Running title: Splicing regulator STA1 in heat stress adaptation

Corresponding Author:
Sang-Dong Yoo
Department of Life Sciences
Division of Life Sciences
KOREA University
145 Anamro, Seongbuk-gu
Seoul, Korea, 02841
Tel: +82-2-3290-3401
Fax: +82-2-927-9028
E-mail: sangdong@korea.ac.kr

Article type: Research Article
Research area: Signaling and Response
One-Sentence Summary:

Heat inducible STA1 activity was found to be involved in the pre-mRNA splicing of heat stress response genes and contributes to the establishment of heat stress tolerance in Arabidopsis.
Abstract

High temperature stress often leads to differential RNA splicing, thus accumulating different types and/or amounts of mature mRNAs in eukaryotic cells. However, regulatory mechanisms underlying plant pre-mRNA splicing in the environmental stress conditions remain elusive. Herein, we describe that a U5-snRNP-interacting protein homolog STABILIZED1 (STA1) has pre-mRNA splicing activity for heat-inducible transcripts including HEAT STRESS TRANSCRIPTION FACTORS and various HEAT SHOCK PROTEINS for the establishment of heat stress tolerance in Arabidopsis thaliana. Our cell-based splicing reporter assay demonstrated STA1 acts on pre-mRNA splicing for specific subsets of stress-related genes. Cellular reconstitution of heat-inducible transcription cascades supported the view that STA1-dependent pre-mRNA splicing plays a role in DREB2A-dependent HSFA3 expression for heat responsive gene expression. Further genetic analysis with a loss-of-function mutant sta1-1, STA1-expressing transgenic plants in Col background and STA1-expressing transgenic plants in sta1-1 background verified that STA1 is essential in expression of necessary genes including HSFA3 for two-step heat stress tolerance in plants. However, constitutive overexpression of cDNA version of HSFA3 in sta1-1 background is unable to execute plant heat stress tolerance in sta1-1. Consistently our global target analysis of STA1 showed that its splicing activity modulates a rather broad range of gene expression in response to heat treatment. The findings of this study reveal that heat-inducible STA1 activity for pre-mRNA splicing serves as a molecular regulatory mechanism underlying the plant stress tolerance to high temperature stress.
INTRODUCTION

The splicing of precursor messenger RNA (pre-mRNA) is a necessary step for intron-containing gene expression in eukaryotic cells in order to produce mature transcripts for protein translation (Wahl et al., 2009). This process is highly ordered and tightly controlled by multi-subunit spliceosome activity to mix and match introns and exons of pre-mRNAs. The high-molecular-weight spliceosome complex comprises small nuclear ribonucleoprotein particles (snRNPs) called U1, U2, U4/U6, and U5 snRNPs. For splicing of pre-mRNA introns, U1 snRNP recognizes the 5′-splicing site (SS), and U2 snRNP binds to the adenosine at the branch point of introns with the assistance of U2 auxiliary factors (U2AFs). U4/U6 and U5 trimeric snRNPs associate with each other and undergo a step-wise 3′-SS cleavage process. Eventually, U5 snRNP dissociates from the complex along with a lariat form of the intron. In this process, U5 snRNP accurately and dynamically swaps interacting partners with other snRNP subunits (Wahl et al., 2009).

The functions of ribonucleoprotein particles are evolutionarily conserved in most eukaryotes (Knowler and Wilks, 1980). Recent advances in functional genomic analysis platforms enabled the inference that alternative splicing of pre-mRNAs is a central regulatory module in extending gene reservoir with a limited number of structural genes encoded in genomes for protein information (Reddy et al., 2013). Consequently, a fewer genomic resources address physiological and developmental complexities through transcript variants produced in cellular and environmental contexts (Reddy et al., 2013; Staiger and Brown, 2013).

The sessile lifestyle of plants facilitates the evolution of diverse adaptation processes at the multidimensional layers from cells to organisms under various environmental stress conditions (Godfray and Garnett, 2014; McClung, 2014). For example, in the cell autonomous manner, low temperature activates the ICE-CBF-COR pathway and establishes cold stress tolerance in Arabidopsis (Zhu et al., 2007). Such cellular adaptation processes also require post-transcriptional regulatory steps in gene expression. For example, COR15A expression was originally reported to be transcriptionally upregulated and further modulated post-transcriptionally upon cold treatment (Artus et al., 1996; Lee et al., 2006). In a recessive loss-of-function mutant sta1-1, pre-mRNA of COR15A accumulated in response to the cold stress and thus STA1 is implicated in controlling pre-mRNA splicing of COR15A (Lee et al., 2006).
STA1 is a stress-inducible U5 snRNP-interacting partner, of which cellular functions involve in the establishment of cold stress and ABA tolerance (Lee et al., 2006). Recently STA1 is also documented for heat stress responses without mechanistic details (Yu et al., 2016). High temperature (heat) is a detrimental environmental stress condition affecting plant biomass yields and its occurrence becomes more prevalent in the cropping fields under today’s climate change (Godfray and Garnett, 2014; McClung, 2014). Evolutionarily conserved heat stress transcription factors (HSFs) and heat shock proteins (HSPs) play key roles in establishing plant basal and/or acquired heat stress tolerance (Kotak et al., 2007; Scharf et al., 2012). For example, the gene regulatory modules of HSF and HSP expression have been well characterized in Arabidopsis. The key stress-related transcription factors DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN (DREB) 2A and DREB2C, induce expression of heat-inducible transcription factor HEAT SHOCK TRANSCRIPTION FACTOR A3 (HSFA3), and its transcription activity induce downstream HSPs expression (Sakuma et al., 2006; Schramm et al., 2008; Chen et al., 2010). A similar transcription cascade is also found in the maize, and thus this transcriptional regulatory module is most likely conserved through the evolution of diverse plant species (Qin et al., 2007). Nevertheless, post-transcriptional regulatory steps of heat-inducible gene expression remain largely unknown in plants.

In this study, we utilized a novel splicing reporter in Arabidopsis leaf mesophyll protoplasts to examine STA1 activity on pre-mRNA splicing of stress-induced genes. STA1 was involved in the pre-mRNA splicing of heat-inducible HSFA3 as well as cold-inducible COR15A and IDD14. Clearly, STA1 splicing activity was indispensable for mature mRNA expression of HSFA3 and its downstream HSPs in a reconstituted DREB2A-dependent gene regulatory module. Further genetic analysis verified that STA1 involved in pre-mRNA splicing of essential genes, including HSFA3 and its target gene HSA32, necessary for in the establishment of plant heat stress tolerance. However, a single heat stress transcription factor HSFA3 was unable to recapitulate the stress tolerance in the absence of STA1 activity. Our global target search of STA1 activity using differential display of RNA-followed by sequencing (DDR-seq) revealed that STA1 played central roles in a broader range of heat inducible gene expression. Our findings unraveled that heat-inducible STA1 activity secures appropriate gene expression under heat stress conditions, of which gene products contribute to plant tolerance.
RESULTS AND DISCUSSION

STA1 Function in Pre-mRNA Splicing

To evaluate the biochemical activity of STA1 in pre-mRNA splicing at a cellular system, a splicing reporter construct was generated using the translational fusion of the GUS reporter gene to the 3’-end of a genomic version of the structure gene under the regulation of a constitutive 35S promoter (Fig. 1A). This GUS reporter activity increases only when the intron of pre-mRNA is spliced out correctly. Otherwise, a premature termination codon (PTC) of the intron retained in the pre-mRNA would interrupt the complete translation of the GUS reporter protein. Therefore, in principle, an increase or decrease in the cellular GUS activity may primarily reflect the amount of mature mRNAs serving as protein translation templates.

