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OsEIN2-OsEIL1/2-OsNAP module regulates rice thermotolerance and leaf senescence

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Abstract

High temperature and leaf senescence are critical factors affecting the growth, development, yield and quality of rice. Ethylene signaling plays an essential role in plant responses to various stresses and leaf senescence. Here, we report that the master components of ethylene signaling, OsEIN2 and OsEIL1/2, regulate rice thermotolerance and leaf senescence at the seedling stage by activating *OsNAP* expression. Physiological analyses showed that OsEIN2 and OsEIL1/2 triggered excessive ROS accumulation, chlorophyll degradation, and severe cell death under heat stress and dark treatment. Transcriptomic analyses revealed that many genes regulated by OsEIL1 under high temperature were involved in ROS homeostasis, leaf senescence and stress response. Our analyses revealed that OsEIL1/2 could directly transcriptionally activate OsNAP-mediated rice thermosensitivity and leaf senescence. Moreover, we found that OsEIL1/2 repressed the expression of *OsEIL5*, a positive regulator of thermotolerance, implying that different EIN3-like homologs might have antagonistic effects on rice response to heat stress. Taken together, our results provide new insights into the OsEIN2-OsEIL1/2-*OsNAP* module involved in regulating rice thermotolerance and leaf senescence, and offer genetic resources for developing heat-tolerant rice cultivars.

1 Introduction

Heat stress is one of the major abiotic stresses associated with climate warming, severely restricting plant growth and development (Zhao et al. 2017). Rice (*Oryza sativa* L.), a staple cereal crop for more than half of the world's population, is sensitive to heat stress. High temperature affects multiple cellular and physiological processes such as membrane dysfunction, protein denaturation, nucleotide damage, metabolic imbalances, excessive accumulation of reactive oxygen species (ROS), chlorophyll degradation, and cell death in rice (Parankusam et al. 2017, Vu et al. 2019). Rice heat response is a complex trait governed by multiple genetic factors. Recently, increasing thermotolerance-related QTLs and candidate genes such as *TT1/2/3* (Li et al. 2015, Kan et al. 2022, Zhang et al. 2022). *OsCBL8* and *OsCIPK17* (Gao et al. 2022), *OsU2AF35a* (Liu et al. 2025b), *Q12* (Li et al. 2025), *NAT1*, *bHLH110* and *CER1* (Lu et al. 2025) have been identified, providing genetic resources for cultivating heat-resistant rice varieties.

Leaf senescence is a pivotal developmental program and an adaptive response to external stimuli, that is typically induced by various stresses and severely affects crop yield and quality (Leng et al. 2017, Lee and Masclaux-Daubresse 2021). In rice, *OsNAP*, *PGL*, *OsPLS4, OsCNGC14/16*, *PSL50* and *PWL1* participate in leaf senescence and tolerance to extreme temperatures (Liang et al. 2014, Yang et al. 2016, Cui et al. 2020, Zhou et al. 2020, He et al. 2021, Xu et al. 2023). Ethylene signaling is essential for the induction of leaf senescence (Qiu et al. 2015, Schippers et al. 2015, Wang et al. 2018). In rice, *SUB1A* and *OsRTH1* delay leaf senescence by repressing the ethylene signal (Fukao et al. 2012, Zhang et al. 2012). OsEIN2 and OsEIL2 promote leaf senescence (Ma et al. 2013, Jin et al. 2020), but their regulatory mechanism were unclear.

Upon sensing environmental stimuli, plants rapidly trigger elaborate hormone signaling networks to cope with various stresses (Verma et al. 2016, Castroverde and Dina 2021). The components of ethylene-signaling pathway such as EIN2, EIN3 and EIN3-LIKE (EIL) play central roles in the regulation of plant development and stress responses (Zhao et al. 2021). In *Arabidopsis*, EIN2, EIN3 and EIL1 positively regulate plant thermotolerance (Huang et al. 2021). In rice, OsEIN2, OsEIL1 and OsEIL2 are essential for the regulation of biotic stresses and abiotic stresses (such as high salinity, drought, and cold) (Yang et al. 2015, Yang et al. 2017, Jin et al. 2020, Ma et al. 2020, Zhao et al. 2022, Zhai et al. 2024). However, their role in rice thermotolerance has not been reported.

Recent study documented that OsEIL5 positively regulated rice heat tolerance by activating the ER stress resistance (Liu et al. 2025a). Here, we found that both ethylene precursor (1-Amino-1-cyclopropanecarboxylic acid, ACC) and ethylene signal components (OsEIN2, OsEIL1 and OsEIL2) negatively regulated rice thermotolerance. At the transcriptional level, OsEIL1/2 could directly activate *OsNAP*, which was key regulators of leaf senescence and thermotolerance by triggering oxidative stress and chlorophyll degradation. Moreover, we confirmed that OsEIL1/2 could repress *OsEIL5* expression, a positive regulator of rice thermotolerance, suggesting that multiple homologs of EIL have different roles in rice response to heat stress. Taken together, our results enrich the molecular mechanism of ethylene signaling in the regulation of rice thermotolerance and leaf senescence, providing useful genetic resources for developing stress-tolerant rice.

2 Results

2.1 OsEIN2 and OsEIL1/2 negatively regulates rice thermotolerance by triggering oxidative stress and cell death

Ethylene and its signaling pathway play central regulatory roles in plant response to various abiotic stresses. Here, we first explored the potential role of ethylene in rice response to heat stress using ACC. When 2-week-old seedlings were subjected to heat stress and followed by recovery, all seedlings treated with ACC showed more severe stress symptoms with leaf curling and withering than those without ACC treatment (Fig. 1A), suggesting that ACC reduced rice thermotolerance. Then, we confirmed that OsEIN2, OsEIL1 and OsEIL2, negatively regulated rice thermotolerance. When 2-week-old plants were exposed to heat stress and followed by recovery, almost all the overexpressing plants (*OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE) showed severe stress symptoms with more leaf yellowing and withering and ultimately died compared with Nip, while the mutant (*osein2* and *oseil1*) and RNA interference (*OsEIL2*-Ri) plants displayed slight stress symptoms and recovered normal growth (Fig. 1B/C). We further confirmed that ACC aggravated stress symptoms of *OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE plants compared with Nip plants, while its effect on *osein2*, *oseil1* and *OsEIL2*-Ri plants was relatively weakened. In contrast, the ethylene biosynthesis inhibitor (aminoethoxyvinyl glycine, AVG) dramatically could enhanced the heat tolerance of all plants, especially in *OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE plants (Fig. S1A /B). These

results indicate that ethylene and its signaling components might negatively regulate rice thermotolerance.

