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Davis, Seth Jon [orcid.org/0000-0001-5928-9046](https://orcid.org/0000-0001-5928-9046) (2024) Diurnal regulation of alternative splicing associated with thermotolerance in rice by two glycine-rich RNA-binding proteins. *Science Bulletin*. pp. 59-71. ISSN 2095-9281

<https://doi.org/10.1016/j.scib.2023.11.046>

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## Article

# Diurnal regulation of alternative splicing associated with thermotolerance in rice by two glycine-rich RNA-binding proteins

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## ARTICLE INFO

## Article history:

Received 28 July 2023

Received in revised form 17 October 2023

Accepted 21 November 2023

Available online 23 November 2023

## Keywords:

Alternative splicing

Diurnal regulation

Glycine-rich protein

*Oryza sativa* L.

Thermotolerance

## ABSTRACT

Rice (*Oryza sativa* L.) production is threatened by global warming associated with extreme high temperatures, and rice heat sensitivity is differed when stress occurs between daytime and nighttime. However, the underlying molecular mechanism are largely unknown. We show here that two glycine-rich RNA binding proteins, OsGRP3 and OsGRP162, are required for thermotolerance in rice, especially at nighttime. The rhythmic expression of *OsGRP3/OsGRP162* peaks at midnight, and at these coincident times, is increased by heat stress. This is largely dependent on the evening complex component OsELF3-2. We next found that the double mutant of *OsGRP3/OsGRP162* is strikingly more sensitive to heat stress in terms of survival rate and seed setting rate when comparing to the wild-type plants. Interestingly, the defect in thermotolerance is more evident when heat stress occurred in nighttime than that in daytime. Upon heat stress, the double mutant of *OsGRP3/OsGRP162* displays globally reduced expression of heat-stress responsive genes, and increases of mRNA alternative splicing dominated by exon-skipping. This study thus reveals the important role of *OsGRP3/OsGRP162* in thermotolerance in rice, and unravels the mechanism on how *OsGRP3/OsGRP162* regulate thermotolerance in a diurnal manner.

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## 1. Introduction

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population. An increasing human population demands more rice productivity, which is challenged by a more adverse climate associated with extreme weather conditions [1]. Based on mathematical modeling, world-wide cereal production is estimated to have a loss of 6%–7% yield per 1 °C increase in seasonal mean temperature associated with extreme heat disasters [2]. In the past century, daily minimum temperature in nighttime increased more than daily maximum temperature in daytime [3]. Crops, such as rice, have different sensitivity to heat stress between day and night and among different varieties [4–6]. However, how this diurnal difference in thermotolerance is acquired in plants remains elusive.

The circadian clock is a ubiquitous controlling system that allows plants to coordinate their growth and development with day and night signals. It maintains a roughly 24-h rhythm of internal biological process [7]. This circadian oscillation is generated by a number of repressors and activators that are integrated into multiple interconnected feedback loops [8,9]. Many circadian clock

regulators are involved in plant responses to warm temperatures [10]. For example, early flowering 3 (ELF3), one of the evening complex (EC) components that are night-time repressors in the circadian circuit, inhibits hypocotyl growth in *Arabidopsis* at ambient temperature, and it is subjected to liquid-liquid separation and proteasome-mediated protein degradation under warm temperature conditions [10–12]. In contrast, the expression of *ELF4*, another important component of EC, is also tightly regulated by reveille 5 (RVE5), RVE7, circadian clock associated 1 (CCA1) and late elongation hypocotyl (LHY), either positively or negatively under warm temperature conditions in *Arabidopsis* [13]. Interestingly, RVE4 and RVE8 regulate the first wave of heat stress responses in the day, but confers thermotolerance in the evening in *Arabidopsis* [14]. These results suggest that plant growth and survival under high temperature conditions are dependent on the time of day when high temperature occurs, however, factors that provide temporal resistance in a diurnal manner await discovery.

In the current study, we show that *OsGRP3/OsGRP162* convey diurnal signals downstream of EC, and regulate diurnal thermotolerance in rice. Furthermore, we show that *OsGRP3/OsGRP162* bind to various mRNAs and interact with spliceosomal components to regulate alternative splicing. Collectively, these results demonstrate that *OsGRP3/OsGRP162* are required for rice plants to maintain high yielding in response to nighttime heat stress.

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## 2. Materials and methods

### 2.1. Plant materials and phenotypic analysis

The wild-type plants (subsp. ZH11 background) were used in the current study. To generate the gene-edited double mutant plants of *OsGRP3/OsGRP162* (*dm-1/dm-2*), gene-specific guide sequences (sgRNAs) were selected and the CRISPR/Cas9 expression cassettes were prepared. The single mutants of *OsGRP3/OsGRP162* were selected from a segregation population derived from the backcrossing of *dm-1* to ZH11. To obtain the FLAG-tagged *OsGRP3/OsGRP162* overexpression plants, the full-length coding sequences of *OsGRP3/OsGRP162* were amplified and cloned into the vector pCAMBIA1300-FLAG. Error-free constructs were introduced into plants by *Agrobacterium*-mediated stable transformation. All the primers are listed in Table S1 (online). The rice seeds were germinated and grown in 96-well plates with Kimura B nutrient solution (vegetative stage) or directly in soil (reproductive stage) under 20,000 lux light conditions (12 h light/12 h dark). High temperature treatments were applied in the growth chambers (CONVIRON PGR15, Canada) for a period of time as mentioned in the text. Survival rate, seed setting rate and other important agronomical traits were measured after recovery at normal growth temperatures. There were 24 plants in each of the three biological replicates for phenotypic analysis. Student's *t*-test or analysis of variance (ANOVA) was used for statistical analysis as indicated in the text.

### 2.2. Subcellular localization

For protein subcellular localization studies, the coding sequences of *OsGRP3/OsGRP162* were amplified and subcloned into the pCAMBIA1300-GFP vector. These C-terminal YFP-tagged fusion proteins were transiently co-expressed with the nuclear protein marker (NLS-mCherry) in rice protoplasts mediated by the PEG methods, and protein subcellular localization of each fusion protein was observed under a confocal microscopy (Zeiss LSM A710). All the primers are listed in Table S1 (online).

### 2.3. Yeast two-hybrid, split-luciferase, and pull-down assays

For yeast two-hybrid assay, the full-length and truncations of *OsGRP3/OsGRP162* were cloned into the bait vector pGBKT7, and full-length of U2AF35a, U2AF35b, U2AF65a, U2AF65b, U1-70K or PRP18 were cloned into the prey vector pGBKT7 (Clontech, Palo Alto, USA) to generate the baits and preys, respectively. Different combination of vectors was transformed into the yeast strain PJ69 using a commercial kit (Zymo Research, Irvine, USA) and grew on different selective plates. For split-luciferase assay, the full-length of *OsGRP3/OsGRP162* were fused in frame with the LUC<sup>C</sup>, and full-length of U2AF35a, U2AF35b, U2AF65a, U2AF65b, or U1-70K were used in frame with the LUC<sup>C</sup>, respectively, and different combination of vectors was transformed into *Agrobacterium tumefaciens* strain GV3101 and infiltrated into *Nicotiana benthamiana* leaves. Luciferase luminescence was detected by a Tanon 5200 Image Analyzer (Tanon, Shanghai, China). For pull-down assays, the full-length of *OsGRP3/OsGRP162* were cloned into pETMALC-H (maltose binding protein (MBP) fusion) vector while the full-length of U2AF35a/U1-70K were cloned into pGEX4T-1 (GST fusion) vector, respectively. These fusion proteins were expressed in transetta cells (Novagen, Madison, USA) at 16 °C overnight. GST-U2AF35a, GST-U1-70K or the GST control was conjugated to glutathione resin (GenScript, Piscataway, USA) and incubated with the purified MBP-GRP3 or MBP-GRP162 in the pull-down buffer (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA

(pH 8.0), 1 mmol/L DTT and 0.5% NP-40) at 4 °C for 2 h, and then the resin was washed 4–6 times, and then the boiled protein extracts were subjected to SDS-PAGE and Western blotting using anti-GST (Abmart, Shanghai, China) and anti-MBP antibody (Abmart), respectively. All the primers are listed in Table S1 (online).

### 2.4. Effector-reporter assay

For effector-reporter assays, promoter sequences of *OsGRP3/OsGRP162*, or four copies of LBS sequences (TAGATACT) derived from the promoter of *OsGRP3/OsGRP162* were synthesized and inserted into the pGreen0800-II vector together with the 35S promoter generate the firefly luciferase reporter in which the *Renilla* luciferase driven by the 35S promoter served as an internal control. A mutated form of LBS (LBSm, CTCGCAGG) was used as a negative control. The coding sequence of OsELF3-2/OsELF4a/OsLUX was inserted into the pSKM36 vector with the MYC tag to generate the effector vector. Different combination of constructs was transiently expressed in tobacco (*Nicotiana benthamiana*) leaves. Three days after infiltration, luciferase activities were measured with a dual-luciferase reporter assay kit (Promega). All the primers are listed in Table S1 (online).