The design principle of the splicing reporter was validated by the splicing activity of STA1 with a gCOR15A-GUS reporter construct using the well-established Arabidopsis leaf mesophyll protoplasts. The GUS-based splicing reporter and a UBQ10 promoter-driven renilla luciferase (rLUC) control reporter constructs were co-transfected to either wild type (Col) or sta1-1 protoplasts with or without a STA1-HA effector construct and then incubated for 6 h under light (Yoo et al., 2007). The splicing reporter activity of intron-containing gCOR15A-GUS construct was clearly induced with STA1 expression when compared to the basal activity obtained without the effector expression (Fig. 1, B and C). In the assay the control reporter activity of intron-free rLUC construct was not altered in the presence or absence of STA1 expression, indicating that STA1 did not modulate transcription activity in this system. To further verify this notion, a control experiment was independently carried out with 35S-driven intron-free fLUC reporter construct in sta1-1 LMPs. Again, STA1 did not affect the LUC reporter activity at all (Supplemental Fig. S1). Then, to re-examine whether the difference in the GUS reporter activity originates from the splicing efficiency depending on STA1 activity, both the intron-retained and intron-free forms of COR15A-GUS were detected using semi-quantitative reverse transcriptase-dependent PCR (RT-PCR) using the RNA extracted from the transfected protoplasts of Col and sta1-1 (Supplemental Fig. S2). The mature mRNA of gCOR15A-GUS was substantially enriched in Col, whereas its pre-mRNA was rather enriched in sta1-1, indicating that a clear shift of pre-mRNA to mature mRNA was made for gCOR15A-GUS by STA1. Taken together, our cell-based assay demonstrated pre-mRNA splicing activity of STA1.
Then, to investigate whether STA1 activity has any preference in pre-mRNA splicing targets, two more splicing reporters were constructed with genomic versions of *INDETERMINATE DOMAIN 14* (gIDD14) and *MITOGEN-ACTIVATED PROTEIN KINASE 10* (gMPK10). *IDD14* is another cold-inducible gene that produces a spliced variant and controls its own gene product activity in response to cold (Seo et al., 2011). The stress-related *MPK10* encodes a gene for a signaling potent kinase, the function of which is largely unknown (Mao et al., 2011). The reporter activity of gIDD14-GUS, but not gMPK10-GUS, was increased in *sta1-1* protoplasts by STA1 expression (Fig. 1, D and E). gIDD14 contains a single intron as gCOR15a but gMPK10 has multiple introns. However, the number of introns is seemingly irrelevant to the target preference of STA1 for pre-mRNA splicing based on the global analysis of STA1-dependent heat inducible gene expression in this study (see below).

To characterize STA1 functions in abiotic stress responsive gene regulation other than cold stress and ABA responses reported in the previous study (Lee et al., 2006), STA1 expression patterns were searched through Arabidopsis eFP database, which integrates high-throughput transcriptome analyses (Winter et al., 2007). High-temperature stress induces STA1 expression in addition to cold stress (Supplemental Fig. S3). To verify the gene expression of STA1 in response to heat, semi-quantitative RT-PCR was performed after mild heat treatment (37 °C). STA1 expression increased within 1 h and maintained up to 3 h, and then decreased in 12 h after heat treatment (Fig. 2A). Since STA1 expression is under the regulation of heat stress, its gene product activity most likely plays a role in the stress-responsive gene expression.

To investigate STA1 functions in heat stress related gene expression, the gene expression of several heat stress factors (HSF) including *HSFA3*, *HSFB1*, and *HSFB2a* were examined upon heat treatment. These HSFs are transcription factors that have important functions in plant heat stress adaptation (Schramm et al., 2008; Scharf et al., 2012). To precisely characterize the regulatory functions of STA1 on splicing of heat inducible transcripts, a heat inducible expression of the HSFs was specifically measured for the total mRNA (primer combination of f1 and r1), pre-mRNA (primer combination of f1 and r2) and mature mRNA (primer combination of f1 and r3) in Col and sta1-1 (Fig. 2B). Total mRNA expression of *HSFA3* was induced and maintained in both Col and sta1-1 seedlings at 37 °C, indicating that total transcript levels of *HSFA3* increased with and without STA1 activity by heat stress (Fig. 2, C and D). Notably, pre-mRNA expression was marginal in Col; however, it was rather significantly induced in sta1-1
indicating that pre-mRNA transcripts of \textit{HSFA3} were not spliced appropriately in \textit{sta1-1} compared to those in \textit{Col}. Mature \textit{HSFA3} mRNA expression was then induced in \textit{Col}, but not in \textit{sta1-1}. The expression patterns of \textit{HSFB1} and \textit{HSFB2a} were similar to those of \textit{HSFA3} (Supplemental Fig. S4).

To examine STA1 activity for pre-mRNA splicing of heat stress responsive genes a new splicing reporter was constructed with the genomic version of \textit{HSFA3} (g\textit{HSFA3}). This time the reporter was generated with Nano-luciferase (nLUC) that is smaller in size and thus more sensitive in cellular responses compared to firefly-luciferase (Hall et al., 2012). To substantiate this splicing assay, evolutionarily conserved splicing sites of the intron in g\textit{HSFA3} (GT-intron-AG) were mutated to be un-spliced at splice donor (Do) or acceptor (Ac) site (Fig. 2E) and subjected to the cell-based assay with and without STA1 in \textit{sta1-1} protoplasts. The reporter activity from wild type g\textit{HSFA3-nLUC} increased with STA1 expression, but those from two mutated forms of g\textit{HSFA3-nLUC} did not (Fig. 2F), confirming that the induction of splicing reporter activity resulted from authentic pre-mRNA splicing of the reporter genes by STA1.

In the loss-of-function allele \textit{sta1-1}, \textit{STA1} transcript is deleted with six nucleotides in-frame and it produces \textit{sta1-1} protein omitting two amino acids (Lee et al., 2006). To investigate whether \textit{sta1-1} protein has any splicing activity, the reporter assay using g\textit{HSFA3-nLUC} was carried out in combination of \textit{STA1} and \textit{sta1-1} expression. Protein blot analysis first showed that \textit{STA1} and \textit{sta1-1} accumulated to a similar level in \textit{Col} protoplasts (Fig. 2G). The reporter activity was induced by \textit{STA1}, but not by \textit{sta1-1}, and its activity by both \textit{STA1} and \textit{sta1-1} was similar to the activity obtained by \textit{STA1} alone (Fig. 2G), indicating \textit{sta1-1} did not carry any noticeable splicing activity of g\textit{HSFA3}. However, \textit{sta1-1} alone reduced the reporter activity to some extent, and this might indicate that \textit{sta1-1} has a little dominant negative (DN) function, although such activity is competitively weaker than \textit{STA1}. This null splicing activity of \textit{sta1-1} is coherent with the fact that \textit{sta1-1} is a recessive loss-of-function mutant (Lee et al., 2006).