Heat stress usually induces excessive accumulation of ROS and oxidative damage in plants. We assessed H_2O_2 accumulation in different materials by DAB staining and quantitative measurement. There was no obvious difference among all materials under normal conditions. Under heat treatment, the H_2O_2 accumulation in the leaves of *osein2*, *oseil1* and *OsEIL2*-Ri was less than that in Nip, but greater in *OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE (Fig. 1D/E). Under normal condition, there was no significant difference in cell death and cell damage among all materials. After treated by heat stress, the leaves of *OsEIN2-OE*, *OsEIL1*-OE and *OsEIL2*-OE exhibited more intensive cell death detected by trypan blue staining and higher electrolyte leakage than those of Nip, but less intensive and lower in *osein2*, *oseil1* and *OsEIL2*-Ri (Fig. 1F/G), indicating that OsEIN2 and OsEIL1/2 reduced rice thermotolerance by triggering ROS accumulation, severe oxidative injury and cell death under heat stress.

2.2 OsEIN2-OsEIL1/2 cascade accelerates leaf senescence under heat stress

OsEIN2 and OsEIL2 promote leaf senescence, but their regulatory mechanism is unclear (Ma et al. 2013, Jin et al. 2020). Here, we confirmed that OsEIN2, OsEIL1 and OsEIL2 accelerated dark-induced leaf senescence under normal condition. After plants exposed to darkness, *OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE plants showed severe leaf yellowing and even death compared with Nip, while *osein2*, *oseil1* and *OsEIL2*-Ri displayed the senescence symptoms to a minor extent only (Fig. 2A). Consistently, the dark-induced ROS accumulation and cell death in *OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE lines were more than those in Nip, but less in *osein2*, *oseil1* and *OsEIL2*-Ri (Fig. 2B).

Heat sensitivity was associated with leaf senescence in rice (Xu et al. 2023). Here, we verified that OsEIN2 and OsEIL1/2 promoted the senescence under heat stress. After exposure to heat for 6 hours, the detached leaves were subjected to darkness. The leaves of *OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE treated by heat started to show obvious yellowing symptoms on the 4nd day compared with those of Nip and their controls. On the 6th day, all the leaves treated by heat showed more severe yellowing symptoms than their controls. In particular, the leaves of all overexpressing plants treated by heat were completely yellowed, whereas those of *osein2*, *oseil1* and *OsEIL2*-Ri only showed slight yellowing (Fig. 2C). These results suggested that *OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE promoted leaf senescence under normal condition and heat stress.

2.3 OsEIL1 transcriptionally regulates many genes involved in ROS homeostasis and leaf senescence under heat stress

To characterize transcriptional responses to heat stress mediated by ethylene signal in rice seedlings, we performed RNA-seq analysis on the leaves of *OsEIL1*-OE, *oseil1* and NiP seedlings under heat stress. Compared with Nip, total 3876 differentially expressed genes (DEGs) were identified, consisting of

2598/576 upregulated and 1092/198 downregulated DEGs in *OsEIL1*-OE/*oseil1*, respectively (Table S1). Among of them, the representative Gene Ontology (GO) categories were the transcriptional regulation and oxidoreductase activities for their molecular functions, and mainly involved in biological processes in response to various stresses and stimulus (Fig. S2). We further analyzed genes associated with ROS metabolism (ROS), leaf senescence (LS) and heat shock transcription factor (HSF) in these DEGs by tree view (heat map), totally 32 genes were subjected to heat map analysis (Fig. 3A). Several candidate genes *OsEBP89* (Zhang et al. 2020), *HSFs*, peroxidase genes (*OsPRXs*), *OsERF101* (Lim et al. 2020), *OsNAC131* and *OsNAP* (Zhou et al. 2013, Liang et al. 2014) were validated by RT-qPCR (Fig. 3B). These results indicate that OsEIL1 affect rice thermotolerance partially by transcriptionally regulating ROS homeostasis and leaf senescence.

2.4 OsEIN2 and OsEIL1/2 trigger OsNAP mediated leaf senescence and thermosensitivity

Previous study showed that OsNAP was a key regulator of rice leaf senescence (Zhou et al. 2013, Liang et al. 2014). Based on the RNA-seq analysis, we further confirmed that *OsNAP* was upregulated in *OsEIN2-OE*, *OsEIL1-OE* and *OsEIL2*-OE lines under normal condition, but downregulated in *osein2*, *oseil1* and *OsEIL2*-Ri (Fig. 4A). Predictive analysis using PlantPAN 3.0 showed that there were twelve putative EIN3-binding sites (EBSs) in *OsNAP* promoter (2 kb upstream of the start codon) (Fig. 4B and Fig. S3A). The Y1H and EMSA assays verified that OsEIL1 and OsEIL2 could directly bind to the P5 fragment of *OsNAP* promoter (Fig. 4B/C). Further ChIP-qPCR analysis validated that the P5 fragment was significantly enriched by GST-OsEIL1 and MBP-OsEIL2 against GST and MBP, respectively. The negative control, P4 fragment, could not be enriched by OsEIL1 and OsEIL2 (Fig. 4D). These results indicate that OsEIL1/2 can directly activate *OsNAP* expression.

The obvious symptom of leaf senescence is yellowing due to the chlorophyll degradation (Iqbal et al. 2017). *OsEIN2* and *OsEIL1/2* could induce rice leaf senescence under normal condition and heat stress (Fig. 2). At the transcriptional level, expression of *OsNAP* and its targeted genes (*OsI57*, *OsI85* and *OsSGR)* could be activated by OsEIN2 and OsEIL1/2 (Fig. 5A). Consistent with these, we found that the accumulations of total chlorophyll, chlorophyll a and b in *osein2*, *oseil1* and *OsEIL2*-Ri were significantly higher than those in Nip after darkness, but lower in *OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE (Fig. 5B), suggesting that OsEIN2 and OsEIL1/2 might affect rice thermotolerance partially by activating OsNAP mediated chlorophyll degradation and leaf senescence.