### 2.5. Gene expression and alternative splicing analysis

For RNA-seq analysis, seven-day-old rice seedlings subjected to heat stress (45 °C vs. 29 °C) for 18 h were harvested for RNA-seq analysis using an Illumina HiSeq 4000 (LC Bio Technology CO., Ltd., Hangzhou, China) in accordance with the standard Illumina protocols. Clean reads of all samples were aligned to the MSU rice reference genome using HISAT2 (<https://daehwankimlab.github.io/hisat2/>, version: hisat2-2.2.1) package, the mapped reads of each sample were assembled using StringTie (<https://ccb.jhu.edu/software/stringtie/>, version: stringtie-2.1.6) with default parameters. Then, the expression abundance for mRNAs was calculated by fragment per kilobase of transcript per million mapped reads (FPKM) value. Differential gene expression analysis was performed by DESeq2 software between two different groups (and by edgeR between two samples). The cutoff for significant differential gene expression was set as: up-regulation  $q < 0.05$ , fold change (FC)  $\geq 2$  and down-regulation  $q < 0.05$ , FC  $\leq 0.5$ . Venn diagrams, heat map, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed with the OmicStudio cloud platform provided by LC Bio Technology CO. For alternative splicing (AS) analysis, rMATS (version 4.1.1) (<https://rnaseq-mats.sourceforge.net>) was used to identify alternative splicing events and analyze differential alternative splicing events between samples. The cutoff for significant differential AS events induced by heat was set as: false discovery rate (FDR)  $< 0.05$ , FC  $\geq 1.1$  in a comparison. Motif significantly overrepresented in the differentially skipped exons was discovered and displayed using MEME with default settings. For quantitative RT-PCR (RT-qPCR), total RNA was extracted from samples using a RNA Prep Pure Plant Kit (Tiangen), and reverse-transcribed using the 5 × PrimeScript RT Master Mix (Takara) and oligo (dT) primers (Takara). RT-qPCR was performed in the CFX96 real-time system (Bio-Rad) using the Super-Real PreMix Plus Kit (Tiangen). The  $\Delta\Delta C_t$  (threshold cycle) method was used to calculate the gene expression level. All the primers are listed in Table S1 (online).

### 2.6. RNA immunoprecipitation-qPCR (RIP-qPCR)

For RIP-qPCR, 2 g of *OsGRP3-FLAG/OsGRP162-FLAG* transgenic plants driven by the constitutive ubiquitin promoter were grown at 29 °C for two weeks and then ground into powder in liquid

nitrogen and suspended in 7.5 mL lysis buffer (20 mmol/L Tris-HCl, 20 mmol/L KCl, 2 mmol/L EDTA, 2.5 mmol/L MgCl<sub>2</sub>, 25% glycerol, 250 mmol/L sucrose, 1 mmol/L PMSF, 1% plant protease inhibitors and 10 U/mL rRNasin) followed by the fixation in 1% formaldehyde for 1 h, which was stopped by adding glycine with the final concentration 0.125 mol/L. Samples were centrifuged at 2500 × g for 2 min at 4 °C and the pellets were resuspended in 3 mL NRBT Buffer (20 mmol/L Tris-HCl, 2.5 mmol/L MgCl<sub>2</sub>, 25% glycerol, 0.2% Triton-100, 1 mmol/L PMSF, 1% plant protease inhibitors and 8 U/mL rRNasin). After two times of repeated resuspending and centrifugation, the pellets were resuspend in 250 μL Nuclei Lysis Buffer (50 mmol/L Tris-HCl, 10 mmol/L EDTA, 1% SDS, 1 mmol/L PMSF, 1% plant protease inhibitors and 160 U/mL rRNasin) and sonicated 5 × 10 s with 10 s pauses. Samples were centrifuged at 16,000 × g for 15 min at 4 °C and the supernatants were diluted ten times with the dilution buffer (16.7 mmol/L Tris-HCl, 167 mmol/L NaCl, 1.2 mmol/L EDTA, 1.1% Triton X-100, 1 mmol/L PMSF, 1% plant protease inhibitors and 360 U/mL rRNasin). The supernatant was incubated with anti-FLAG antibody (Abmart) or IgG control at 4 °C overnight, subsequently the mixture was precipitated by pre-washed protein A beads. In parallel, the supernatant (10%) before dilution was kept as the input. RNA was extracted from the input and immuno-precipitated samples and used for quantitative PCR (qPCR). All the primers are listed in Table S1 (online).

### 3. Results

#### 3.1. *OsGRP3/OsGRP162* are rhythmically induced by heat stress

In our previous RNA-seq analysis of heat-stress responses, two class IVa glycine-rich proteins with RNA recognition motifs, *OsGRP3* (LOC\_Os03g46770) and *OsGRP162* (LOC\_Os12g43600) (Fig. S1a online), were found to be induced by heat stress treatment in rice plants [15]. To examine the temporal pattern of their expression, we examined diurnal transcript accumulation of *OsGRP3/OsGRP162* in seedling tissues. For this we grew ZH11 plants at control (29 °C) and high (45 °C) growth temperatures (12-h light/12-h dark) and checked the expression of *OsGRP3/OsGRP162* every six hours by RT-qPCR. Interestingly, the transcripts of *OsGRP3/OsGRP162* peaked at midnight (ZT 18 h) when plants were grown at 29 °C (Fig. 1a), and both *OsGRP3* and *OsGRP162* were highly induced at midnight when ZH11 plants were grown at 45 °C under the same photoperiod conditions (Fig. 1a). These results support that *OsGRP3/OsGRP162* are rhythmic and heat-induced in rice.

#### 3.2. *OsELF3-2* is the upstream regulator of *OsGRP3/OsGRP162* under heat stress conditions

To identify upstream regulators of *OsGRP3/OsGRP162* expression, we surveyed the diurnal expression of core circadian clock genes under control (29 °C) and high (45 °C) growth temperatures in ZH11 plants (Fig. 1b and Fig. S2 online). The EC complex genes just preceded *OsGRP3/OsGRP162* expression (Fig. 1b). Specifically, among the four rice EC genes, the transcript of *OsELF3-2* peaked at ZT 18 h, while the transcript of *OsELF4a* and *OsLUX* peaked at ZT 12 h. In contrast the transcript of *OsELF3-1* had a modest expression amplitude (Fig. 1b). Notably, the transcript of *OsELF3-2* was reduced by heat at ZT 18 h while the transcript of *OsELF4a* and *OsLUX* was reduced by heat at ZT 12 h (Fig. 1b). Since the rhythmic expression pattern of *OsELF3-2* negatively correlates with *OsGRP3/OsGRP162*, we were interested in possible regulation of *OsGRP3/OsGRP162* expression by EC under both normal growth and heat stress conditions. We obtained the loss-of-function mutants of *OsELF3-1* and *OsELF3-2* [16,17], and checked the diurnal expression

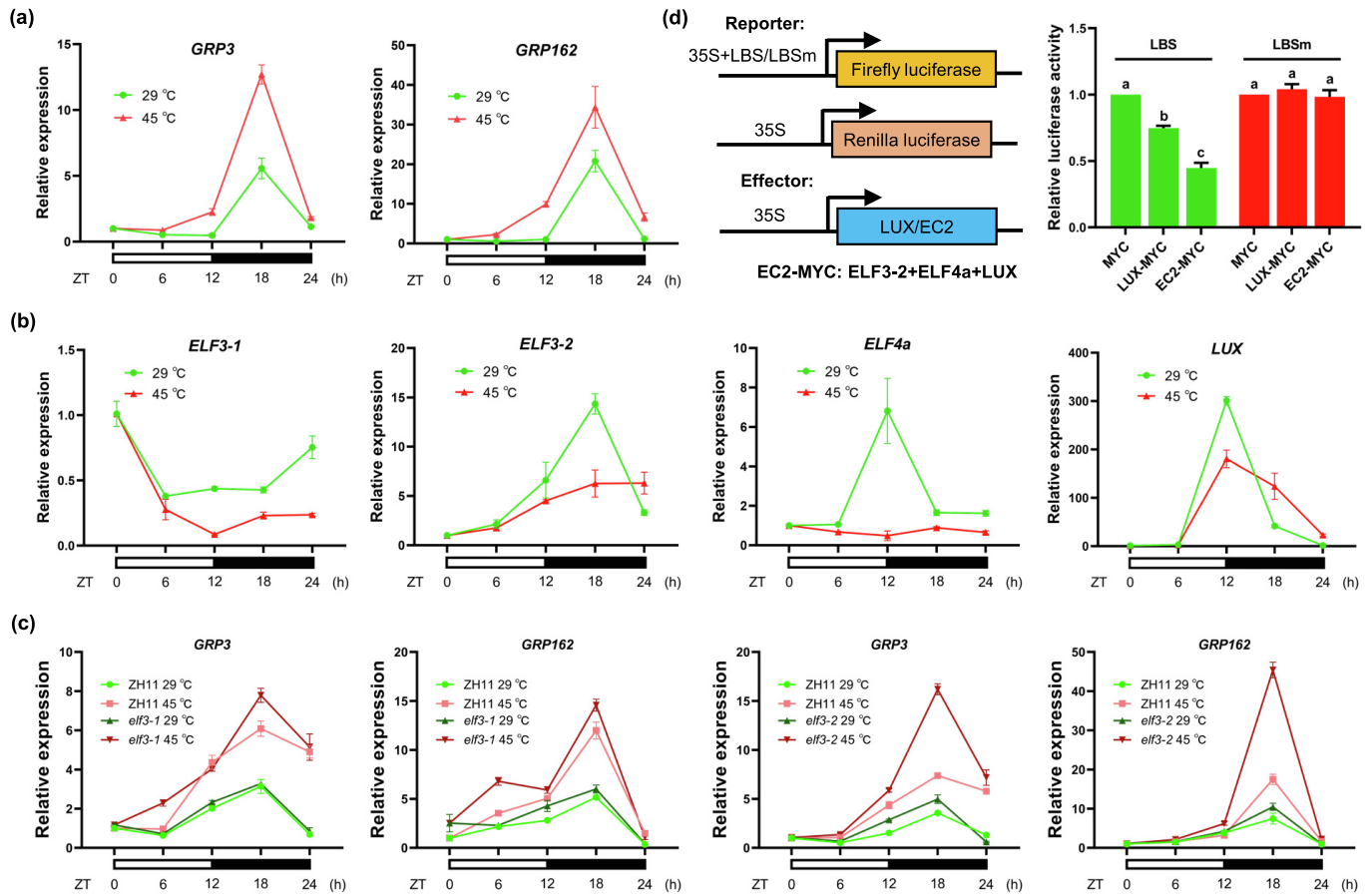
of *OsGRP3/OsGRP162* in these mutant plants. The results showed that comparing to that in ZH11 plants, the expression level of *OsGRP3/OsGRP162* was dramatically increased in *elf3-2* mutant plants at ZT 18 h, especially at high temperatures, but only slightly increased in *elf3-1* mutant plants, which is consistent with the expression pattern of *OsELF3-1* as mentioned above (Fig. 1c). Taken together, these results suggest that rhythmical induction of *OsGRP3/OsGRP162* at midnight by heat stress is largely dependent on *OsELF3-2*, one of the EC components.