Reconstitution of Heat Inducible Transcription Cascades by DREB2A and STA1

To substantiate this finding, we reconstituted transcription regulatory circuits of heat-response gene expression in Arabidopsis protoplasts. Namely, DREB2A responsible for \textit{HSFA3} transcription under heat stress conditions was transiently expressed in \textit{Col} or \textit{sta1-1} protoplasts with and without \textit{STA1} (Fig. 3A; Sakuma et al., 2006; Schramm et al., 2008). Prior to the
measurement of HSFA3 pre-mRNA, DREB2A and STA1 expressions were verified by RT-qPCR with the RNA extracted from the transfected protoplasts (Supplemental Fig. S5). With forward and reverse primers specifically designed to recognize the pre-mRNA transcripts of HSFA3 (Fig. 3B), the endogenous expression of pre-mRNA of HSFA3 exhibited a basal level and it was not induced by STA1 (Figs. 3, C and D). In contrast the pre-mRNA expression was induced by DREB2A and its accumulation was rather pronounced in sta1-1 protoplasts when compared to that in Col protoplasts. Furthermore the pre-mRNA of HSFA3 decreased in the presence of STA1, implicating its role in the splicing of HSFA3 pre-mRNA. This was consistent with our observation that pre-mRNA splicing of HSFA3 was dependent on the STA1 activity (Figs. 2, C and D).

To examine whether DREB2A-driven HSFA3 becomes a functional transcription activator in the presence of STA1 activity, mature mRNA expression of HSP23.6, HEAT STRESS-ASSOCIATED PROTEIN 32 (HSA32), and HSP70T-2 was measured by RT-qPCR as potential target genes of HSFA3. Either STA1 or DREB2A alone could not induce mature mRNA of HSP23.6, HSA32 and HSP70T-2 in sta1-1 protoplasts that are disconnected in the process from pre-mRNA to mature-mRNA (Fig. 3E). The co-expression of STA1 and DREB2A did induce mature mRNA accumulation of the HSPs. Evidently, pre-mRNA of HSFA3 induced by DREB2A was processed by STA1, and then its mature mRNA products could induce the HSP gene expression appropriately. These results suggested that STA1 activity served an important regulatory step in mature mRNA expression of HSFA3, whose transcription was driven by DREB2A.

Pre-mRNA Splicing Function of STA1 for Plant Thermotolerance

The STA1 splicing activity was then examined for plant heat stress tolerance with wild type (Col), a loss-of-function sta1-1, STA1-expressing transgenic plants in Col background (STA1/Col) and STA1-expressing transgenic plants in sta1-1 background (STA1/sta1-1). Before heat inducible gene expression analysis endogenous and transgene expression of STA1 was confirmed by using semi-quantitative RT-PCR (Supplemental Fig. S6). For two-step acquired heat stress tolerance assay, Arabidopsis seedlings were grown for 5 d at room temperature and exposed to heat at 38 °C for 2 h and then at 45 °C for another 2 h. Seedling viability was examined at 7 d after heat treatment. Col seedlings acclimated and survived from the step-wise heat treatment, whereas
sta1-1 seedlings grew slowly, had shorter primary roots, displayed completely bleached shoots, and eventually died (Fig. 4A, upper panel). Both STA1/Col and STA1/sta1-1 seedlings showed their tolerance at a level similar to Col in response to the step-wise heat treatment, confirming that a genetic defect of STA1 led to the lack of heat stress tolerance in sta1-1. These seedling phenotypes were examined repeatedly with multiple independent STA1-expressing transgenic Col and transgenic sta1-1 lines (Supplemental Fig. S7). In contrast, after one-step basal heat tolerance assay conducted with 5-d-old seedlings exposed to 45 °C for 2 h, none of these genotypes including Col executed heat stress tolerance (Fig. 4A, lower panel). Seedling survival and lethal phenotypes of Col displayed respectively for one-step basal and two-step acquired heat stress treatments were consistent with a previous report (Silva-Correia et al., 2014). In summary heat inducible STA1 has a regulatory role in the establishment of the two-step acquired heat stress tolerance, but not that of the one-step basal heat stress, perhaps through its splicing function of heat responsive gene expression.

To further understand STA1 function in heat responsive gene expression, total RNA was extracted from whole seedlings before and at 1 d after the step-wise heat treatment, and gene expression was measured by RT-qPCR. To specifically measure the expression of wild-type STA1 and mutant sta1 transcripts we designed primers based on the 6-nucleotide-in-frame deletion in sta1-1 (Lee et al., 2006). Total STA1 (tSTA1) expression combining STA1 and sta1 expression was comparatively high in STA1/sta1-1 seedlings before heat treatment (Fig. 4B) reflecting the fact that transgene expression was under the regulation of 35S constitutive promoter. In response to heat treatment, tSTA1 expression was highly induced in Col, sta1-1 and STA1/sta1-1. However, STA1 was highly induced only in Col by heat treatment, but not in sta1-1 and STA1/sta1-1 (Fig. 4C). The results suggested that the heat-induced tSTA1 expression resulted from the induction of sta1 expression in STA1/sta1-1, and further implicated that STA1 and/or STA1-dependent splicing products do not seem to involve in the intron-free STA1 and/or sta1 expression.

Then, expression of our model transcript HSFA3 was monitored specifically for total mRNA (primer combination of f1 and r1), pre-mRNA (primer combination of f1 and r2) and mature mRNA (primer combination of f1 and r3) under heat stress conditions. HSFA3 expression in any type of mRNA was low in all genotypes before heat treatment (Fig. 4D). Total mRNA expression of HSFA3 was highly induced in all genotypes by heat treatment (Fig. 4D, upper
Pre-mRNA of HSFA3 was accumulated to a high level in sta1-1 by heat treatment, but not to the same level in Col and STA1/sta1-1 (Fig. 4D, middle panel). On the contrary mature HSFA3 mRNA expression level was high in Col and STA1/sta1-1 by heat treatment, but such high expression level was not reached in sta1-1 (Fig. 4D, lower panel). These results indicated that HSFA3 transcription was driven by heat treatment regardless of STA1 activity, resulting in the synthesis and accumulation of its pre-mRNAs. However, HSFA3 splicing was carried out appropriately only in the presence of STA1 activity. To verify the RT-qPCR data at a technical point, STA1 and HSFA3 expression was monitored with total RNA in the absence of reverse-transcription as a negative control, resulting in null amplification of target genes (Supplemental Fig. S8). Taken together, mature mRNA expression of HSFA3 resulted from STA1-independent transcription and STA1-dependent pre-mRNA splicing in response to heat stress.

The higher induction HSFA3 expression in Col and STA1/sta1-1 correlated well with their seedling viability after the two-step heat treatment (Fig. 4A, upper panel). All these experiments thus clearly indicated that STA1-dependent splicing played an important regulatory step for HSFA3 expression in the establishment of Arabidopsis heat stress tolerance. To examine whether STA1-dependent splicing of HSFA3 pre-mRNA led to the expression of its downstream genes, HSA32 expression was monitored using RT-qPCR. Total mRNA expression of HSA32 was highly induced in Col-0 and STA1/sta1-1 by heat treatment, but unlike HSFA3, it was never induced to the same level in sta1-1 (Fig. 4E, upper panel) suggesting full induction of HSA32 expression requires STA1 activity. Pre-mRNA of HSA32 accumulated in sta1-1 to a certain level that was higher than those in Col and STA1/sta1-1 under the stress conditions (Fig. 4E, middle panel) and thus pre-mRNA splicing of HSA32 again required STA1 activity as HSFA3. Consequently, mature HSA32 mRNA expression level was higher in Col and STA1/sta1-1 than sta1-1 under the stress conditions (Fig. 4E, lower panel). These results indicated that heat-induced HSA32 expression is seemingly under the transcriptional regulation of HSFA3, splicing of which is also dependent on STA1 activity. In addition its mature mRNA expression is also under the post-transcriptional regulation of STA1 activity. In case of intron-free HSP18.2 that is another target gene of HSFA3 (Schramm et al., 2008) the gene expression was highly induced in Col and STA1/sta1-1 in response to heat treatment, but such induction was clearly compromised in sta1-1 (Supplemental Fig. S9) again suggesting that HSP18.2 induction requires functional HSFA3. Taken all together, these results suggest that STA1 plays a necessary regulatory step in
heat response gene expression, and its gene products have important roles in establishing plant
two-step acquired heat stress tolerance.