To check the effect of OsNAP on rice thermotolerance, we generated the knockout mutants of *OsNAP* (*osnap*) with frameshift mutations using CRISPR-Cas9 (Fig. S4). After 2-week-old plants treated by heat stress, two *osnap* lines showed better growth status with less withering and yellowing leaves than Nip. After recovered growth, almost all Nip plants died, while most *osnap* plants were still alive, suggesting that *OsNAP* negatively affects rice thermotolerance (Fig. 5C). Taken together, our results indicate that OsNAP is a key downstream regulator of OsEIN2-OsEIL1/2 cascade mediated leaf senescence and thermosensitivity.

2.5 OsEIL1/2 might antagonize *OsEIL5* mediated rice thermotolerance

Another EIL homolog, OsEIL5, positively regulates rice thermotolerance by activating *OsPP91* and multiple ER stress response related genes such as *OsUbc46*, *ZFP* (zinc-finger protein), and *DDC* (dimerization domain containing protein) (Liu et al. 2025a). Here, we found that the expression of *OsEIL5*, *OsUbc46*, *ZFP*, and *DDC* were downregulated in *OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE lines, but upregulated in *osein2*, *oseil1* and *OsEIL2*-Ri lines (Fig. 6A). Predictive analysis showed that there several EBSs in *OsEIL5* promoter (Fig. S3B). Further Y1H and EMSA analyses verified that OsEIL1/2 could bind the specific EBS present in the P3 fragment (Fig. 6B/C), indicating that OsEIL1/2 might transcriptionally repress *OsEIL5* expression and show antagonistic effect on rice thermotolerance.

3 Discussion

Heat stress is a serious threat to the global crop production. Rice (*Oryza sativa* L.) is susceptible to high temperature, especially at the seedling stage, reproductive stage and grain-filling stage. High temperature usually causes delayed growth, reduced tiller and plant height, abnormal seed development, and decreased grain yield and quality in rice (Shrestha et al. 2022, Li et al. 2023). Plant response to heat stress is a complex process governed by multiple intrinsic genetic factors, hormone signals and environmental stimuli (Sharma et al. 2020, Chen et al. 2022). Thus, clarifying the regulatory mechanisms of rice thermotolerance will help us develop stress-tolerant varieties to cope with severe climate challenges.

As a gaseous hormone, ethylene plays dual roles in plant response to environmental stresses. In *Arabidopsis*, ethylene and its signaling components positively regulate its tolerance to heat, cold, drought, and high salinity, while their role in rice is opposite to that in *Arabidopsis* (Shi et al. 2012, Ma et al. 2013, Yang et al. 2013, Yang et al. 2015, Lee et al. 2016, Yang et al. 2017, Jin et al. 2020, Ma et al. 2020, Huang et al. 2021, Zhai et al. 2024). Moreover, except a canonical and conserved ethylene signaling pathway shared in *Arabidopsis* and rice, an alternative ethylene receptor-mediated phosphor-relay pathway has been reported in rice (Zhang et al. 2026), suggesting that different plants have evolved distinct adaptation mechanism to their respective environments in a species-dependent manner mediated by ethylene signal. Ethylene and its signal participate in the control of thermotolerance and leaf senescence in different plants (Poor et al. 2022, Zhang et al. 2026). In *Arabidopsis*, AtEIN2 and AtEIN3 were reported to positively regulate heat stress tolerance and leaf senescence (Zhang et al. 2026), but the related report in rice is little. In this study, we confirmed that exogenous ethylene precursor (ACC) and ethylene biosynthetic inhibitor (AVG) resulted in the decreased and increased rice thermotolerance, respectively. Consistently, OsEIN2 and OsEIL1/2 negatively regulated thermotolerance (Fig. 1A/B and Fig.S1). These results suggest that ethylene and its signal have a negative regulatory effect on the temperature tolerance of rice.

Heat stress induces a series of changes including hormone signal, gene transcriptional expression, excessive ROS accumulation, disordered photosynthesis, stunted growth and development, and even death (Wang et al. 2024). Our results showed that OsEIN2 and OsEIL1/2 could trigger ROS accumulation, cell damage and chlorophyll degradation under heat stress and darkness (Fig. 1C-F, Fi.2B and Fig. 5A) by transcriptionally regulated many genes involved in ROS homeostasis and leaf senescence (Fig. 3), ultimately promoted leaf senescence and thermosensitivity.

Leaf senescence is an important developmental process and physiological event, and closely associated with rice tolerance to high salinity, drought, heat stress (Sakuraba et al. 2015, Huang et al. 2021, Xu et al. 2023). OsEIN2 and OsEIL2 were reported to promote leaf senescence in rice (Ma et al. 2013, Jin et al. 2020), but their regulatory mechanism is unclear. Here, we confirmed that OsEIN2 and OsEIL1/2 accelerated leaf senescence under normal condition and heat stress by activating *OsNAP*, a positive regulator of rice leaf senescence (Liang et al. 2014). Our analysis showed that knocking out *OsNAP* conferred rice with the enhanced tolerance to heat stress, suggesting it is a negative regulator of thermotolerance (Fig. 5B). At the transcriptional level, OsEIN2 and OsEIL1/2 could activate *OsNAP* and its target genes, which might be responsible for the excessive ROS accumulation, chlorophyll degradation, and leaf senescence under dark and heat stress. Moreover, OsEIL1/2 could directly target *OsNAP* by binding the specific EBS within its promoter (Fig. 4). These results demonstrate that OsNAP function as a downstream regulator of OsEIN2-OsEIL1/2 signaling pathway mediating rice thermotolerance and leaf senescence.

Unlike OsEIL1 and OsEIL2, OsEIL5 was reported to positively regulate rice thermotolerance (Liu et al. 2025a). In this study, we found that OsEIN2 and OsEIL1/2 repressed expression of *OsEIL5* and its regulated genes (*OsUbc46*, *ZFP*, and *DDC)* (Fi. 6A). At the transcriptional level, OsEIL1/2 could directly target *OsEIL5* by binding the specific EBS within its promoter (Fig. 6B), indicating that different EIN3 homologs have their unique effects on rice response to heat stress. Thus, we speculated that OsEIL1/2 might antagonize OsEIL5 effect on rice tolerance to heat stress by transcriptionally repressing its expression, and form a subtle regulatory network to balance rice thermosensitivity. Moreover, it has been reported that EIN3 mediates transcriptional repression through interacting with different transcriptional repressors in *Arabidopsis* (Liu et al. 2017, Wang et al. 2020). Thus, to identify OsEIL1/2 interacting proteins will be helpful to enrich their molecular mechanisms involved in regulating plant growth and development and stress responses.

