Here, we cloned and characterized the gene responsible for the *heat-sensitive albino1* (*hsa1*) mutation in rice (*Oryza sativa*). The *hsa1* mutant harbors a recessive mutation in a gene encoding fructokinase-like protein2 (FLN2); the mutation causes a premature stop codon and results in a severe albino phenotype, with defects in early chloroplast development. The color of *hsa1* mutant plants gradually changed from albino to green at later stages of development at various temperatures and chloroplast biogenesis was strongly delayed at high temperature (32 °C). *HSA1* expression was strongly reduced in *hsa1* plants compared to wild type (WT). HSA1 localizes to the chloroplast and regulates chloroplast development. An *HSA1* deletion mutant induced by CRISPR/Cas9 was heat sensitive but had a faster greening phenotype than the original *hsa1* allele at all temperatures. RNA and protein levels of plastid-encoded RNA polymerase-dependent plastid genes were markedly reduced in *hsa1* plants compared to WT. These results demonstrated that HSA1 plays important roles in chloroplast development at early stages, and functions in protecting chloroplasts under heat stress at later stages in rice.

## 1. Introduction

As a major abiotic stress, high temperature affects the yield and quality of crops in many parts of the world. Photosystem II (PSII) is the most thermolabile component within the chloroplast thylakoid membrane and heat stress mainly causes dysfunction of the oxygen-evolving complex, disruption of electron transport, and cleavage of the reaction center-binding protein D1 of PSII [1–5]. Moreover, elevated temperatures affect carbon assimilation metabolism in the chloroplast stroma, as Rubisco activase is inactivated at elevated temperatures, although Rubisco of higher plants is heat stable [6,7]. In addition to the effects on photosynthesis and carbon assimilation, heat stress usually leads to a loss of thylakoid membrane integrity, especially destacking of thylakoid membranes, suggesting that the maintenance of thylakoid structure in response to heat stress is a key sign of heat tolerance in plants [8–10].Given that components of the photosynthetic apparatus are highly susceptible to thermal damage, the chloroplasts may function as sensors to detect increases in temperature [11].

Chloroplast function is required throughout the life cycle of the plant and chloroplast biogenesis and development must be coordinated with seedling growth to ensure optimal rates of photosynthesis, appropriate plant growth, and high yield for crops. Chloroplast gene transcription depends on two types of RNA polymerase [12,13]. The plastid-encoded RNA polymerase (PEP) has sequence similarity to bacterial RNA polymerase and transcribes genes involved in photosynthesis. PEP is composed of four core subunits α, β, β′ and β″, which are encoded by the *rpoA*, *rpoB*, *rpoC1* and *rpoC2* genes in the plastid genome; additional sigma factors and PEP-associated proteins are encoded in the nuclear genome. In higher plants, nuclear-encoded RNA polymerase (NEP) has sequence similarity to the single-peptide RNA polymerase of the T3/T7 bacterio phage and transcribes housekeeping genes in non-photosynthetic plastids [13–15]. Only one NEP subunit has been identified [16].

PEP and NEP recognize different promoters and PEP requires sigma factors for promoter recognition and initiation of transcription [17].

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Many plastid genes have both PEP and NEP promoters. Based on their promoter structures, chloroplast genes can be divided into three classes. Most photosynthesis-related genes of class I are transcribed by PEP, non-photosynthetic housekeeping genes of class II are generally transcribed by both PEP and NEP, and class III genes are exclusively transcribed by NEP [14,18]. NEP provides the major transcriptional activity in the proplastid; later in chloroplast development, NEP is replaced by PEP, which selectively transcribes photosynthesis-related genes in the mature chloroplast [14,19]. The transcriptional activity of PEP and NEP affect non-green plastids and chloroplast development at all stages of plant growth.

Many studies have aimed to identify nuclear-encoded proteins involved in plastid transcription. One study used different biochemical purification schemes to enrich distinct RNA polymerase complexes from chloroplasts; this distinguished three major types: the soluble RNA polymerase, insoluble TAC (Transcriptionally Active Chromosome), and nucleoids [18] The TAC contains the core PEP subunits, DNA, RNA, and a large number of other proteins [16,18]. Work in *Arabidopsis thaliana* and mustard (*Sinapis alba*) identified 35 components of the TAC complex, and 127 proteins have been reported as strong candidates for being components of nucleoids in maize [20,21]. Accumulating evidence shows that in *Arabidopsis*, the presence of defective TACs in developing chloroplasts results in an albino phenotype, lethality, and reduced or delayed greening, along with inhibition of PEP-dependent plastid gene expression and chloroplast development [22–29].

Recent work in rice identified several genes involved in PEP-related chloroplast biogenesis. *OspTAC2* encodes a chloroplast protein consisting of pentratricopeptide repeat domains and a C-terminal small MutS-related domain. The *osptac2* mutants exhibited albino and seedling-lethal phenotypes [30]. *WSL3* encodes a SAP domain protein that interacts with subunits of the TAC complex and is essential for chloroplast development [31]. *TCD5/TSV*, whose Arabidopsis TAC homolog has not been previously identified, encodes a putative oxidoreductase. The *tcd5* mutant displayed defective chloroplasts and is extremely sensitive to cold stress [32,33].