The higher accumulation of HSFA3 pre-mRNA in sta1-1 (Fig. 4D, middle panel) could
reflect the possibility that nonsense-mediated mRNA decay (NMD) activity is compromised in
the absence of STA1 activity (Maquat, 2004). To address whether STA1 functions in NMD, the
seedling survival assay in response to two-step heat treatment was carried out with well-
characterized loss-of-function NMD mutants, upf1 and upf3 (Jeong et al., 2011) together with Col,
sta1-1, and STA1/sta1-1. Consistent with the previous growth phenotype responses to heat stress
treatment (Fig. 4A), Col and STA1/sta1-1 showed heat stress tolerance, but sta1-1 did not
(Supplemental Fig, S10). upf1 and upf3 also showed phenotype similar to Col before and after
the step-wise heat treatment indicating that STA1 most unlikely functions in NMD at least for the
establishment of heat stress tolerance.

HSFA3 plays a key role in expression of many HSP genes, gene products of which
contribute to either positive or negative feedback of heat stress responses and eventually provides
efficient plant heat stress adaptation (Chang et al., 2006). Since mature mRNA expression of
HSFA3 correlated well with plant heat tolerance in our two-step acquired heat assay, we further
examined whether HSFA3 could drive plant heat stress tolerance for itself downstream of STA1.
Two transgenic Arabidopsis lines in sta1-1 background were generated to constitutively express
cDNA version of HSFA3 that does not require splicing activity for its mature mRNA expression
(HSFA3/sta1-1). The HSFA3/sta1-1 lines with single homozygous transgene insertion were
selected and used for further analysis. The expression of HSFA3 was monitored in two transgenic
HSFA3/sta1-1 lines by RT-qPCR (Supplemental Fig. S11). There was no drastic phenotypic
difference among Col, sta1-1, STA1/sta1-1, and HSFA3/sta1-1, except seedlings in the sta1-1
background showed a relatively slower growth and simpler root architecture than Col
(Supplemental Fig. S12). These transgenic lines were tested for heat stress tolerance under both
mild and two-step acquired heat stress conditions together with Col and sta1-1. For mild heat
stress tolerance assay, seedlings were exposed at 38 °C for 1 d and seedling viability was
monitored at 7 d after the heat treatment. Approximately two-thirds of seedlings of Col and
transgenic STA1/sta1-1 complementation lines were survived under mild heat stress conditions,
but transgenic HSFA3/sta1-1 lines were unable to keep their viability as like Col and transgenic
STA1/sta1-1 complementation lines (Fig. 5, A and B). In consistent with previous data (Fig. 4A),
seedlings of Col and \( STA1/sta1-1 \) showed stress tolerance to the step-wise heat treatment, but transgenic \( HSFA3/sta1-1 \) lines again failed to survive under the stress conditions (Fig. 5C).

To monitor protein functions of transgene \( HSFA3 \) in gene regulation, its target gene expression was measured by RT-qPCR. Expression of \( HSFA3 \) target genes \( HSP18.2 \) and \( HSA32 \) was highly induced in Col and \( STA1/sta1-1 \) under two-step acquired heat stress conditions (Fig. 5, D and E). Although \( HSFA3 \) expression was detected in transgenic \( HSFA3/sta1-1 \) lines before heat treatment because of the nature of \( 35S \) promoter used in the transgenic line construction (Supplemental Fig. S11), \( HSP18.2 \) and \( HSA32 \) expression was not any further induced in \( HSFA3/sta1-1 \) compared to \( sta1-1 \) after heat treatment. Furthermore, mature mRNA transcripts of two well characterized heat stress responsive genes \( HSP23.6 \) and \( HSC70-5 \) were failed to accumulate in \( HSFA3/sta1-1 \) lines after heat treatment (Fig. 5, F and G). These results indicated that \( HSFA3 \) expresses in response to heat stress and its gene products induce \( HSP \) expression, but its sole activity is not sufficient to drive plant heat tolerance. This implicated that \( STA1 \) involves in pre-mRNA splicing of a broader range of genome responses to heat stress.

### Global Target Analysis for \( STA1 \)-dependent Splicing Activity

To search for genome-wide global targets of \( STA1 \)-dependent splicing activity in heat inducible transcriptomes, differential display of RNA-followed by sequencing (DDR-seq) was conducted with Col and \( sta1-1 \). Arabidopsis seedlings were first grown on half strength MS agar containing 0.5% sucrose under a cycle of 16 h light and 8 h dark at 23-25 °C for 5 days. For heat-induced DDR-seq analysis, seedlings were exposed to heat treatment at 38 °C for 2 h and then immediately at 45 °C for 2 h, and harvested 24 h after heat treatment. Because two genotypes showed some differences in growth phenotypes, we identified heat inducible genes within each genotype and compared these identified sets of genes to learn similarities and differences in heat responsive gene expression in Col and \( sta1-1 \). DDR-seq analysis was thus carried out with Col (driver) vs. heat-treated Col (tester) and \( sta1-1 \) (driver) vs. heat-treated \( sta1-1 \) (tester) and sequence reads were normalized and analyzed using CLC Genomics Workbench.

After mapping sequence reads to genome information 7762 and 8529 genes were uniquely identified from 398,370 and 436,311 base pairs from Col and \( sta1-1 \), respectively (Supplemental Table S1, Supplemental Fig. S6A). These corresponded to approximately 30–35% of protein coding genes in Arabidopsis genome. In an ordinary process of differentially expressed
gene analysis, transcripts of which expression was affected in opposite direction in wild type and a loss-of function mutant would be recognized as primary responsive genes under the regulation of genetic factors of interests. However, we rather tried to analyze 5745 genes that were commonly sequenced in Col and sta1-1 (Supplemental Fig. S13A), since genes under STA1 regulation seem to accumulate their pre-mRNAs in sta1-1 under induction conditions. Consistently twice more sequence reads matched to introns in the raw data of sta1-1 compared to Col (Supplemental Table S1). In results roughly two thirds of heat-inducible genes in Col-0 overlapped with those in sta1-1 suggesting that STA1 conveys pre-mRNA splicing activity for a significant portion of transcriptome responses to heat stress.

In the commonly identified DDR-seq data 268 genes overlapped with those found in microarray-based data for heat stress responses to 38 °C for 3 h (Supplemental Dataset; http://Arabidopsis.org/info/expression/ATGenExpress.jsp). Since both DDR-seq and microarray experiments were carried out with different heat stress conditions, only a certain level of heat responses presented commonly in both datasets. Despite the limitation, gene ontology (GO) term analysis was carried out for this set of genes to understand representative cellular pathways under heat stress conditions. First genes for responses to heat (GO:0009408, 2.09e-19) and protein folding (GO:0006457, 6.02e-15) were recognized being enriched significantly (Fig. 6A). In addition genes for responses to high light intensity, hydrogen peroxide, cold and regulation of programmed cell death were enriched with equal or less than 1% of false discovery rates (FDR), which perhaps implicated that reactive oxygen species could play a role in heat responsive gene expression.

Furthermore 14 HSP genes were noticed in the category of genes for response to heat and those for protein folding that are known to be the major function of HSPs in stressed cells (Supplemental Fig. S13B). Twelve of them have more than one intron, of which splicing is most likely under STA1 regulation. HSP90-2 expression could not be measured by RT-qPCR with limited analysis and thus it was excluded from further analysis. We focused on these 11 intron-containing heat stress-related genes in the beginning.