Based on our results, we propose a regulatory model illustrating the role of the OsEIN2-OsEIL1/2 pathway in rice thermotolerance and leaf senescence (Fig. 7). Once sensing the heat stress, the signal was transduced by OsEIN2-OsEIL1/2 pathway rapidly and initiates a series of transcriptional reprograming of many genes involved in ROS homeostasis and leaf senescence such as *OsNAP*, which then triggers excessive ROS accumulation, chlorophyll degradation and oxidative damage, ultimately promoting rice thermosesnsitivity and leaf senescence.

4 Materials and methods

4.1 Plant materials and growth conditions

Seeds of *Oryza sativa* L. ssp. japonica cv. Nipponbare (Nip), overexpressing lines (*OsEIN2*-OE, *OsEIL1*-OE and *OsEIL2*-OE), the knockout mutants (*osein2* and *oseil1*) and knockdown of *OsEIL2* (*OsEIL2*-Ri) were described previously (Ma et al. 2013, Yang et al. 2015). *osnap* lines were generated by CRISPR-Cas9. The germinated seeds were cultured in Hoagland Solution or soil in green house.

4.2 Heat and dark treatment

For heat treatments, 2-week-old plants were subjected to 45°C for 2 days and then recovered under normal conditions, and their stress symptoms were photographed. The leaves treated for different time courses were harvested for physiological indices and gene expression.

For the analysis of leaf yellowing, after recovery from heat treatment, the etiolated leaf area exceeding 50% was determined as yellow leaves, and a yellow leaf proportion was calculated as the number of yellow leaves/total number of leaves *100%.

For dark-induced leaf senescence, 2-week-old plants were subjected to darkness at 28°C for 8 days. For the detached dark-induced-leaf senescence, after 2-week-old plants were treated by heat for 6 hours, the detached leaves were incubated on 3 mM MES buffer in darkness at 28°C for 4 days. The senescence symptoms were photographed.

4.3 RNA extraction and quantitative real-time PCR

Total RNA from different tissues were extracted using RNAiso Plus (Takara, 9108), 1 μg Total RNA was reversely transcribed into cDNA with HiScript III RT SuperMix for qPCR (Vazyme, R323-01). Quantitative real-time PCR (qRT-PCR) was performed with a Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Q712-03) on a CFX96 real-time PCR system (Bio-Rad) using specific primers (Table S2).

4.4 Histochemical assays

The DAB and trypan blue staining were performed as described previously (Shen et al. 2015). Briefly, leaves from 2-week-old plants treated by heat (45℃ for 12 hours) were collected and stained in DAB solution (1 mg/ml, 50 mM Tris-HCl, pH = 3.8) for ten hours. The stained leaves washed with 95% ethanol were photographed. For trypan blue staining, the leaves from 2-week-old plants treated by heat (45℃ for 24 hours) were collected and boiled in solution (0.1 mg/ml trypan blue, lactic acid: phenol: glycerol: H_2O = 1:1:1:1) for 10 min, after transferred to chloral hydrate (25 mg/ml) solution for 3 days, the blue spots were photographed.

4.5 H_2O_2 content measurement

The H_2O_2 content of leaves without or with heat treatment (45℃ for 12 hours) were measured using H_2O_2 content Kit (Suzhou Comin Biotechnology Company, H_2O_2-1-Y) based on the manufacturer's instruction. More than three biological replicates were performed.

4.6 Electrolyte leakage and chlorophyll measurement

Electrolyte leakage was measured with modification. Leaves of 2-week-old rice seedlings were harvested and immersed in 10 ml of deionized water, shaking at room temperature for 24 hours, then measured the electrical conductivity (C1) and the total conductivity (C2) after boiling for 30 minutes, the electrical conductivity of deionized water was defined as C0. The electrolyte leakage was calculated as (C1-C0)/(C2-C0)*100%. At least three replicates were performed.

For chlorophyll content measurement, 2-week-old plants with or without dark treatment were harvested for chlorophyll detection using a chlorophyll content determination kit (Suzhou Comin Biotechnology Company, CPL-1-G) based on the manufacturer's instruction. At least three replicates were performed.

4.7 Yeast one-hybrid assay

The full-length cDNA of OsEIL1 and OsEIL2 were cloned into the prey vectors pB42AD. Fragments containing EBS elements from *OsNAP* and *OsEIL5* promoters were fused into the bait vector pLacZi, respectively. The prey and the bait vectors were co-transformed into the EGY48 yeast strain, and the empty pB42AD and pLacZi vectors were used as negative controls. The transformed yeasts were grown on SD selective medium (SD/-Ura/-Trp) with X-Gal at 30°C for 3–5 days, and the blue coloration was photographed.

4.8 Electrophoretic mobility shift assay

A recombinant protein GST-OsEIL1 and MBP-OsEIL2 were generated according to Zhai et al. The biotin-3' labeled and unlabeled DNA fragments of promoters were synthesized by Biomed company (Table S1). The DNA probes and proteins were co-incubated using a Chemiluminescent EMSA Kit (Beyotime Biotechnology, GS009) according to the manufacturer's instructions.

4.9 Semi-in vivo chromatin immunoprecipitation (ChIP)

In vitro ChIP was performed as described previously (Li et al. 2017). The genomic DNA of 3-week-old Nip seedlings was sonicated to 100–500 bp fragments. DNA fragments were co-incubated with glutathione agarose Resin-GST-OsEIL1, glutathione agarose Resin-GST, amylose beads-MBP-OsEIL2 and amylose beads-MBP for 4 hours. After the beads were washed and broken down, the enriched DNA fragments were eluted for ChIP-qPCR analysis with specific primers (Table S1).

4.10 ACC and AVG treatment

Rice seeds were grown on normal hydroponic cultivation, and transferred to hydroponic cultivation with different concentrations of ACC and AVG at 10-day-old for heat treatment.