LUX arrhythmo (LUX) processes a single MYB domain and recognizes the *cis*-element called LUX binding site (LBS, AGAT(A/T)CG) [18,19]. We scanned 2 kb upstream promoter regions of *OsGRP3/OsGRP162* and found that a palindrome sequence AGATACT is present in both the promoters of *OsGRP3* and *OsGRP162*. To examine whether EC can repress the promoter activity of *OsGRP3/OsGRP162*, we carried out effector-reporter assays. We linked the promoter sequence of *OsGRP3* or *OsGRP162* (Fig. S3 online), or LBS (AGATACT) (Fig. 1d) and a constitutive 35S promoter sequence to the firefly luciferase (LUC) gene to serve as a reporter, and co-expressed it with the constitutively expressed effector *OsLUX* or *OsEC2* (*OsELF3-2/OsELF4a/OsLUX*) in tobacco leaves. The results showed that co-expressing LUX suppressed the LUC reporter activity and expression *OsEC2* further suppressed the reporter activity (Fig. 1d and Fig. S3 online). These results support that the diurnal induction of *OsGRP3/OsGRP162* by heat stress is due to the diurnal reduction of *OsELF3-2* expression (*OsEC2* activity) under high temperature conditions.

#### 3.3. *OsGRP3/OsGRP162* are required for thermotolerance

To explore the biological function of *OsGRP3/OsGRP162* in heat-stress tolerance in rice, we generated several alleles of gene-edited single mutants and double mutants of *OsGRP3/OsGRP162* using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system, of which the full-length *OsGRP3/OsGRP162* proteins are predicted to be prematurely truncated (Fig. S1b online). We characterized two double mutants (*dm-1/dm-2*) in details in this study. Both *grp3-1 grp162-1* (*dm-1*) and *grp3-2 grp162-2* (*dm-2*) mutant plants grew similarly as wild-type plants (variety ZH11) at normal growth temperature (29 °C) (Fig. 2a). However, the survival rates of *grp3-1 grp162-1/grp3-2 grp162-2* plants were much reduced comparing to that of ZH11 plants after 3 d of heat stress (45 °C) and a subsequent recovery at control temperature (29 °C for 14 d) (Fig. 2b, i). Further analysis of the heat-stress phenotypes of the respective single mutants showed that the survival rates of the single mutants of *OsGRP3/OsGRP162* (*grp3-1/grp162-1*) were not much differed to that of ZH11 (Fig. S4a–c online). These results demonstrate that *OsGRP3* and *OsGRP162* are functionally redundant and are required for heat stress tolerance at vegetative stage.

To examine the role of *OsGRP3/OsGRP162* in heat stress tolerance at reproductive stage, we carried out phenotypic assays at flowering. We transferred the double mutants as well as the wild-type plants grown at 30 °C to high temperature condition (38 °C in the light for 12 h and 30 °C in the dark for 12 h) for 3 d starting at flowering stage, and then brought them back to normal growth temperature (30 °C). At maturity, several agronomic traits were measured. The results showed that seed setting rate (grain filling percentage) of *grp3-1 grp162-1/grp3-2 grp162-2* double mutant plants was similar to ZH11 plants when growing constantly at 30 °C, while for the heat-stressed plants, the seed setting rate of *grp3-1 grp162-1/grp3-2 grp162-2* double mutant plants was much reduced comparing to that of ZH11 plants (Fig. 2c, d, and k). Thus, the *OsGRP3/OsGRP162* genes work together to ensure proper seed yields after a heat-treatment at flowering. Other agronomic traits such as plant height, panicle length, number of spikelet per



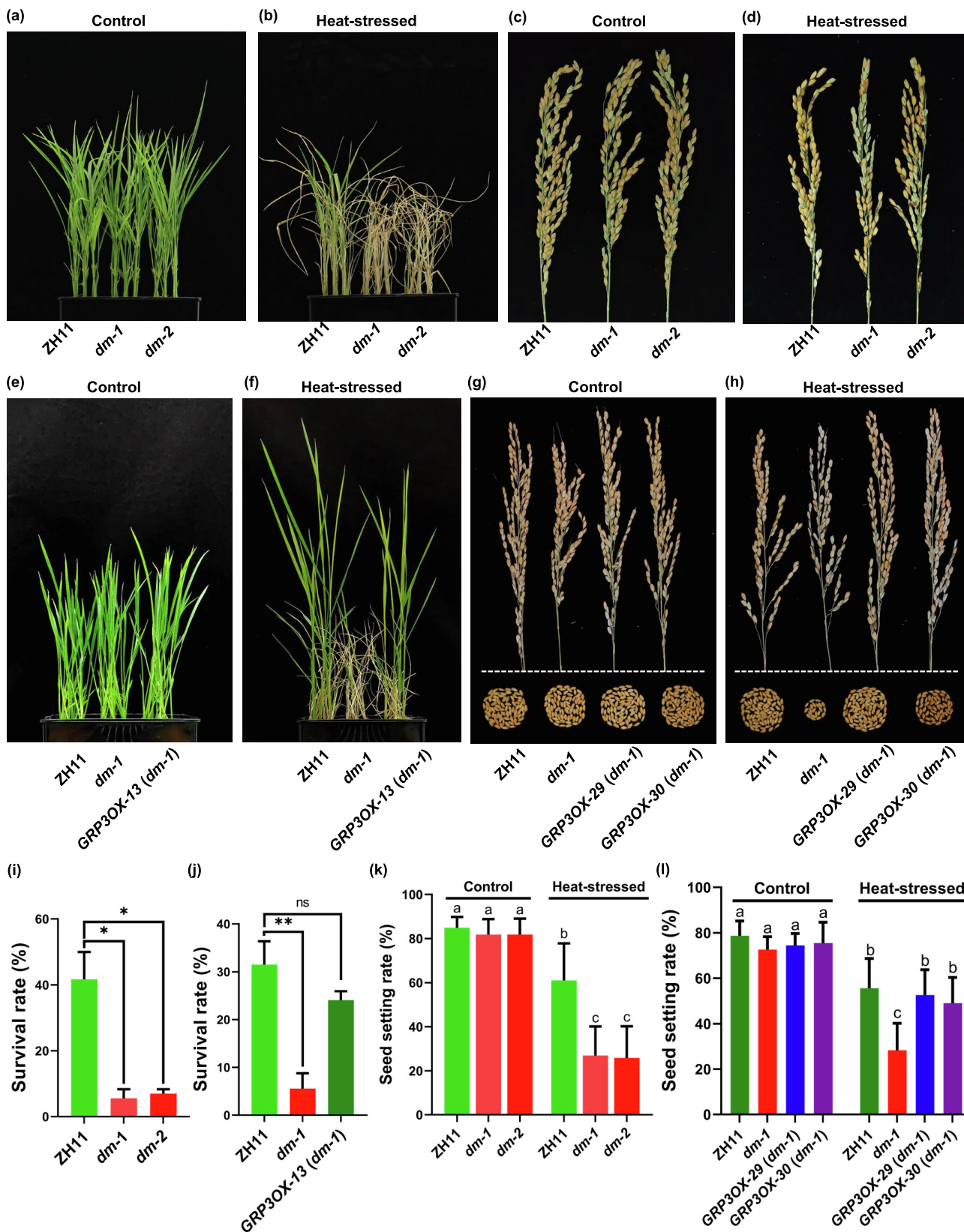
**Fig. 1.** EC controls the rhythmically expression of *OsGRP3* and *OsGRP162* at high temperatures. (a, b) Up-regulation of *OsGRP3*/*OsGRP162* (a) and down-regulation of *OsELF3-2* (b) by heat stress at midnight. (c) Increased diurnal expression of *OsGRP3*/*OsGRP162* at high temperatures in *elf3-2* mutant plants. Seven-day-old wild-type (ZH11), mutant plants of *OsELF3-1* (*elf3-1*) or *OsELF3-2* (*elf3-2*) plants grown consistently at 29 °C were kept at 29 °C or transferred to 45 °C, and total seedlings were sampled every 6 h at indicated zeitgeber time (ZT, X-axis) for RT-qPCR analysis. Relative expression is the expression level of *OsGRP3* or *OsGRP162* or evening complex (EC) genes at different sampling time normalized to that at ZT 0 h at 29 °C, both of which were normalized to that of *PP2A*.  $n = 3$ . Open and closed bars given in each panel represent light and dark periods, respectively. (d) Effector-reporter assays. Schematic design for the effectors and reporters is shown in which *OsLUX*/*OsEC2* driven by the CaMV 35S constitutive promoter linked with a native or mutated form (m) of LBS sequence derived from the *OsGRP3*/*OsGRP162* promoter was used as a reporter. Renilla luciferase driven by the CaMV 35S promoter in the same reporter vector was used as an internal control. Different combination of vectors was transiently expressed in tobacco leaves. Relative luciferase activity is the firefly luciferase activity normalized to the Renilla luciferase activity, which was then normalized to the empty vector control (MYC).  $n = 5$  leaf discs. Different letters above the bars indicate significant differences for comparisons between two groups as determined by analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test ( $P < 0.05$ ), and the same letters indicate no significant differences between two groups. Error bars depict standard error (SE).