When these intron-containing HSP genes were analyzed for their gene expression patterns under heat stress conditions, mature mRNA expression of HSP23.6, HSC70-5, BOB1, CPHSC70-1 and HSP60-3B was highly induced in Col, but much less in sta1-1 (Fig. 6B and Supplemental Fig. S14A). This pattern of mature mRNA accumulation was similar to that of HSFA3 (Fig. 4D).
However, mature mRNA expression of HSP70-3, HSP70-4, HSP70-16 and HSP89.1 was induced similarly in both Col and sta1-1 or even higher in sta1-1 (Supplemental Fig. S14A), indicating that mature transcript accumulation of these genes was likely independent of STA1 activity. Taken together, mature mRNA expression of HSP23.6, HSC70-5, BOB1, CPHSC70-1 and HSP60-3B is most likely under the control of STA1-dependent pre-mRNA splicing.

To verify this notion, accumulation of total mRNA and pre-mRNA of HSP23.6, HSC70-5, BOB1, CPHSC70-1 and HSP60-3B was measured separately by RT-qPCR. Total mRNA expression of HSP23.6, HSC70-5 and CPHSC70-1 was induced in Col and sta1-1 by heat treatment (Fig. 6C and Supplemental Fig. S14B). However, their pre-mRNA transcripts were higher in sta1-1 than Col (Fig. 6D and Supplemental Fig. S14C). These results indicated that induction of HSP23.6, HSC70-5 and CPHSC70-1 expression by heat treatment was mainly regulated at post-transcriptional level by STA1 splicing activity as shown with HSFA3 expression (Fig. 4D). In cases of BOB1 and HSP60-3B, total mRNA accumulation was relatively less induced in sta1-1 compared to Col by heat treatment (Fig. 6C and Supplemental Fig. S14B), but their pre-mRNA levels were still higher in sta1-1 than Col (Fig. 6D and Supplemental Fig. S14C). These results implicated that STA1 not only modulated splicing of BOB1 and HSP60-3B at post-transcriptional level, but also influenced their transcription perhaps by the function of STA1 splicing products as shown with HSA32 expression (Fig. 4E). Taken all together STA1 splicing activity is indispensable for mature mRNA accumulation of a subset of HSPs under heat stress conditions.

In this study, STA1, a putative component of the U5 snRNP complex, was characterized as an essential regulatory factor involved in the splicing of a specific subset of pre-mRNAs including HSFA3 and its target gene HSA32 in response to high temperature stresses. Phenome and molecular analyses in response to heat treatment demonstrated that STA1 functions in splicing, but not in NMD, as a regulator in heat stress tolerance. HSFA3-dependent transcript cascades that were reconstituted using transient expression of the stress-related DREB2A and STA1 unambiguously linked to the STA1 activity with pre-mRNA splicing of HSFA3, although it is not a sufficient factor for the establishment of plant heat stress tolerance. Consistently STA1 controls pre-mRNA splicing of a broader range of heat inducible transcriptomes. In conclusion, heat-inducible STA1-dependent post-transcriptional regulation of the stress responsive gene expression plays a role in the establishment of the acquired heat stress tolerance in Arabidopsis.
More recently STA1 has been reported to play a role in miRNA biogenesis and RNA-directed DNA methylation based on analyses of relevant molecular and cellular phenotypes of sta1-1 (Chaabane et al., 2013; Dou et al., 2013). Moreover, heat stress seemingly alters splicing of miRNA, perhaps linking STA1 activity to miRNA splicing (Yan et al., 2012). This new STA1 functions in small RNA expression deserve more attentions to elucidate specific STA1 functions in gene regulation.

Our findings unravel that STA1 has specific targets in pre-mRNA splicing for the post-transcriptional regulation of gene expression response to heat stress. Even so, how STA1 modulates heat stress tolerance remains unclear. Identification of genetic components and elucidation of their molecular mechanisms in the STA1-containing spliceosome complex that is responsible for its splicing specificity will enlighten the molecular basis underlying stress-specific gene expression, and eventually stress adaptive responses in plants.
MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plants were grown in soil for 22 to 24 d under a photoperiod of 13 h light/11 h dark (60 μmol/m²/s) at 25 °C. Arabidopsis thaliana Columbia-0 (Col) plants were used as the wild-type, *sta1-1* (Lee et al., 2006), *upf1-5* (Jeong et al., 2011) and *upf3-1* (Jeong et al., 2011) mutants were used for experiments. Plasmid constructs for transgenic plants were generated by inserting the cDNA of *STA1* or *HSFA3* between the 35SC4PPDK promoter (designated as HBT) and the NOS terminator in a mini-binary vector, pCB302 (Cho et al., 2012). The constructs were expressed in Col or *sta1-1* plants. Transgenic lines with similar transgene expression levels were selected and used for further analyses. Multiple independent transgenic lines were generated and analyzed to identify consistent genetic effects. Transgenic plant phenotypes from at least two independent lines of the T3 generation were analyzed.

Heat Stress Survival Assay

To examine plant heat stress tolerance, surface-sterilized seeds were stratified for 4 d at 4°C in the dark, and plants were vertically grown on half-strength Murashige and Skoog (MS) agar medium containing 0.5 % sucrose for 5 d under a photoperiod of 16 h light/8 h dark (60 μmol/m²/s) at 25 °C. For the acquired heat stress tolerance assay, seedlings were subjected to a step-wise heat treatment of 38 °C for 2 h followed by 45 °C for 2 h. For the basal heat stress tolerance assay, seedlings were subjected to 45 °C for 2 h. For the mild heat stress tolerance assay, horizontally grown 3-day-old seedlings were subjected to 38 °C for 24 h. Plant survival was observed 7 d after the heat treatment and scored based on the retention of green shoots.

Arabidopsis Mesophyll Protoplast Transient Expression Assay

Protoplast isolation and transient expression assays were carried out as previously described by Yoo et al. (2007) and Cho and Yoo (2010). The effector construct (*STA1*) was generated by inserting cDNA between the HBT promoter and the NOS terminator in a plant expression vector. All the reporter constructs (g*COR15A*, g*HSFA3*, g*IDD14*, and g*MPK10*) were generated by inserting genomic DNA fused with GUS between the *HBT* (modified 35S) promoter and the *NOS* terminator in a plant expression vector. All the constructs were verified by DNA sequencing. The renilla luciferase driven by UBQ10 promoter (*UBQ10-rLUC*) was included as an internal control.
in the protoplast transient expression assay. The experiments were repeated, indicating consistent
results among the replicates. The primers used for cloning are listed in Supplemental Table S2.

In the functional splicing assay, reporter activities were calculated based on the
GUS/renilla-LUC ratio and normalized to the values obtained without the effector expression. To
measure the GUS activity, the transfected protoplasts with designated constructs were lysed using
a passive lysis buffer (Promega) containing 1% Triton-X (USB) and briefly mixed by vortexing.
The lysate was incubated at room temperature for 10 min and centrifuged at 13,000 rpm for 30 s.
The protoplast lysate was mixed with 10 mM MUG (Gold Biotechnology) and incubated at 37 °C
for 90 min. The reaction mixture was frozen at -80 °C to quench the reaction. The mixture was
then diluted with 0.2 M Na₂CO₃, and the GUS activity was measured using the Glomax
(Promega) single tube system with a UV module, following the manufacturer’s instructions.