4.11 Transcriptome analysis

Two-week-old rice leaves with heat treatment (45°C for 6h) were collected and subjected to transcriptome analysis with three biological replicates. cDNA library construction and RNA sequencing

were performed by Bluescape Scientific CO (Beijing, China). Clean reads (after removing adaptor and low-quality reads) were mapped to the rice genome MSU7.0 using TopHat and analyzed using Cufflinks (Trapnell et al. 2012). The criteria for differentially expressed genes (DEGs) were $P < 0.05$ and fold change > |1|. GO enrichment analysis of DEGs was performed by clusterProfiler R package (v4.2.2).

4.12 Statistical analysis

Mean values and standard deviations were calculated using the SPSS System (IBM SPSS Statistics 19). Student's t test was used for comparing two datasets, and one-way ANOVA with a Tukey's multiple range test was performed for multiple comparisons. Quantification analyses of all the measurements were conducted in GraphPad Prism 8.

Declarations

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Founding

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Z H, P X and X X planned and designed the projects. Z M, X K, C Y, and J X performed the experiments and analyzed the data. Z M, P X, X X and Z H wrote the paper. All authors read and commented on the final manuscript.

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Figures

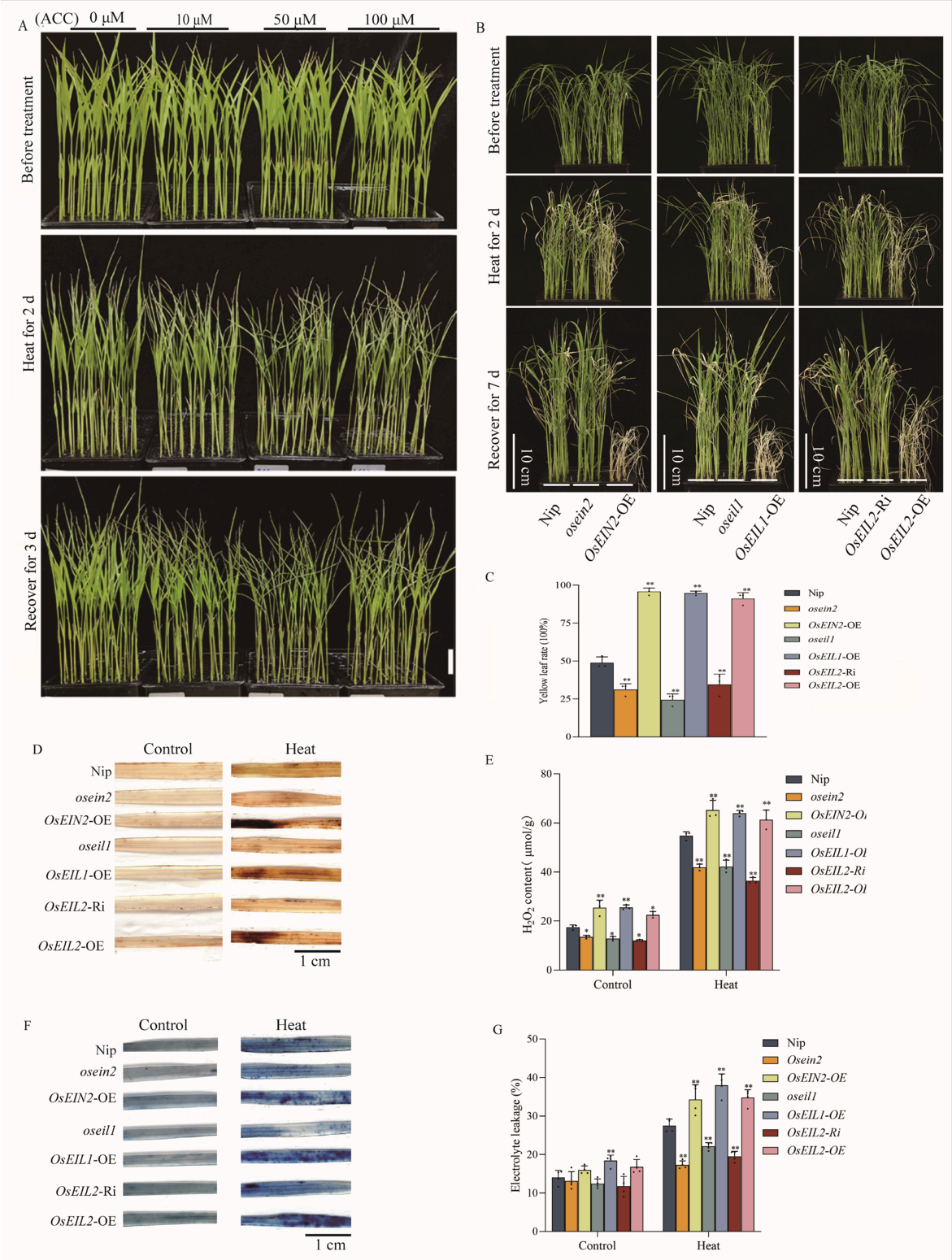


Figure 1

OsEIN2 and *OsEIL1/2* negatively regulate rice tolerance to heat.

(A) Thermotolerance phenotypes of Nip under 0, 50 μM and 100 μM ACC treatment. 10-day-old seedlings subjected to 42°C for 2 days and followed by a recovery growth for 3 days. Bars =2.5 cm. (B) Thermotolerance phenotypes of *OsEIN2*-OE, *osein2*, *OsEIL1*-OE, *oseil1*, *OsEIL2*-OE, *OsEIL2*-Ri, and the wild

type (Nip). Two-week-old seedlings were treated at 45°C for 2 days, followed by a recovery growth for 7 days. Bars = 10 cm. (C) Proportion of yellow leaves after recovery. Data means ± SD for three biological replicates (*n* = 16 for each replicate). Asterisks indicate significant differences by the Student's *t*-test (*P < 0.05, **P < 0.01). (D) DAB staining of leaves for checking H_2O_2 under heat and normal conditions. Bar = 1 cm. (E) Relative H_2O_2 content in leaves under normal and heat stress. Data are the means ± SD of three replicates. Asterisks indicate significant differences by the Student's *t*-test (*P < 0.05, **P < 0.01). (F) Trypan blue staining of leaves for detecting cell death. Bar = 1 cm. (G) Electrolyte leakage of the leaves under normal and heat stress. Data are the means ± SD of three replicates. Asterisks indicate significant differences by the Student's *t*-test (*P < 0.05, **P < 0.01).