panicle were similar between ZH11 and *grp3-1 grp162-1/grp3-2 grp162-2* plants, and 1000-grain weight was slightly reduced in *grp3-1 grp162-1/grp3-2 grp162-2* plants comparing to that of ZH11 plants after heat stress (Fig. S5 online). Therefore, *OsGRP3* and *OsGRP162* are required for heat stress tolerance at reproductive stage.

To further confirm the function of *OsGRP3* and *OsGRP162* in thermotolerance, we constitutively expressed *OsGRP3* or *OsGRP162* in the *grp3-1 grp162-1* double mutant background, and performed phenotypic analysis at seedling or reproductive stages. We treated these *OsGRP3/162* overexpression plants and ZH11 plants with heat stress similarly as mentioned above, and found that survival rate and seed setting rate of the *grp3-1 grp162-1* double mutant were more reduced by heat stress than that of the ZH11 plants as observed before, in contrast, overexpression of either *OsGRP3* or *GRP162* rescued the *grp3-1 grp162-1* mutant phenotype at seedling stage (Fig. 2e–f, 2j and Fig. S4d–k online) and at reproductive stage (Fig. 2g, h and 1l). Taken together, we conclude that *OsGRP3* and *OsGRP162* are required for thermotolerance in terms of survival rate and seed setting rate in rice.

#### 3.4. *OsGRP3*/*OsGRP162* are involved in diurnal regulation of thermotolerance

To investigate whether *OsGRP3*/*OsGRP162* contribute to diurnal regulation of heat-stress tolerance, we examined the *grp3-1 grp162-1/grp3-2 grp162-2* double mutant responses to timed heat pulses, either during daytime or at nighttime. We firstly checked the heat-stress tolerance response between daytime and nighttime with ZH11 and the double mutant of *OsGRP3*/*OsGRP162* at reproductive stage. As previously reported [6], wild-type rice (ZH11) were more sensitive to heat stress in terms of seed setting rate when high temperature occurred at nighttime than that at daytime (Fig. 3a–c). We reduced the heat stress period so that under such conditions, the seed setting rate of ZH11 was similar between under warm-day (38 °C in the light for 12 h and 30 °C in the dark for 12 h) and warm-night (30 °C in the light for 12 h and 38 °C in the dark for 12 h) conditions (Fig. 3d). In agreement with aforementioned results, the *grp3-1 grp162-1* and *grp3-2 grp162-2* plants were more sensitive to heat stress than ZH11 plants in terms of seed setting rate and grain weight per panicle (Fig. 3d, e). Impor-



tantly, the seed setting rate and grain weight per panicle of *grp3-1 grp162-1/grp3-2 grp162-2* plants was more reduced by heat stress when heat stress occurred at nighttime than that at daytime (Fig. 3d, e). The number of spikelets per panicle and thousand grain weight were not much affected by heat stress (Fig. 3e), because these traits are genetically determined before or after the developmental stage when heat stress occurred in our study. We also checked vegetative seedling growth under high temperature in the daytime and at nighttime for the same period of time (12-h light/12-h dark photoperiod), and the results showed that the difference between *grp3-1 grp162-1/grp3-2 grp162-2* and ZH11 plants in terms of plant height and fresh/dry weight was only observed when high temperature (38 °C, 7 d) was applied at nighttime (Fig. S6 online). Thus, *OsGRP3/OsGRP162* are involved in diurnal regulation of thermotolerance in rice.

### 3.5. Mutation of *OsGRP3/OsGRP162* affects heat stress responsive gene expression

To probe the underlying mechanism of *OsGRP3/OsGRP162* in regulation of the heat-stress tolerance, we carried out RNA-seq analysis. For this we transferred seven-day-old *grp3-1 grp162-1* and ZH11 seedlings grown at 29 °C to 29 °C and 45 °C, and collected seedling samples at ZT 18 h, respectively, for RNA extraction and RNA-seq. Besides the commonly regulated genes by heat stress, we identified 744 and 713 genes that were specifically up-regulated and down-regulated, respectively, in ZH11 plants (Fig. 4a, b). In contrast, there were 844 and 735 genes that were specifically up-regulated and down-regulated, respectively, in *grp3-1 grp162-1* plants (Fig. 4a, b).

Gene ontology (GO) enrichment analysis revealed that among the specifically up-regulated genes in ZH11, a number of stress responsive processes, such as “response to heat”, “response to hydrogen peroxide”, “response to unfolded protein”, “response to water deprivation” were enriched; among the specifically up-regulated genes in *grp3-1 grp162-1* plants, GO terms related to RNA processing and modification, including “group II intron splicing”, “U5 snRNA binding”, “U2 snRNA binding”, “polycistronic mRNA processing” were found to be enriched (Fig. 4a and Fig. S7 online). In comparison, among the specifically down-regulated genes in ZH11, GO terms such as “photosynthesis”, “light reaction”, “amino acid transmembrane transporter activity” and “protein metabolic process”; were enriched, among the specifically down-regulated genes in *grp3-1 grp162-1*, GO terms, such as “transporter activity”, “regulation of stomata movement”, “adenylsulfate kinase activity” were enriched (Fig. 4a and Fig. S8 online). To validate the differential expression results obtained from the RNA-seq, we selected four heat shock transcription factor genes and four heat shock protein genes and performed qRT-PCR. The results showed that all of these genes were highly up-regulated by heat stress in ZH11 plants, but they were little up-regulated by heat stress in *grp3-1 grp162-1* plants (Fig. 4c). Together, these results support

that mutation of *OsGRP3/OsGRP162* globally affects the expression of heat stress responsive genes.