Although PEP-related chloroplast biogenesis has been well studied, the mechanisms of the response to heat stress in chloroplast biogenesis remain elusive. In the present study, we cloned and characterized a novel gene responsible for the *heat sensitive albino1* (*hsa1*) mutant phenotype in rice (*Oryza sativa*). Map-based cloning, sequencing, and complementation analysis indicated that the *hsa1* phenotype is caused by the mutation of a gene encoding fructokinase-like protein 2. In comparison with WT, in the *hsa1* mutants, the transcript and protein levels of PEP-dependent genes were lower and this inhibition was stronger at high temperature. We demonstrated that HSA1 is necessary for chloroplast development in seedlings and loss-of-function of HSA1 strongly delays chloroplast biogenesis in rice, especially under heat stress.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

The *hsa1* mutant was isolated from the *japonica* rice cultivar Nipponbare (NPB), which was subjected to ethyl methane sulfonate (EMS) treatment [34]. The $F_1$ and $F_2$ generations of a cross between the *hsa1* mutant and NPB was used for genetic and phenotypic analysis. The *hsa1* mutant individuals from the $F_2$ generation of across between the *hsa1* mutant and the *indica* cultivar 'PA64S' were used for fine mapping of the *hsa1* mutation. All plants were cultivated in an experimental field at the China National Rice Research Institute located in Fuyang, Zhejiang Province (119°6′E, 30°0′N).

The temperatures of the plant incubators (SANYO, Versatile Environmental Test Chamber, MLR-351) were set at 24 °C, 28 °C, and 32 °C under 14-h light: 10-h dark, with light conditions of 300 μmol photons$m^{-2}$·$s^{-1}$. For the lower temperature treatment (24 °C), 60 days after germination (DAG) *hsa1* and WT plants were cultured in a growth chamber at constant 24 °C to the heading stage. The youngest fully expanded leaves at the third-leaf stage were used for all analyses unless otherwise noted.

### 2.2. Investigation of agronomic traits and measurement of chlorophyll concentration

The leaf samples were cut into 1-cm segments, soaked in 10 ml of 80% acetone, and cultured in the dark for 48 h. The optical density (OD) of the sample solutions was measured to determine the concentrations of chlorophyll *a* and *b* and carotenoid at wavelengths of 662 nm (maximum absorption peak of chlorophyll *a*), 646 nm (maximum absorption peak of chlorophyll *b*), and 470 nm (maximum absorption peak of carotenoid), respectively, using an ultraviolet spectrophotometer (DU800, Beckman). Three biological replicates per sample were prepared and analyzed, and 80% acetone was used as a blank. The concentrations of chlorophyll *a* and *b* and carotenoid were calculated according to the methods of Arnon (1949) and Wellburn (1994) [35,36]. Important agronomic traits such as plant height, number of grains per panicle, and 1000-grain weight of 30 replicates of mutant and WT plants at the mature stage were measured.

### 2.3. Transmission electron microscopy

WT and *hsa1* leaf samples from third-leaf stage seedlings grown in 14-h light:10-h dark (light 300 μmol photons$m^{-2}$·$s^{-1}$) at 24 °C and 32 °C were collected for transmission electron microscopy. The leaf samples were cut into small pieces, fixed in 2.5% glutaraldehyde with phosphate buffer (pH7.2), and centrifuged until they were completely immersed in the buffer. The leaf materials were then fixed with osmium tetroxide and examined under a Hitachi H-7650 electron microscope [37].

### 2.4. Gene cloning and vector construction

Based on the initial mapping of *hsa1* to chromosome 3 using 2885 $F_2$ individuals, additional sequence-tagged site (STS) markers and 1450 $F_2$ individuals were used to fine-map *hsa1*. The PCR-based molecular markers and sequencing used in this study are shown in Supplemental Table S1. The PCR conditions were: denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30s, annealing for 30 s (annealing temperature determined by primer pair sequence), 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products were analyzed on 5–6% agarose gels. The specific fragments within the 48-kb target region from the *hsa1* mutant were amplified and then sequenced by Hangzhou Tsingke Biological Engineering Technology and Service Co. Ltd (Tsingke, Hangzhou, China).

For mutant complementation, the following amplification primers were designed based on the *HSA1* genomic sequence, comprising 2029-bp upstream sequence, the entire coding region of *HSA1*, and 1208-bp downstream sequence: 40550-1F (aaggtaccTGGCCCATATCTTAGCAG CTT) and 40550-2R (cctctagaGTATGGCCAGTTGACCACGA). Binary vector constructs containing 7353-bp of the WT *HSA1* genomic sequence (pCOM) and the control vector (pCAMBIA1300, pCK) were introduced into *hsa1* plants via *Agrobacterium tumefaciens*-mediated transformation. Hygromycin-resistant transgenic lines were selected. The targeted deletion vector was constructed via the CRISPR/Cas9 system [38], using the target sequence TCAAGTGATGATGAGAGTGA for *HSA1*, and introduced into NPB via *A. tumefaciens*-mediated transformation.

### 2.5. Phylogenetic analysis

The sequences used for phylogenetic analysis were found using NCBI BLAST searches. Using FLN protein sequences as queries,