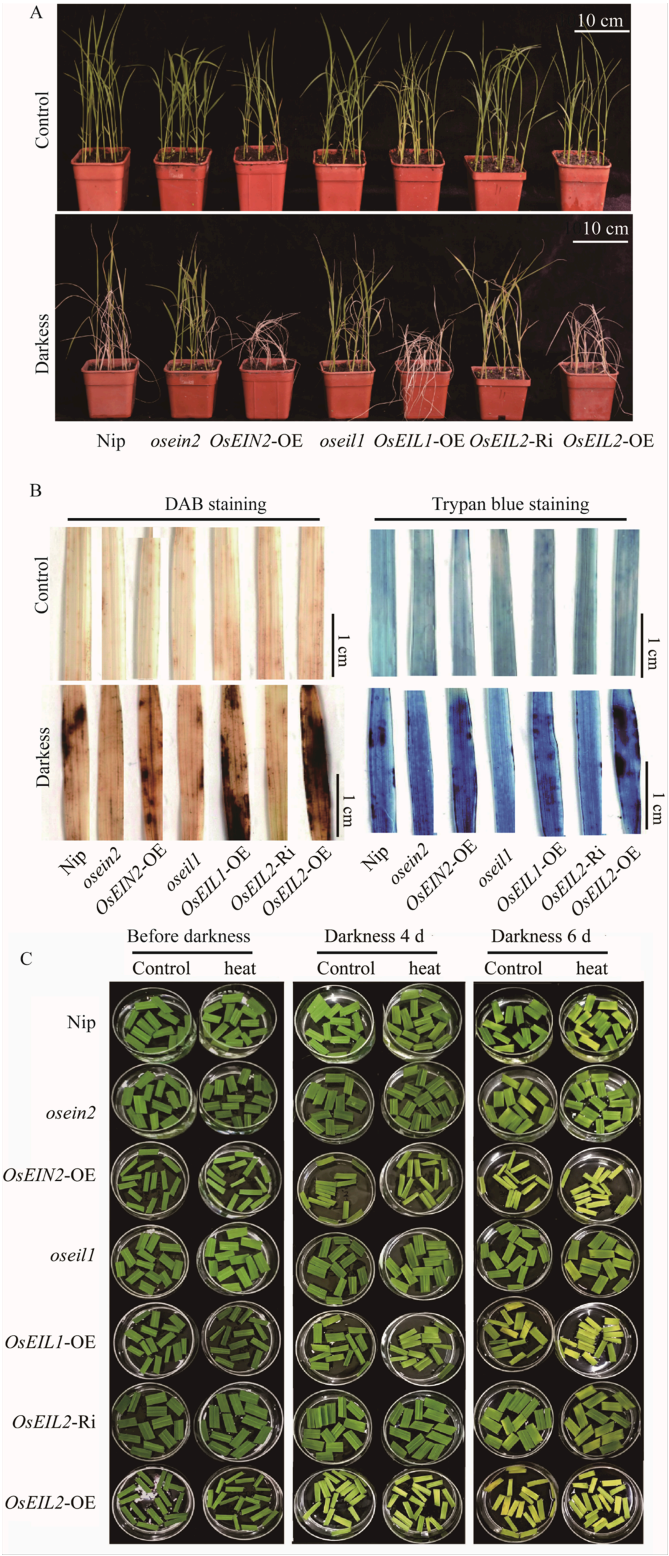


Figure 2

OsEIN2 and OsEIL1/2 accelerate leaf senescence under heat.

(A) Leaf senescence phenotypes of *OsEIN2*-OE, *osein2*, *OsEIL1*-OE, *oseil1*, *OsEIL2*-OE, *OsEIL2*-Ri, and the wild type (Nip). The 2-week-old seedlings were subjected to darkness for 6 days to induce leaf senescence. Bar = 10 cm. (B) Two-week-old seedlings were treated by darkness for 3 days, H_2O_2

accumulation and cell death were detected by DAB staining and trypan blue staining, respectively. Bar = 1 cm. (C) OsEIN2 and OsEIL1/2 accelerate leaf senescence under heat. After 3-week-old seedlings treated by heat (45℃ for 6 h), the detached leaves from all materials were incubated on 3 mM MES buffer in darkness at 28°C for 4 days. The phenotypes were photographed on the 2nd and 4th day.