### 3.6. *OsGRP3/OsGRP162* regulate alternative splicing under heat stress conditions

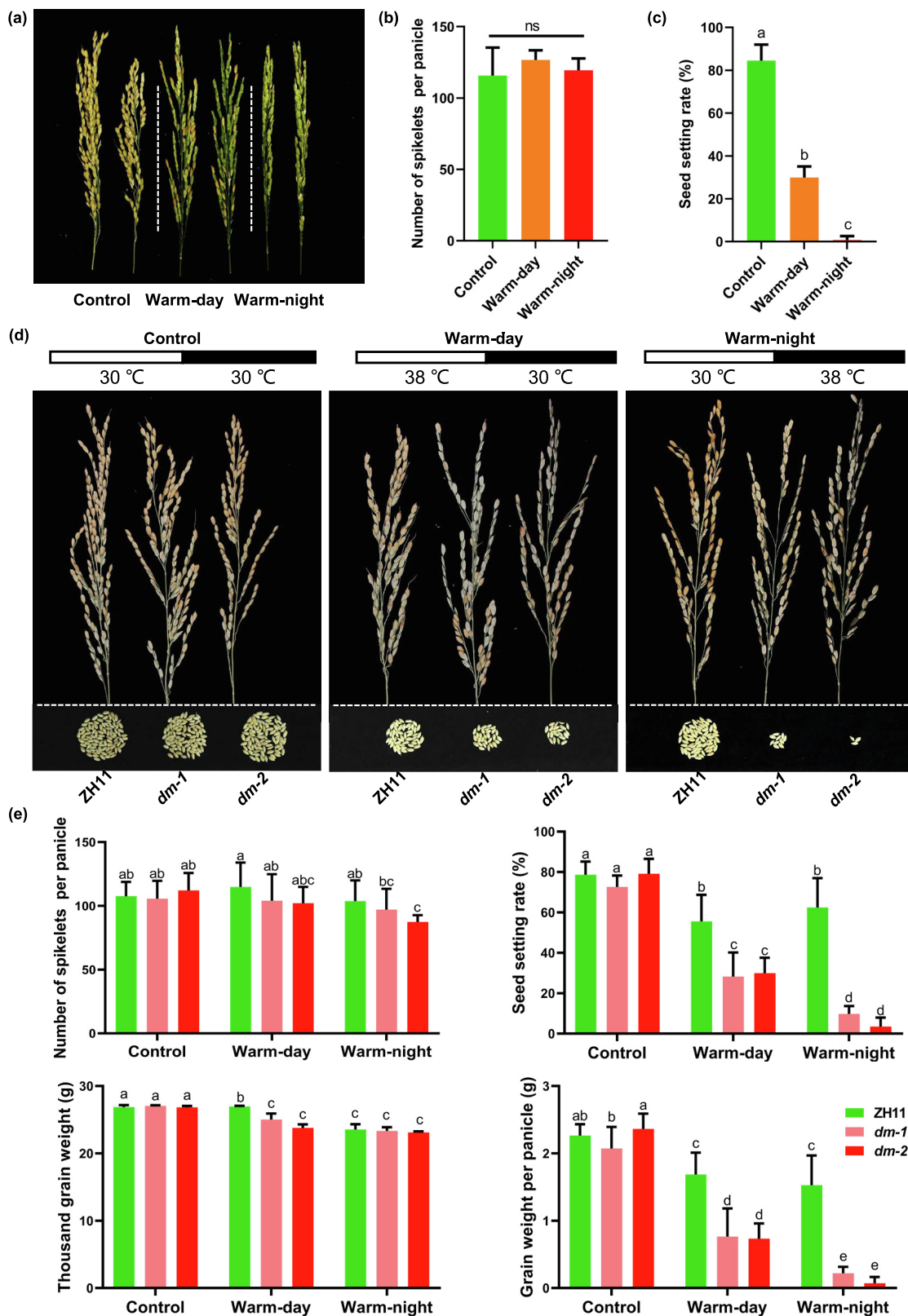
Alternative splicing (AS) generates multiple transcripts from the same gene, and it is markedly induced in response to heat stress in plants [20]. Since GO term of “RNA processing” was enriched in the genes that specially up-regulated by heat stress in *grp3-1 grp162-1* plants, we examined AS events in ZH11 and *grp3-1 grp162-1* plants grown under two different temperature conditions. In total 45,991 to 50,255 AS events were observed in these experiments (Fig. S9a online). Notably, there were 453 AS events (corresponding to 410 genes) induced by heat stress in ZH11 plants while there were 1095 AS events (corresponding to 880 genes) induced by heat stress in *grp3-1 grp162-1* plants (Fig. 5a and S9b online), in which 681 genes were specifically induced with AS events by heat stress in *grp3-1 grp162-1* plants (Fig. S9b online), and they were enriched in “RNA binding”, “chloroplast”, et al. (Fig. S9c online). Among different types of AS events, the number of exon-skipping (ES) events was dramatically differed between ZH11 (206 ES events corresponding to 197 genes) and *grp3-1 grp162-1* plants (622 ES events corresponding to 543 genes) (Fig. 5b, c and S10a online), in which 448 genes were specifically induced with ES events by heat stress in *grp3-1 grp162-1* plants (Fig. S10a online). Further analysis of the differentially skipped exon sequences showed that one common motif “WUSUUG” was significantly ( $E = 6.2 \times 10^{-110}$ ) overrepresented (Fig. S10b). GO and KEGG enrichments showed that these genes were enriched in “RNA binding”, “mRNA processing”, “chloroplast”, “metallo-endopeptidase activity”, “aspartate family amino acid biosynthetic process”, “homoserine dehydrogenase activity”, “threonine biosynthetic process”, “carotenoid biosynthetic process”, “plastid-encoded plastid RNA polymerase complex”, “spliceosome”, et al. (Fig. S10c, d online).

To validate the differential AS results obtained from the RNA-seq, we selected nine genes including the key abiotic stress transcriptional regulators *OsDREB2B* and *OsHsfA2c* from the above-mentioned differential ES events in *grp3-1 grp162-1* plants, and checked the level of different spliced transcripts by RT-qPCR in ZH11 and *grp3-1 grp162-1* plants under two temperature conditions. The results showed that the level of ES transcripts of all of these nine genes was higher in *grp3-1 grp162-1* plants than that in ZH11 plants at 45 °C (Fig. 5d). These results support that *OsGRP3/OsGRP162* regulate alternative splicing, especially ES events, under heat stress conditions.

### 3.7. *OsGRP3/OsGRP162* bind to target mRNAs and interact with U1/U2 components of the spliceosome

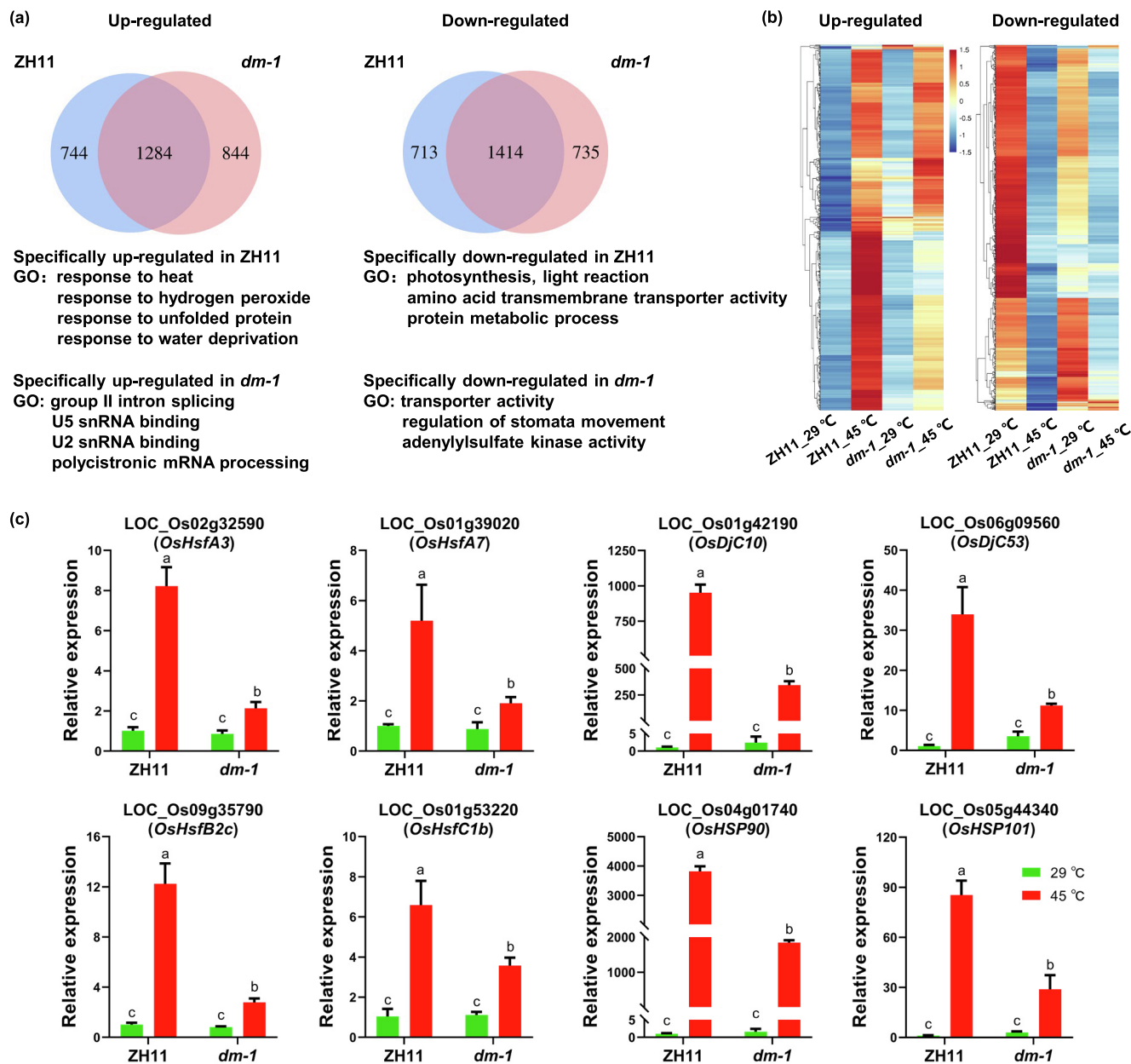
To clarify the molecular mechanism by which *OsGRP3/OsGRP162* regulate AS, we firstly checked the subcellular localiza-

**Fig. 2.** *OsGRP3* and *OsGRP162* are required for thermotolerance in rice. (a–l) Phenotypic analysis of the double mutant of *OsGRP3/OsGRP162* and the overexpression plants of *OsGRP3* at seedling stage (a, b, e, f, i, and j) and reproductive stage (c, d, g, h, k, and l). For survival test, seven-day-old wild-type (ZH11) and two double mutant plants *grp3-1 grp162-1 (dm-1)* and *grp3-2 grp162-2 (dm-2)*, and *OsGRP3* overexpression plants (*GRP3OX-13*) in *dm-1* background grown at 29 °C were subjected to heat stress (45 °C) treatment for 3 d and then recovered at 29 °C for 14 d, and plants were photographed and survival rate was calculated. For thermotolerance test at reproductive stage, the above-mentioned plants grown at 30 °C were subjected to heat stress at flowering (38 °C in the light for 12 h and 30 °C in the dark for 12 h) for 3 d, and then recovered at 30 °C until maturity, and panicles and filled grains per panicle were photographed, seed setting rate was measured. The control plants were constantly placed at normal growth temperature. Error bars represent SE,  $n = 3$  in i, j and  $n = 9$  in k, l. Asterisks indicate significance levels when comparing to ZH11 in Student's *t*-test. (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, not significant). (k, l) Different letters above the bars indicate significant differences for comparisons between two groups as determined by analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test ( $P < 0.05$ ), and the same letters indicate no significant differences between two groups.