**RNA Isolation and Transcript Measurement**

For *STA1* expression test, Col-RD29A-LUC was used as the WT (Lee et al., 2006). Seeds were
sown on 1× MS medium (2% sucrose and 0.3% gelrite) after surface sterilization with sodium
hypochlorite (4%). The seeds were stratified at 4 °C for 2 d and grown at 22 °C under continuous
light. For heat treatment, the plants were placed in a 37 °C incubator.

For gene expression analysis, total RNA was isolated by the Trizol method (Invitrogen),
and 1 μg of total RNA was used for cDNA synthesis using M-MLV reverse transcriptase
(Promega). Gene expression was quantitatively measured using real-time PCR (Bio-Rad) with
the SYBR Green dye-added PCR mix (Bio-Rad). *PROTEIN PHOSPHATASE 2A (PP2A*,
*AT1G13320), TUBULIN4 (TUB4, At1g04820), and ELONGATION INITIATION FACTOR 4a
(ELF4a, At3g13920) transcripts were used as the controls with gene-specific primers. Detailed
primer sequences are listed in Supplemental Table S2 of the Supplementary material. Each
primer set was pre-tested by PCR for a single gene product. The experiments were repeated thrice,
and consistent results were obtained.

**Differential display of RNA-followed by sequencing (DDR-seq)**

For sample preparation, plants were grown on half-strength MS agar medium containing 0.5 %
sucrose for 5 d and subjected to an acquired heat stress as described in material and method. Heat
treated (tester) and control (driver) seedlings were harvested 1 d after heat treatment.
Total RNA was extracted based on the Trizol method (Invitrogen) and poly-(A)-RNA was isolated using Dynabead mRNA DIRECT™ Kit (Thermo Scientific). Fifty ng of poly-(A)-RNA was used to synthesize double stranded cDNA. After ethanol precipitation, double stranded cDNA was digested with Dpn II enzyme (NEB) for 2 h, phenol/CHCl₃ extracted, ethanol precipitated and resuspended with H₂O. cDNA was ligated with pre-annealed R-24/12 adaptor (2 mg/ml) at 16 °C for 12 h. R adaptor ligated cDNA was phenol/CHCl₃ extracted, ethanol precipitated and resuspended with H₂O. To generate tester and driver amplicons, cDNA was amplified with multiple PCR reactions using high-fidelity DNA polymerase (Phusion® High-fidelity DNA Polymerase, NEB) and R-24 primer (72 °C 5 min, 20 cycles of 94 °C 1 min; 72 °C 3 min, 72 °C 10 min). The PCR products were phenol/CHCl₃ extracted and isopropanol precipitated. To remove R-24/12 adaptor, the PCR products were digested with Dpn II for 2 h, phenol/CHCl₃ extracted twice, ethanol precipitated and resuspended with H₂O. The concentrations of the tester and driver products were quantified using Quibit® 2.0 Fluorometer (Invitrogen). Five hundred ng of the tester was further ligated with pre-annealed J-24/12 adaptor (2 mg/ml) at 14 °C or 12 h.

For the subtractive hybridization, 25 μg of driver and 250 ng of J-adaptor ligated tester cDNA were combined and phenol/CHCl₃ extracted, CHCl₃ extracted and ethanol precipitated. The pellet was resuspended with 4 μl of 3x buffer solution containing 30 mM HEPES (USB) pH 8.0 at 20 °C and 3 mM EDTA (USB). The solution was incubated at 98 °C for 5 min for DNA denaturation. One μl of 5 M NaCl was added into the solution and the mixture was further incubated at 67 °C for 20 h to hybridize tester and driver. The hybridization solution was mixed well with 8 μl of 5 mg/ml of yeast RNA and further diluted with 367 μl H₂O. For each subtraction, eight 100 μl PCR reactions were prepared including 20 μl of hybridization mix without the primer. The PCR mixtures were incubated at 72 °C for 5 min and 0.8 μl of J-24 primer (1 mg/ml) was added. Ten cycles of PCR reaction were performed (94 °C 1 min, 70 °C 3 min) and incubated at 72 °C for 10 min. PCR products were combined, phenol/CHCl₃ extracted twice, ethanol precipitated and resuspended with 40 μl H₂O. 20 μl PCR products were digested with Mung Bean Nuclease (NEB) at room temperature for 30 min. To stop the reaction, 160 μl of 50 mM Tris pH 8.9 was added and incubated at 70 °C for 10 min. For amplification, eight 100 μl PCR reactions were prepared including 20 μl of Mung Bean Nuclease-treated products and 0.8 μl of J-24 primer (1 mg/ml) without polymerase. The PCR mixtures were incubated at 95 °C for 1
min and cooled down to 80 °C. 2.5 U of polymerase was added and eighteen cycles of PCR reaction were performed (94 °C 1 min, 70 °C 3 min). After further incubation at 70 °C for 10 min, PCR products were combined, phenol/CHCl3 extracted twice, CHCl3 extracted, ethanol precipitated and resuspended with 50 μl H2O, generating difference product (DP).

For next generation sequencing, library was prepared according to Ion Xpress™ Plus Fragment Library Kit (Thermo Scientific) and sequencing were conducted with the manufacturer’s instruction with Ion PGM™ (Thermo Scientific). Raw reads were normalized and analyzed using trial version of CLC Genomics Workbench (QIAGEN, Valencia, CA, USA) platform.

**Gene Ontology Analysis**
Gene ontology (GO) analysis was carried out using agriGO web-based tool (Du et al., 2010). Complete _GO_ was used for identification of enriched gene ontology with Fisher’s test. For multiple significance tests, a Benjamini and Yekutieli FDR correction was used and GO terms with FDR<0.01 were visualized with graphical view.

**ACCESSION NUMBERS**
Sequence data from this study can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: _STA1_, AT4g03430; _COR15A_, AT2G42540; _IDD14_, AT1G68130; _MPK10_, AT3G59790; _DREB2A_, AT5G05410; _HSFA3_, AT5G05410; _HSFB1_, AT4G36990; _HSFB2a_, AT5G62020; _UPF1_, AT5G47010; _UPF3_, AT1G33980; _HSA32_, AT4G21320; _HSP23.6_, AT4G25200; _HSP70T-2_, AT2G32120; _HSP70-16_, AT1G11660; _HSP70-4_, AT3G12580; _HSP60-3B_, AT3G23990; _HSP70-1_, AT5G02500; _BOB1_, AT5G53400; _HSP70-3_, AT3G09440; _CPHSC70-1_, AT4G24280; _HSP70-7_, AT5G49910; _HSC70-5_, AT5G09590; _HSP89.1_ and AT3G07770.

**ACKNOWLEDGEMENTS**
We thank for Jungwoo Hong for initial technical support.

**SUPPLEMENTAL MATERIALS**
Supplemental Figure S1. Splicing activity was measured in the presence and absence of STA1 for 35S-fLUC activity in leaf mesophyll protoplasts (LMPs) of sta1-1.

Supplemental Figure S2. Intron-retained pre-mRNA and exon-only mature mRNA of gCOR15A-GUS were discerned using semi-quantitative RT-PCR with RNA extracted from transfected LMPs of Col and sta1-1.

Supplemental Figure S3. STA1 expression upon cold and heat treatment.

Supplemental Figure S4. Different types of HSF transcripts were measured in Col and sta1-1 in response to heat treatment (37°C) using semi-quantitative RT-PCR.

Supplemental Figure S5. Analysis of STA1 and DREB2A transcripts.

Supplemental Figure S6. Analysis of the endogenous and transgene expression of STA1 in transgenic plants.