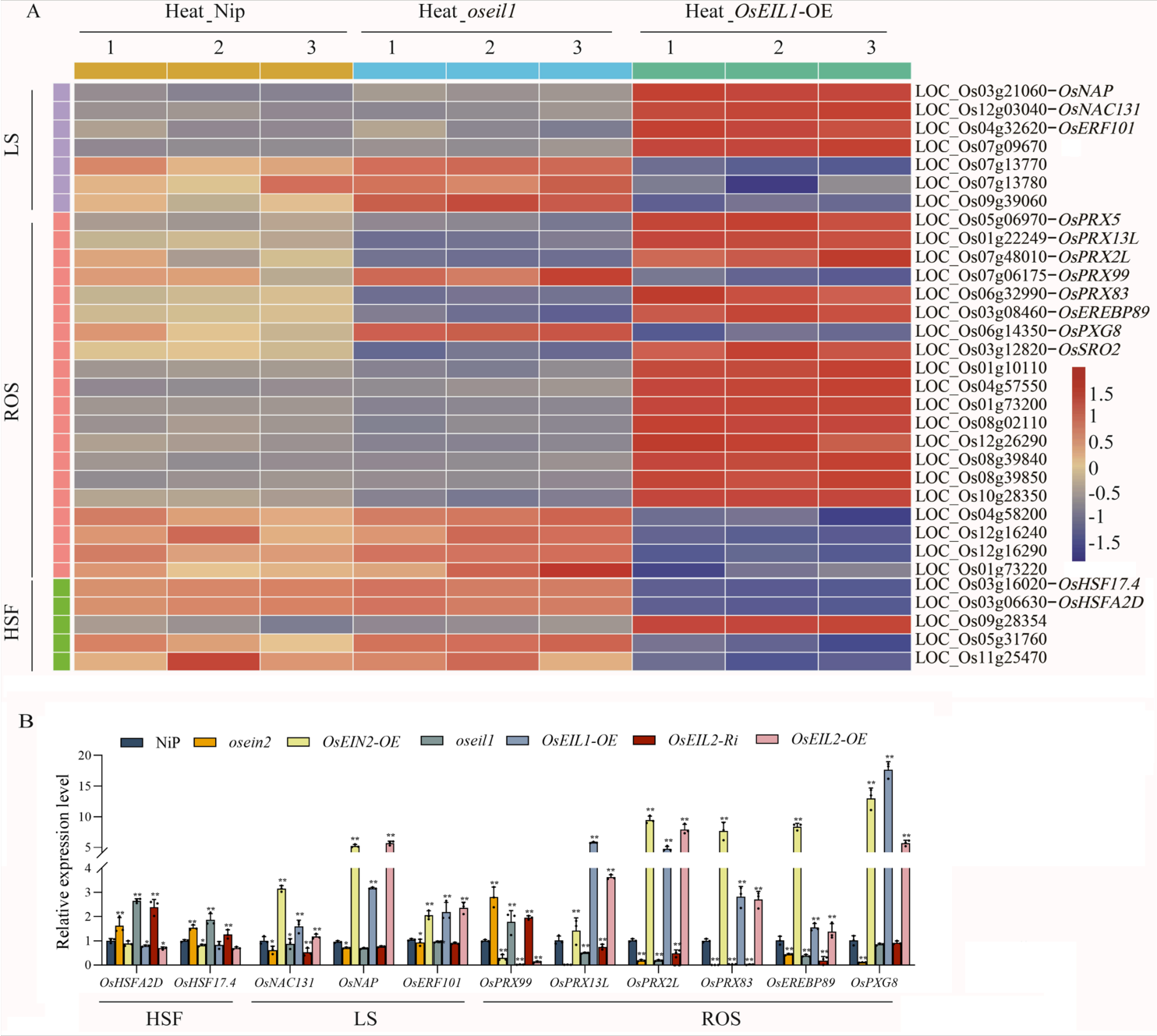


Figure 3

Transcriptome analysis and qPCR analysis of differentially expression genes (DEGs) in Nip, *oseil1* and *OsEIL1*-OE plants under heat treatment.

(A) Heat map of microarray expression profiles for genes associated with ROS metabolism (ROS), leaf senescence (LS) and heat shock transcription factor (HSF). (B) Expression of *HSFs*,*OsOPR5*, *OsERF101*,

OsNAC131, *OsNAP*, *OsEBP89* and*OsPRXs* gene in Nip, *oseil1* and *OsEIL1*-OE plants under heat treatment. *OsActin* was used as an internal control gene. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

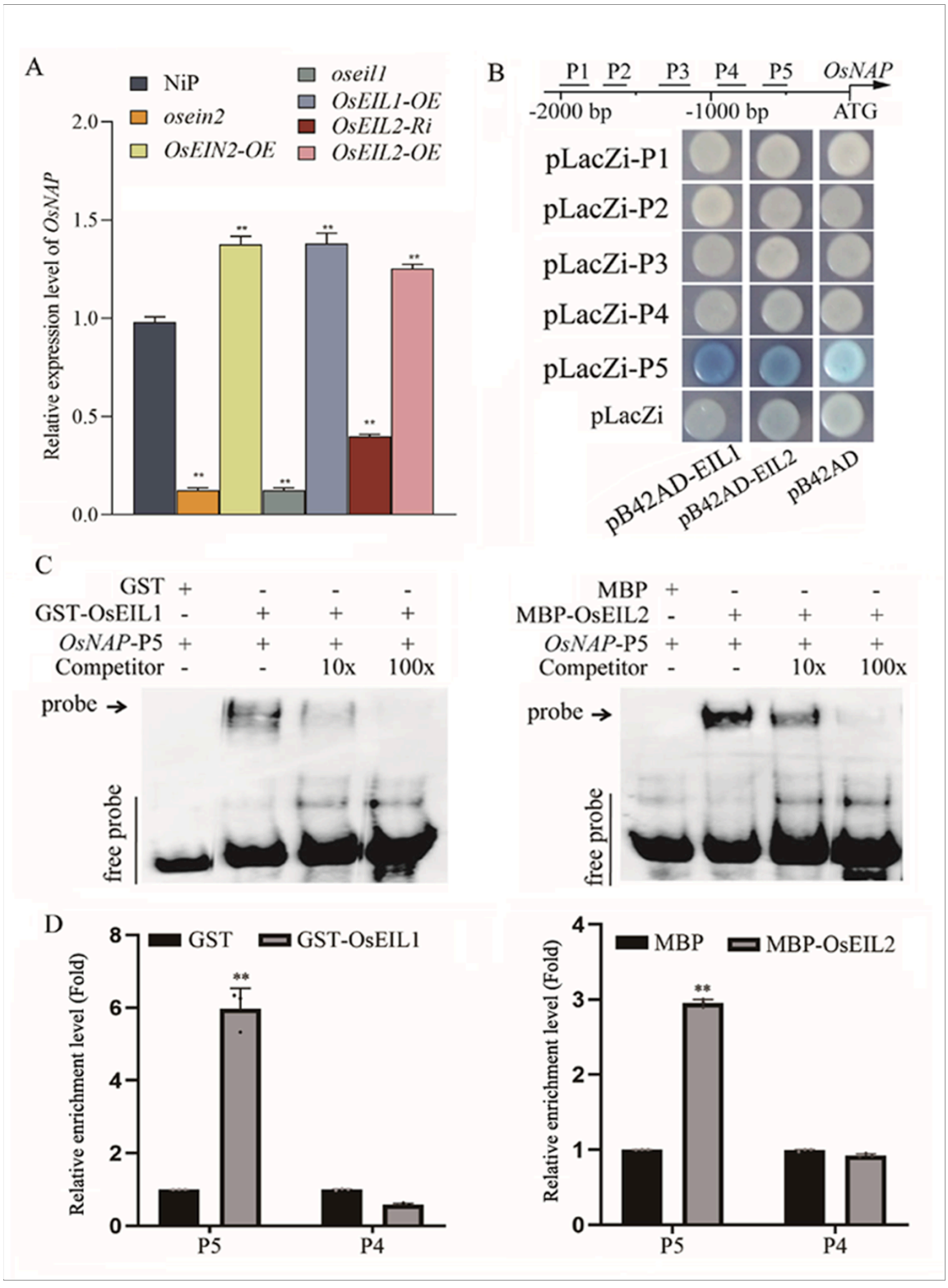


Figure 4

OsEIL1/2 directly activate the *OsNAP*expression .