**Fig. 3.** OsGRP3 and OsGRP162 regulate diurnal thermotolerance in rice. (a–c) Heat stress sensitivity is differed between daytime and nighttime in rice. Wild-type (ZH11) plants grown at 30 °C were subjected to heat stress (38 °C) at flowering for 7 d, and then recovered at 30 °C until maturity. Heat stress was set in warm-day (38 °C in the light for 12 h and 30 °C in the dark for 12 h) and warm-night (30 °C in the light for 12 h and 38 °C in the dark for 12 h), respectively. Panicles were photographed, and agronomic traits were measured. (d, e) Diurnal regulation of thermotolerance by OsGRP3/OsGRP162. ZH11 and two lines of *OsGRP3/OsGRP162* double mutant plants *grp3-1 grp162-1* (*dm-1*) and *grp3-2 grp162-2* (*dm-2*) grown at 30 °C were subjected to heat stress (38 °C) at flowering for 3 days, and then recovered at 30 °C until maturity. The bars depict SE ( $n = 9$ ). Open and closed bars given in each panel represent light and dark periods. Different letters above the bars indicate significant differences for comparisons between two groups as determined by analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test ( $P < 0.05$ ), and the same letters indicate no significant differences between two groups.

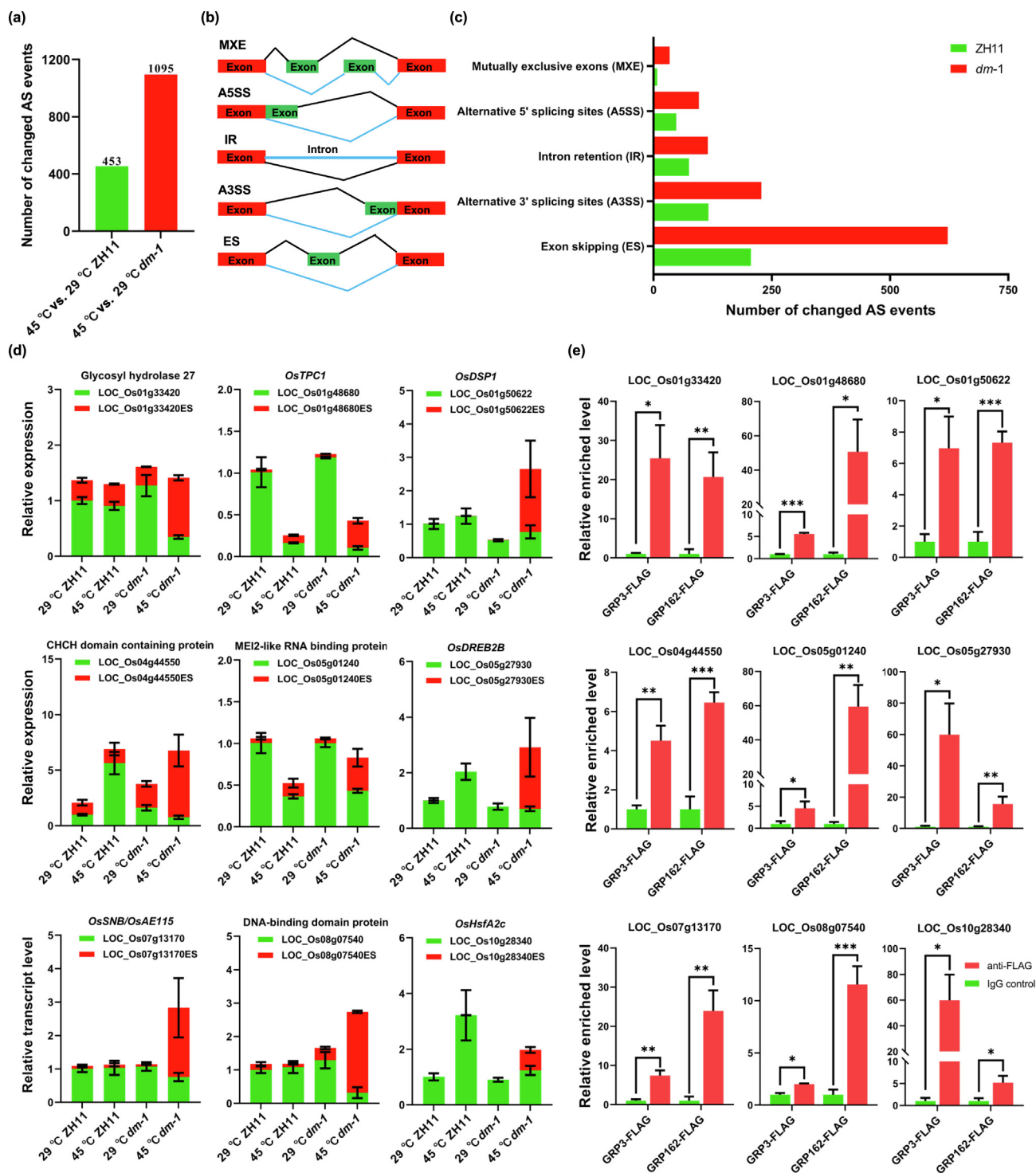




**Fig. 4.** Mutation of *OsGRP3* and *OsGRP162* impairs the expression of heat stress responsive genes. (a, b) Venn diagrams (a) and heat maps (b) showing differential regulation of genes by heat stress in wild-type (ZH11) and the *OsGRP3/OsGRP162* double mutant *grp3-1 grp162-1* (*dm-1*), respectively. Seven-day-old rice seedlings were subjected to heat stress at 45 °C for 18 h and gene expression were checked by RNA-seq analysis. Up-regulation was set as  $P \leq 0.05$ , fold change (FC)  $\geq 2$  and down-regulation was set as  $P \leq 0.05$ ,  $FC \leq 0.5$  (a). The full list of GO enrichment is presented in Fig. S4 and Fig. S5 (online). The expression level of ZH11-specific up-regulated (744) and down-regulated (713) genes was shown in heat maps (b). (c) Validation of *OsGRP3/OsGRP162*-dependent gene expression under heat stress conditions by RT-qPCR. Relative expression is the expression level of target gene normalized to that in ZH11 at 29 °C, both of which were normalized to that of *PP2A*. Error bars represent SE,  $n = 3$ . Different letters above the bars indicate significant differences for comparisons between two groups as determined by analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test ( $P < 0.05$ ), and the same letters indicate no significant differences between two groups.

tion of *OsGRP3/OsGRP162*. C-terminal YFP fusion proteins of *OsGRP3/OsGRP162* were mainly found in nuclei and co-localized with the nuclear marker in rice protoplasts (Fig. 6a), which is agreed with their roles in AS. We then performed RNA immunoprecipitation (RIP) assays coupled with qPCR, with *OsGRP3/OsGRP162* overexpression plants driven by the constitutive ubiquitin promoter, and found that above mentioned nine *OsGRP3/OsGRP162*-regulated RNAs were enriched when *OsGRP3/OsGRP162* were immunoprecipitated (Fig. 5e). These results demonstrate that *OsGRP3/OsGRP162* bind to target mRNAs.