Supplemental Figure S7. Seedling survival assay was carried out with Col, sta1-1, two lines of STA1-expressing Col (STA1/Col), and two lines of STA1-expressing sta1-1 (STA1/sta1-1) for two-step acquired and one-step basal heat treatments.

Supplemental Figure S8. The expression levels of STA1 and HSFA3 mRNA were monitored using RT-qPCR without reverse transcription as control.

Supplemental Figure S9. The expression levels of HSP18.2 in Col, sta1-1 and STA1-expressing sta1-1 (STA1/sta1-1) were monitored using RT-qPCR.

Supplemental Figure S10. STA1 involves in pre-mRNA splicing but not in nonsense-mediated mRNA decay for the establishment of plant heat stress tolerance.

Supplemental Figure S11. Analysis of HSFA3 expression in two HSFA3/sta1-1 transgenic plants.

Supplemental Figure S12. Non-heat treated twelve-day-old seedlings of Col, sta1-1, two lines of STA1/sta1-1 and two lines of HSFA3/sta1-1 are shown.

Supplemental Figure S13. Analysis of DDR-seq of Col and sta1-1 in response to heat stress.

Supplemental Figure S14. Analysis of heat inducible genes enriched in DDR-seq analysis.

Supplemental Table S1. DDR-seq information.

Supplemental Table S2. Primers used in the study.

Supplemental Dataset. GO analysis of commonly enriched 268 genes in DDR-seq data of Col and sta1-1.
FIGURE LEGENDS

Figure 1. STA1 induces splicing activity of specific pre-mRNA.

A, A schematic diagram of the functional splicing assay is shown. The genomic version of splicing target gene was cloned into the GUS reporter construct with a translational fusion. B, C, Splicing activity was measured in the presence and absence of STA1 for gCOR15A-GUS in leaf mesophyll protoplasts of Col (B) and sta1-1 (C). UBQ10-rLUC activity served as an internal control. STA1 protein expression was shown using protein blot analysis with anti-epitope specific antibody. Rubisco small subunit proteins (RBC) served as a protein loading control using coomassie blue staining. The means of three replicates are shown with standard error bars. Asterisks represent Paired t-test significance between samples (**P < 0.01, *P < 0.05). D, E, Splicing activity was measured in the presence and absence of STA1 for gIDD14-GUS (D) and gMPK10-GUS (E) in leaf mesophyll protoplasts of sta1-1. UBQ10-rLUC activity served as an internal control. The means of three replicates are shown with standard error bars.

Figure 2. Heat-inducible STA1 involves in pre-mRNA splicing of HSFA3.

A, STA1 expression was measured in response to heat treatment (37°C) using semi-quantitative RT-PCR. PP2A served as a RNA control. B, A schematic diagram of genomic DNA of HSFA3 is shown with primer positions. C, D, Different types of HSFA3 transcripts were measured in Col (C) and sta1-1 (D) in response to heat treatment (37°C) using semi-quantitative RT-PCR. Heat inducible total mRNA (f1/r1), pre-mRNA (f1/f2) and mature mRNA (f1/f3) of HSFA3 were measured. Experiments were triplicated with consistent results. Representative data are shown. E, Schematic diagrams WT (wild type), mDo (mutation on splice donor) and mAc (mutation on splice acceptor) of gHSFA3-nLUC are shown. F, Splicing reporter activities from WT gHSFA3-nLUC or mDo and mAC forms of gHSFA3-nLUC were measured in the presence and absence of STA1. Expression of HA-tagged STA1 variants was shown by protein blot analysis using anti-HA antibody. RBC used as a loading control. G, Splicing reporter activity from gHSFA3-nLUC was measured in combination of STA1 and sta1-1 expression. Expression of HA-tagged STA1 and Flag-tagged sta1-1 was shown by protein blot analysis using anti-epitope specific antibody. Rubisco small subunit proteins (RBC) served as a protein loading control using coomassie blue staining. 35S-fLUC activity served as an internal control. All of the experiments were repeated three times with consistent results. The means of three replicates are shown with
standard error bars. Different letters indicate a significance difference by Tukey-Kramer test (p<0.05).

**Figure 3. STA1 induces pre-mRNA splicing of HSFA3 driven by DREB2A.**

A, A working model is proposed for DREB2A-dependent heat-inducible gene expression. B, A schematic diagram of HSFA3 is shown with a set of primers used for detecting intron-retained pre-mRNA. C, D, Intron-retained HSFA3 pre-mRNA, gene expression of which was driven by DREBA2, was discerned in LMPs of Col and sta1-1 with/without STA1. The pre-mRNA accumulation was monitored using semi-quantitative RT-PCR (C) and RT-qPCR (D). E, Expression of HSP23.6, HSA32, and HSP70T-2 in sta1-1 was monitored in combination of DREB2A and STA1 expression using RT-qPCR. Quantitative values were normalized with an internal control EIF4a and presented in comparison to those values in Col without effector transfection. All of the experiments were repeated three times and produced consistent results. The means of triplicates are shown with standard error bars. Asterisks represent Paired t-test significance between samples (***P < 0.001, **P < 0.01, and *P < 0.05).

**Figure 4. STA1 involves in the establishment of stress tolerance in response to two-step heat treatment in Arabidopsis.**

A, Seedling survival assay was carried out with Col, sta1-1, STA1-expressing Col (STA1/Col), and STA1-expressing sta1-1 (STA1/sta1-1) for two-step acquired and one-step basal heat treatments. B, C, The expression levels of tSTA1 (B) and STA1 (C) were quantitatively monitored before (Cont) and at 1d after heat treatment (Heat) using RT-qPCR. D, E, The expression levels of total mRNA, pre-mRNA and mature-mRNA of HSFA3 (D) and HSA32 (E) were quantitatively monitored using RT-qPCR in Col, sta1-1 and STA1/sta1-1 before (Cont) and at 1 d after heat treatment (Heat). Primer sets for total mRNA (f1/r1), pre-mRNA (f1/r2) and mature-mRNA (f1/r3) were described with schematic diagrams. Quantitative values were normalized with internal controls ELF4a. The means of triplicates are shown with standard error bars. Different letters indicate a significance difference by Tukey-Kramer test (p<0.05).

**Figure 5. STA1, but not HSFA3, establishes plant stress tolerance for mild heat and two-step acquired heat stresses.**
A, Seedling survival assay was carried out with Col, *sta1-1*, two lines of *STA1*-expressing *sta1-1* (*STA1/sta1-1*), two lines of *HSFA3*-expressing *sta1-1* (*HSFA3/sta1-1*) for mild heat treatment. B, Viable seedlings was measured to calculate survival rate. The means of triplicates are shown with standard error bars. C, Seedling survival assay was carried out for two-step acquired heat treatment. D-G, The expression levels of mature mRNA of *HSP18.2* (D), *HSA32* (E), *HSP23.6* (F) and *HSC70-5* (G) were monitored in Col, *sta1-1*, two lines of *STA1/sta1-1* and two lines of *HSFA3/sta1-1* before and 1 d after the step-wise heat treatment using RT-qPCR. The means of triplicates are shown with standard error bars. Different letters indicate a significance difference by Tukey-Kramer test (p<0.05).

Figure 6. Heat inducible genes enriched in DDR-seq analysis.
A, Graphical view of gene ontology (GO) term analysis for gene (see Supplemental Dataset). B, Mature mRNA expression of *HSP23.6*, *HSC70-5* and *BOB1* was measured in Col and *sta1-1* using RT-qPCR. C, D, Total mRNA (C) and premature mRNA (D) of *HSP23.6*, *HSC70-5* and *BOB1* were measured using RT-qPCR in Col and *sta1-1*. The means of three replicates are shown with standard error bars. Different letters indicate a significance difference by Tukey-Kramer test (p<0.05).
LITERATURE CITED


luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate.