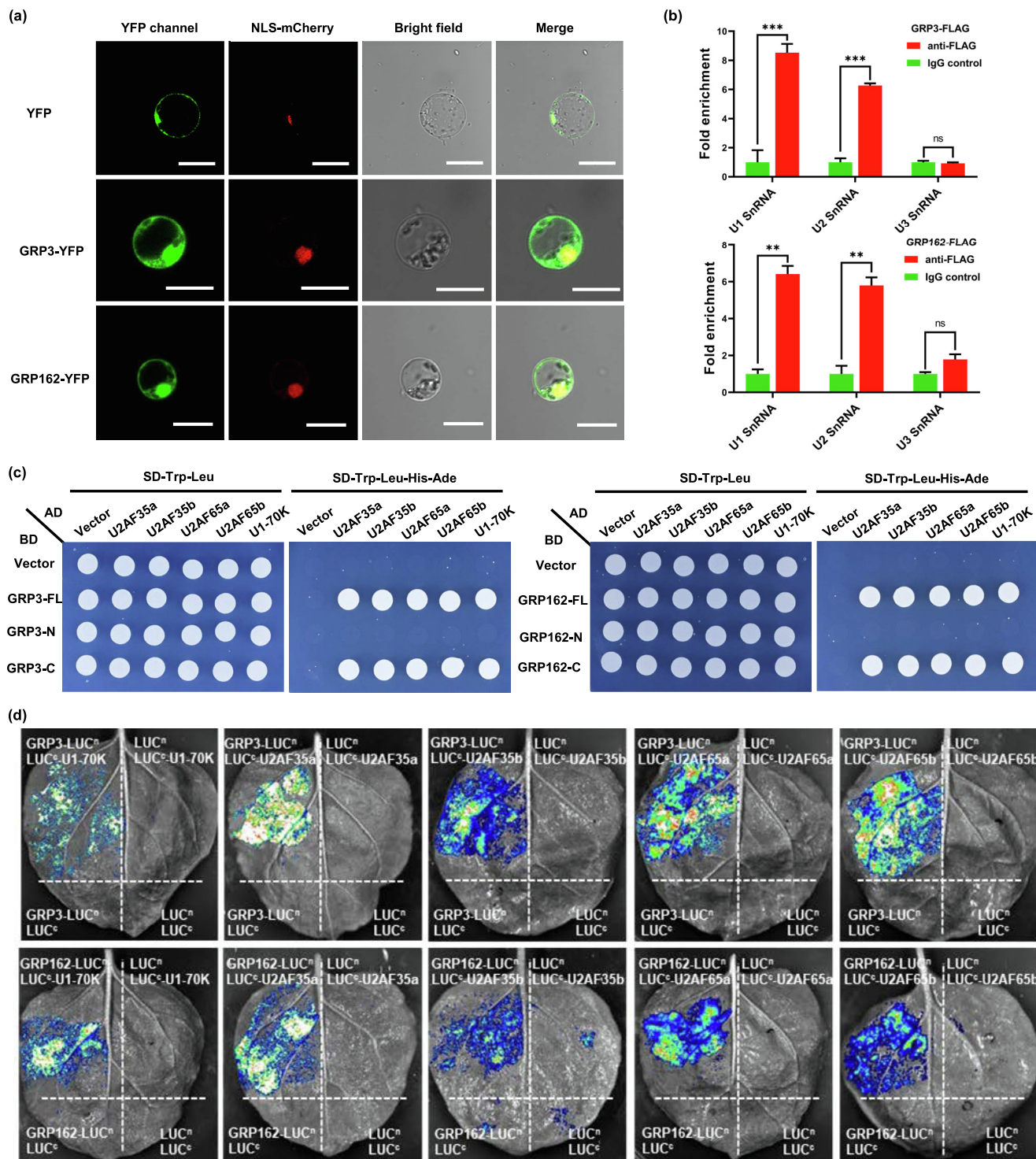
The core spliceosome consists of five small nuclear ribonucleoproteins (SnRNPs) and numerous spliceosome-associated proteins assembled in sequential order [21]. As *OsGRP3/OsGRP162* are RNA binding proteins, we next checked their association of with SnRNAs of SnRNPs and spliceosomal protein factors. RIP-qPCR analysis showed that *OsGRP3/OsGRP162* was associated with U1 and U2 SnRNA, but not U3 SnRNA (Fig. 6b). Yeast two-hybrid assays showed that C-terminal regions of *OsGRP3/OsGRP162* interacted with the small and large subunits of U2 auxiliary factor (U2AF), *OsU2AF35a/b*, *OsU2AF65a/b*, respectively, and the U1



**Fig. 5.** OsGRP3 and OsGRP162 regulate alternative splicing under heat stress conditions. (a–c) Differential alternative splicing (AS) induced by heat stress between wild-type (ZH11) and the *OsGRP3/OsGRP162* double mutant *grp3-1 grp162-1* (*dm-1*). Total number of changed AS events is shown in (a), AS events are grouped into five major types (b) and the number of changed AS events in each group is shown in (c). (d) Validation of exon skipping events by RT-qPCR. Seven-day-old ZH11 and *dm-1* plants were subjected to heat stress at 45 °C for 18 h and the expression of exon-skipped (ES) and non-skipped isoforms were checked by RT-qPCR analysis. (e) *In vivo* binding of OsGRP3/OsGRP162 to target RNAs. Transgenic rice plants constitutively overexpressing *OsGRP3-FLAG/OsGRP162-FLAG* were harvested for RIP-qPCR using anti-FLAG antibody. Relative enrichment level is the RNA level of each anti-FLAG sample normalized to that of the IgG control sample, both of which were normalized to that of the input control. Error bar represents SE,  $n = 3$ . Asterisks indicate significance levels between each comparison in Student's *t*-test. (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

SnRNP 70K (OsU1-70K) (Fig. 6c), but not with OsPRP18 (Fig. S11 online), a protein required for the 2nd reaction in pre-mRNA splicing [22]. The interaction between OsGRP3/OsGRP162 and U1/U2

subunits of the spliceosome was further confirmed in split luciferase assays in tobacco (*Nicotiana benthamiana*) plants (Fig. 6d) and in pull-down assays (Fig. S12 online). Together, these results



**Fig. 6.** OsGRP3 and OsGRP162 interact with U1/U2 components of the spliceosome. (a) Subcellular localization analysis of GRP3/GRP162. The YFP-tagged OsGRP3/OsGRP162 were co-transformed with the nuclear marker (NLS-mCherry) in rice protoplasts and observed under the confocal microscope. Bar = 50  $\mu$ m. (b) Association of OsGRP3/OsGRP162 with U1/U2 SnRNA. Transgenic rice plants overexpressing *OsGRP3-FLAG/OsGRP162-FLAG* grown at 29 °C were harvested and RIP-qPCR was performed using anti-FLAG antibody. Relative enrichment level is the RNA level of each anti-FLAG sample normalized to that of the IgG control sample, both of which were normalized to that of the input control. Error bar represents SE,  $n = 3$ . Asterisks indicate significance levels between each comparison in  $t$ -test (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , ns, not significant). (c) Yeast two-hybrid assays. The full-length (FL), N-terminal fragment and C-terminal fragment of OsGRP3/OsGRP162 were fused with the DNA-binding domain (BD) of GAL4, while the full-length of U1 (OsU1-70 K), and U2 (OsU2AF35a, OsU2AF35b, OsU2AF65a, and OsU2AF65b) of the spliceosomal components were fused with the activation domain (AD) of GAL4, respectively. Activation of the histidine biosynthetic gene *HIS3* or the AIR-carboxylase *ADE2* in the purine biosynthetic pathway was used for protein–protein interaction markers. SD, synthetic dropout medium. (d) Split-luciferase assays. OsGRP3/OsGRP162 were fused with the N-terminal (LUC<sup>n</sup>), and U1/U2 spliceosomal components were fused to the C-terminal (LUC<sup>c</sup>) portion of firefly luciferases, respectively. Empty vectors were used as controls. Different combinations of constructs were expressed in tobacco leaves and chemiluminescence was detected.

demonstrate that OsGRP3/OsGRP162 directly bind to target mRNAs and are involved in AS through interacting with U1/U2 spliceosomal factors.

#### 4. Discussion

Warm temperatures as night have detrimental effects on plant growth and development, leading to global yield reduction, but the underlying mechanisms are still poorly understood. Previous studies showed that warm nighttime temperature globally disrupts the timing of gene expression in natural field conditions [23]. However, the key regulators controlling diurnal thermotolerance under extreme high temperature conditions has not yet been demonstrated in plants. The circadian clock integrates environmental signals and coordinates plant growth and development throughout the day and night [24]. In this study, we showed that OsELF4a-OsELF3-3-OsLUX complex (OsEC2) represses the promoter activity of *OsGRP3/OsGRP162* in effector-reporter assays. *OsELF3-2* is rhythmically expressed at midnight, with its expression level down-regulated by heat stress, leading to the up-regulation of *OsGRP3/OsGRP162* at midnight. Given that the *OsGRP3/OsGRP162* double mutant plants are more sensitive to heat stress when the stress occurs in nighttime than that in daytime, we conclude that *OsGRP3/OsGRP162* are required for diurnal regulation of thermotolerance in rice.

The EC is highly conserved in plants and is a critical component within circadian clock. It is consisted of ELF3, ELF4, and LUX and well-conserved in plants [9,10]. The rice genome encodes two ELF3 paralogues, namely OsELF3-1 and OsELF3-2. Previous studies have shown that OsELF3-1 plays a predominant role in controlling photoperiodic flowering and repressing phytochrome B signaling in the daytime [25,26], while OsELF3-2 is involved in plant immunity [17]. There is redundancy between them, as the double ELF3 mutant in rice has more extreme phenotypes than either single [27]. In that the EC directly suppresses the expression of flowering genes such as OsPRR37 and OsGhd7 [27]. Previously, *OsELF4a* expression was shown to be up-regulated by salt stress and the OsELF4a-OsELF3-1-OsLUX complex (OsEC1) represses the expression of *OsGI* to modulate salt stress tolerance and heading date in rice [28]. In the current study, we showed that OsELF3, especially OsELF3-2, regulates the diurnal expression of *OsGRP3/OsGRP162* under heat stress conditions to modulate thermotolerance in rice, emphasizing its role in circadian regulation of stress tolerance (Fig. 7a). Because the *OsELF3-1* and *OsELF3-2* double mutant has a severe phenotype (never flowered) [27], we did not examine the expression of *OsGRP3/OsGRP162* in *OsELF3-1* and *OsELF3-2* double mutant plants.

In plants, alternative splicing (AS) is widespread and induced by abiotic stresses [29]. For example, under normal temperature growth conditions, *HSFA2d* is mainly spliced into the inactive form HSFA2dII; under heat stress conditions, *HSFA2d* is alternatively spliced into the active form HSFA2dI to activate downstream heat stress responsive genes in rice [30]. The molecular mechanism underlying AS regulation under heat stress conditions is little understood in plants. In contrast to constitutively splicing, AS is usually acts in competition with at least one other 5' or 3' splice site [31]. Therefore, in addition to the classical core components of spliceosome consisting of five snRNPs and numerous spliceosome-associated proteins which assemble in a sequential order [21]; other RNA-binding proteins, serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) are also required for specifically recognizing and binding to the *cis*-regulatory elements located in either introns or exons collectively known as splicing regulatory elements (SREs) [29]. For example, AtRS40/AtRS41/AtSR45a regulate salt stress responses

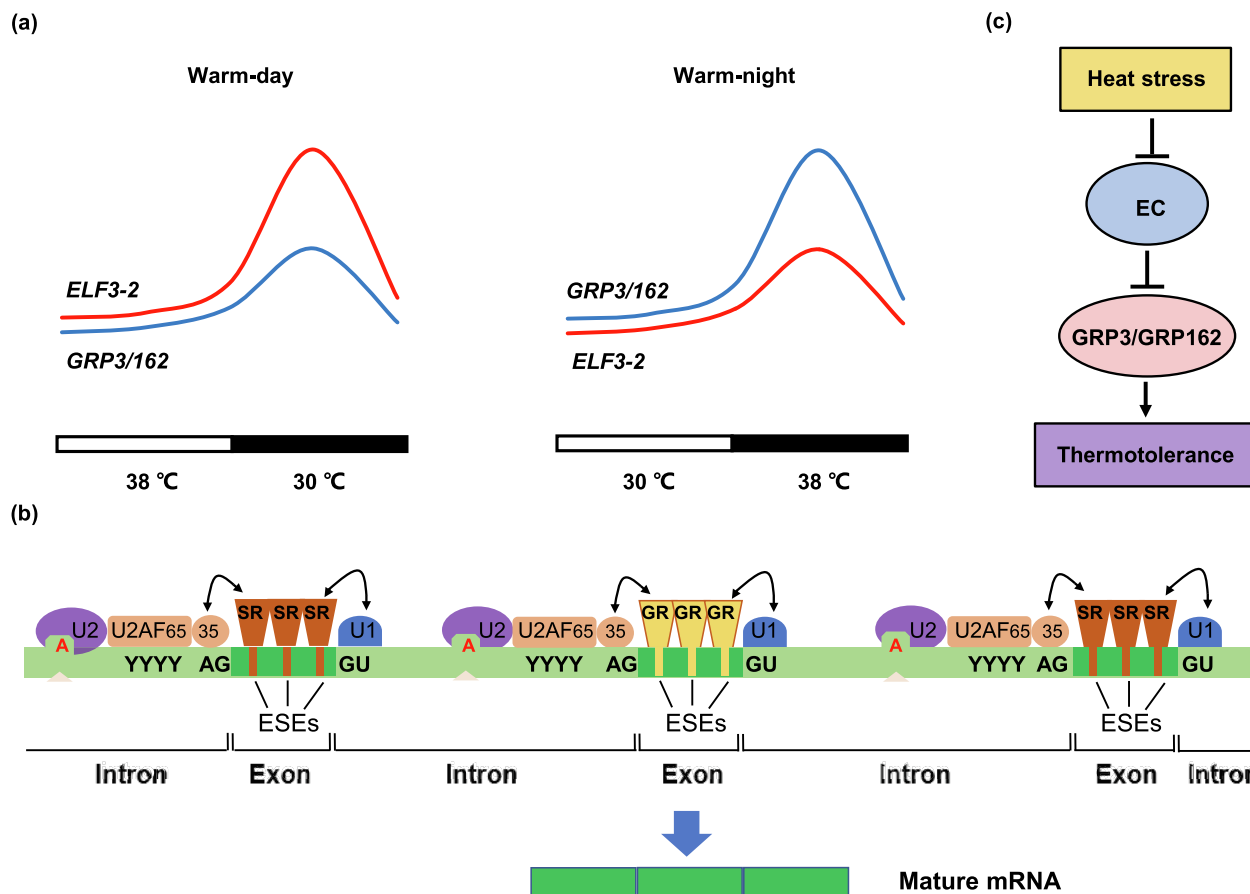
by regulating AS in *Arabidopsis* [32,33]. In contrast, hnRNP-like glycine-rich proteins AtGRP7/AtGRP8 bind to target mRNA and affect in particular the choice of alternative 5' splicing sites in *Arabidopsis* [34,35]. In the past decades, circadian-controlled AS has been suggested to regulate plant fitness under adverse environmental conditions [36], however, the underlying molecular mechanism are not much explored. In our study, we revealed that two glycine-rich proteins OsGRP3/OsGRP162 regulate AS under heat stress conditions, integrating circadian clock information and high temperature signals into mRNA alternative splicing and heat stress tolerance.

Unlike that exon skipping is the major type in animals and human, intron retention is the prevalent AS events in wild-type plants under normal growth conditions [31]. Interestingly, exon skipping events are predominantly found in the *OsGRP3/OsGRP162* double mutant plants, especially under heat stress conditions, suggesting that *OsGRP3/OsGRP162* prevent exon skipping at high temperature in rice. The *cis*-acting SREs can be classified as exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs) [37]. In our working model (Fig. 7b), *OsGRP3/OsGRP162* directly bind to ESEs presented on the exons of targeting mRNAs, and interact with U1/U2 components of the spliceosome in early steps for enhancing exon inclusion (preventing exon skipping) in WT plants under heat stress conditions. In the *grp3-1 grp162-1* mutant plants, due to the defect in exon inclusion, exon skipping is prevalent, in which SR proteins recognize ESEs and interact with U1/U2 components, stimulating splicing and joining two adjacent exons under heat stress conditions. We do not exclude the possibility that *OsGRP3/OsGRP162* play multiple roles during AS, since other types of AS events are also found in the heat-stressed double mutant of *OsGRP3/OsGRP162*. Nevertheless, our results demonstrate that *OsGRP3/OsGRP162* are important for AS under heat stress conditions in rice.

Being sessile, plants have evolved multiple strategies to cope with the changing environmental temperature conditions, ranging from signaling, gene regulation and physiological responses [38–41]. Heat shock transcription factors (HSFs) are widely recognized as the central regulators of heat shock proteins (HSPs) to boost the cytosolic protein folding machinery in plants under heat stress conditions [42]. Recently, we identified a plasma membrane-localized NAC transcription factor OsNTL3 that relocates to nucleus under heat stress conditions to up-regulate the expression of genes involved in protein folding in endoplasmic reticulum [15]. In the current study, we demonstrate that *OsGRP3/OsGRP162* are important not only for plant survival at seedling stage, but also for yield production at reproductive stage. Several transcription factors including HSFs are differentially spliced in *grp3-1 grp162-1* mutant plants, which may lead to impaired protein folding under heat stress conditions. Indeed, the expression level of many heat-responsive HSFs and HSPs are reduced in *grp3-1 grp162-1* mutant plants at high temperatures. Taken together, the current results support our working model that heat stress down-regulates the level of ELF3-2/EC to release the inhibitory effects of EC and promote diurnal expression of *OsGRP3/OsGRP162* (Fig. 7a); *OsGRP3/OsGRP162* regulate diurnal thermotolerance through controlling mRNA alternative splicing, predominantly preventing exon skipping under heat stress conditions (Fig. 7b, c). Since plant growth and yield production are constantly subjected to environmental stresses under natural conditions, the current study sheds light on how circadian clock regulates diurnal thermotolerance in plants.

#### Conflict of interest

The authors declare that they have no conflict of interest.



**Fig. 7.** Working models for the function of *OsGRP3* and *OsGRP162* in diurnal thermotolerance. (a) Diurnal expression of *OsGRP3/OsGRP162* regulated by *OsELF3-2*. Under warm day conditions, the expression of *OsGRP3/OsGRP162* (blue line) is largely suppressed by EC at midnight because *OsELF3-2* (red line) is highly expressed at this timepoint. Under warm night conditions, the expression of *OsELF3-2* is down-regulated by heat stress, lifting the expression level of *OsGRP3/OsGRP162* at midnight. (b) Promoting exon inclusion by *OsGRP3/OsGRP162* under heat stress conditions. Serine-rich (SR) proteins bind to exonic splicing enhancers (ESEs) and interact with U1/U2 components to stimulate RNA splicing and joining two adjacent exons, in contrast, glycine-rich (GR) proteins *OsGRP3/OsGRP162* bind to ESEs and interact with U1/U2 components to promote exon inclusion (prevent exon skipping) in rice under heat stress conditions. (c) Diurnal regulation of thermotolerance by *OsGRP3/OsGRP162*. Heat stress rhythmically down-regulates the expression of *OsELF3*, one of the EC components, to release the inhibitory effect of EC on the expression of *OsGRP3/OsGRP162*, leading to rhythmical expression peak of *OsGRP3/OsGRP162* at midnight (ZT 18 h) and diurnal thermotolerance in rice.

## Acknowledgments

This work was supported by the State Key Project of Research and Development Plan of China (2022YFF1001603 and 2021YFF1000404), and the Natural Science Foundation of Zhejiang, China (LD21C020001). We would like to thank Drs. Chang-Ying Wu and Yue-Se Ning for kindly providing the rice *elf3-1* and *elf3-2* mutant seeds.

## Author contributions

Jian-Xiang Liu and Chuang Yang designed the experiments; Chuang Yang, Anni Luo and Hai-Ping Lu performed the experiments; Jian-Xiang Liu and Chuang Yang analyzed the data; Jian-Xiang Liu, Chuang Yang and Seth Jon Davis wrote the paper.

## Appendix A. Supplementary materials

Supplementary materials to this article can be found online at <https://doi.org/10.1016/j.scib.2023.11.046>.

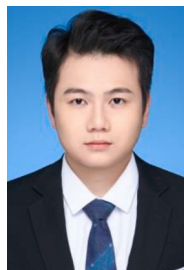
## Data availability

The RNA-seq data is deposited in the Gene Expression Omnibus (GEO) under accession number GSE222671.

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