ACS Chem Biol 7: 1848-1857


Fig. 1. STA1 induces splicing activity of specific pre-mRNA. A, A schematic diagram of the functional splicing assay is shown. The genomic version of splicing target gene was cloned into the GUS reporter construct with a translational fusion. B, C, Splicing activity was measured in the presence and absence of STA1 for gCOR15A-GUS in leaf mesophyll protoplasts of Col (B) and sta1-1 (C). UBQ10-rLUC activity served as an internal control. STA1 protein expression was shown using protein blot analysis with anti-epitope specific antibody. Rubisco small subunit proteins (RBC) served as a protein loading control using coomassie blue staining. The means of three replicates are shown with standard error bars. Asterisks represent Paired t-test significance between samples (***P < 0.001, **P < 0.01, and *P < 0.05). D, E, Splicing activity was measured in the presence and absence of STA1 for gIDD14-GUS (D) and gMPK10-GUS (E) in leaf mesophyll protoplasts of sta1-1. UBQ10-rLUC activity served as an internal control. The means of three replicates are shown with standard error bars.
Fig. 2. Heat-inducible STA1 involves in pre-mRNA splicing of HSFA3. A, STA1 expression was measured in response to heat treatment (37°C) using semi-quantitative RT-PCR. PP2A served as a RNA control. B, A schematic diagram of genomic DNA of HSFA3 is shown with primer positions. C, D, Different types of HSFA3 transcripts were measured in Col (C) and sta1-1 (D) in response to heat treatment (37°C) using semi-quantitative RT-PCR. Heat inducible total mRNA (f1/r1), pre-mRNA (f1/f2) and mature mRNA (f1/f3) of HSFA3 were measured. Experiments were triplicated with consistent results. Representative data are shown. E, Schematic diagrams WT (wild type), mDo (mutation on splice donor) and mAc (mutation on splice acceptor) of gHSFA3-nLUC are shown. F, Splicing reporter activities from WT gHSFA3-nLUC or mDo and mAC forms of gHSFA3-nLUC were measured in the presence and absence of STA1. Expression of HA-tagged STA1 variants was shown by protein blot analysis using anti-HA antibody. RBC used as a loading control. G, Splicing reporter activity from gHSFA3-nLUC was measured in combination of STA1 and sta1-1 expression. Expression of HA-tagged STA1 and Flag-tagged sta1-1 was shown by protein blot analysis using with anti-epitope specific antibody. Rubisco small subunit proteins (RBC) served as a protein loading control using coomassie blue staining. 35S-fLUC activity served as an internal control. All of the experiments were repeated three times with consistent results. The means of three replicates are shown with standard error bars. Different letters indicate a significance difference by Tukey-Kramer test (p<0.05).
**Fig. 3.** STA1 induces pre-mRNA splicing of *HSFA3* driven by DREB2A. **A,** A working model is proposed for DREB2A-dependent heat-inducible gene expression. **B,** A schematic diagram of *HSFA3* is shown with a set of primers used for detecting intron-retained pre-mRNA. **C, D,** Intron-retained *HSFA3* pre-mRNA, gene expression of which was driven by DREB2A, was discerned in LMPs of Col and *sta1-1* with/without STA1. The pre-mRNA accumulation was monitored using semi-quantitative RT-PCR (C) and RT-qPCR (D). **E,** Expression of *HSP23.6*, *HSA32*, and *HSP70T-2* in *sta1-1* was monitored in combination of DREB2A and STA1 expression using RT-qPCR. Quantitative values were normalized with an internal control *EIF4a* and presented in comparison to those values in Col without effector transfection. All of the experiments were repeated three times and produced consistent results. The means of triplicates are shown with standard error bars. Asterisks represent Paired t-test significance between samples (****P < 0.001, **P < 0.01, and *P < 0.05**).
A

Acquired heat resistance

Basal heat resistance

Col    sta1-1    STA1/Col    STA1/sta1-1    sta1-1

B

C

Relative gene expression

Cont    Heat

0   5   10   15   20

STA1

Relative gene expression

Cont    Heat

0   2   4   6   8   10

tSTA1    Col    sta1-1    STA1/sta1-1

www.plantphysiol.org on March 13, 2017 - Published by www.plantphysiol.org
Downloaded from www.plantphysiol.org on March 13, 2017 - Published by www.plantphysiol.org
Copyright © 2017 American Society of Plant Biologists. All rights reserved.
Fig. 4. STA1 involves in the establishment of stress tolerance in response to two-step heat treatment in Arabidopsis. A, Seedling survival assay was carried out with Col, sta1-1, STA1-expressing Col (STA1/Col), and STA1-expressing sta1-1 (STA1/sta1-1) for two-step acquired and one-step basal heat treatments. B, C, The expression levels of tSTA1 (B) and STA1 (C) were quantitatively monitored before (Cont) and at 1d after heat treatment (Heat) using RT-qPCR. D, E, The expression levels of total mRNA, pre-mRNA and mature-mRNA of HSFA3 (D) and HSA32 (E) were quantitatively monitored using RT-qPCR in Col, sta1-1 and STA1/sta1-1 before (Cont) and at 1 d after heat treatment (Heat). Primer sets for total mRNA (f1/r1), pre-mRNA (f1/r2) and mature-mRNA (f1/r3) were described with schematic diagrams. Quantitative values were normalized with internal controls ELF4a. The means of triplicates are shown with standard error bars. Different letters indicate a significance difference by Tukey-Kramer test (p<0.05).
A

Cont Mild heat resistance

B

![Graph showing survival rate across different conditions](image)

C

Acquired heat resistance

D

Relative gene expression for HSP18.2

E

Relative gene expression for HSA32_f1/r3
Fig. 5. STA1, but not HSFA3, establishes plant stress tolerance for mild heat and two-step acquired heat stresses. A, Seedling survival assay was carried out with Col, sta1-1, two lines of STA1-expressing sta1-1 (STA1/sta1-1), two lines of HSFA3-expressing sta1-1 (HSFA3/sta1-1) for mild heat treatment. B, Viable seedlings was measured to calculate survival rate. The means of triplicates are shown with standard error bars. C, Seedling survival assay was carried out for two-step acquired heat treatment. D-G, The expression levels of mature mRNA of HSP18.2 (D), HSA32 (E), HSP23.6 (F) and HSC70-5 (G) were monitored in Col, sta1-1, two lines of STA1/sta1-1 and two lines of HSFA3/sta1-1 before and 1 d after the step-wise heat treatment using RT-qPCR. The means of triplicates are shown with standard error bars. Different letters indicate a significance difference by Tukey-Kramer test (p<0.05).
**Fig. 6.** Heat inducible genes enriched in DDR-seq analysis. A, Graphical view of gene ontology (GO) term analysis for gene (see Supplemental Dataset). B, Mature mRNA expression of *HSP23.6, HSC70-5* and *BOB1* was measured in Col and *sta1-1* using RT-qPCR. C, D, Total mRNA (C) and premature mRNA (D) of *HSP23.6, HSC70-5* and *BOB1* were measured using RT-qPCR in Col and *sta1-1*. The means of three replicates are shown with standard error bars. Different letters indicate a significance difference by Tukey-Kramer test (p<0.05).


and analyzing large-scale biological data sets. PLoS ONE 2: e718