(A) Expression of *OsNAP*in different materials under normal condition. The leaves of 2-week-old plants were used for the RT-qPCR analysis. Asterisks indicate significant differences by the Student's *t*-test (**$P < 0.01$). (B) The interaction of OsEIL1 and OsEIL2 with the *OsNAP* promoter revealed by yeast one-hybrid assay. The upper panel is a schematic diagram of the EBS-containing regions in the promoter. The putative EBS sequences are listed in Fig. S3A. (C) EMSA with GST-OsEIL1 or MBP-OsEIL2 protein was performed with P5 of *OsNAP* promoter. Competition for the labelled P5 was tested by adding an excess of unlabelled fragment (competitor) as indicated, and GST or MBP protein was used as negative control. The symbols + and − indicate the presence and absence of the corresponding proteins or probes, respectively. (D) ChIP-qPCR assays verified the binding of OsEIL1/2 to the *OsNAP* promoter. The genome DNA enriched by GST-OsEIL1, MBP-OsEIL2, GST or MBP proteins were used for qPCR analyses. The P4 fragment was used as a negative control. *OsActin* was used as an internal reference. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's *t*-test (**$P < 0.01$).

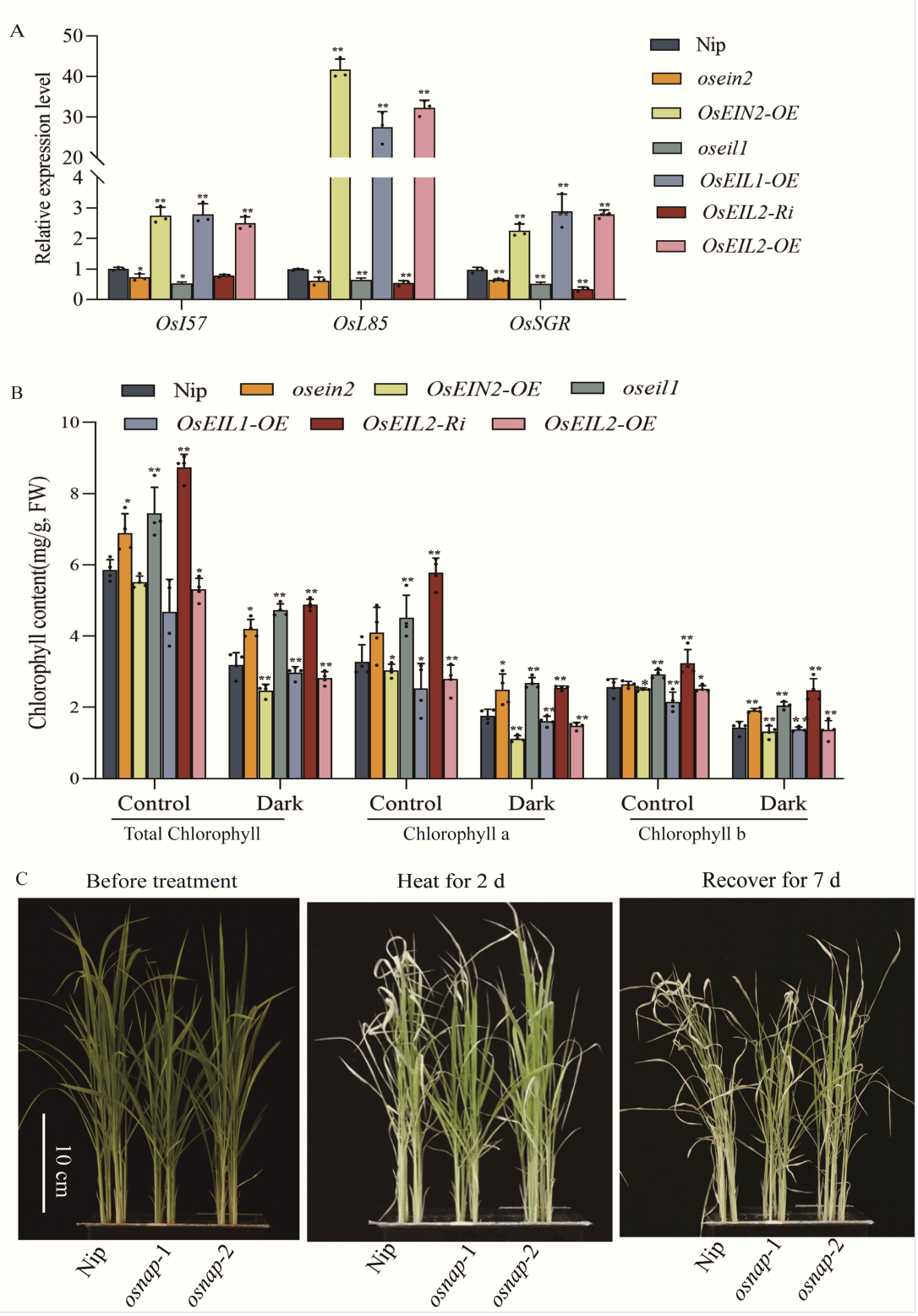


Figure 5

OsNAP negatively regulates rice thermotolerance.

(A) Expression of *OsI57*, *OsL85* and *OsSGR* in different materials under normal condition. *OsActin* was used as an internal control gene. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's *t*-test (*$P < 0.05$, **$P < 0.01$). (B) 2-week-old plants with or

without dark treatment were harvested for chlorophyll a, chlorophyll b and total chlorophyll content detection. FW means fresh weight. More than three replicates were performed. Data are the means ± SD of three biological replicates. Asterisks indicate significant differences by the Student's *t*-test (**P < 0.01). (C) Thermotolerance phenotypes of *osnap* and the wild type (Nip). Two-week-old seedlings were treated at 45°C for 2 days, followed by a recovery growth for 7 days. Bars = 10 cm.

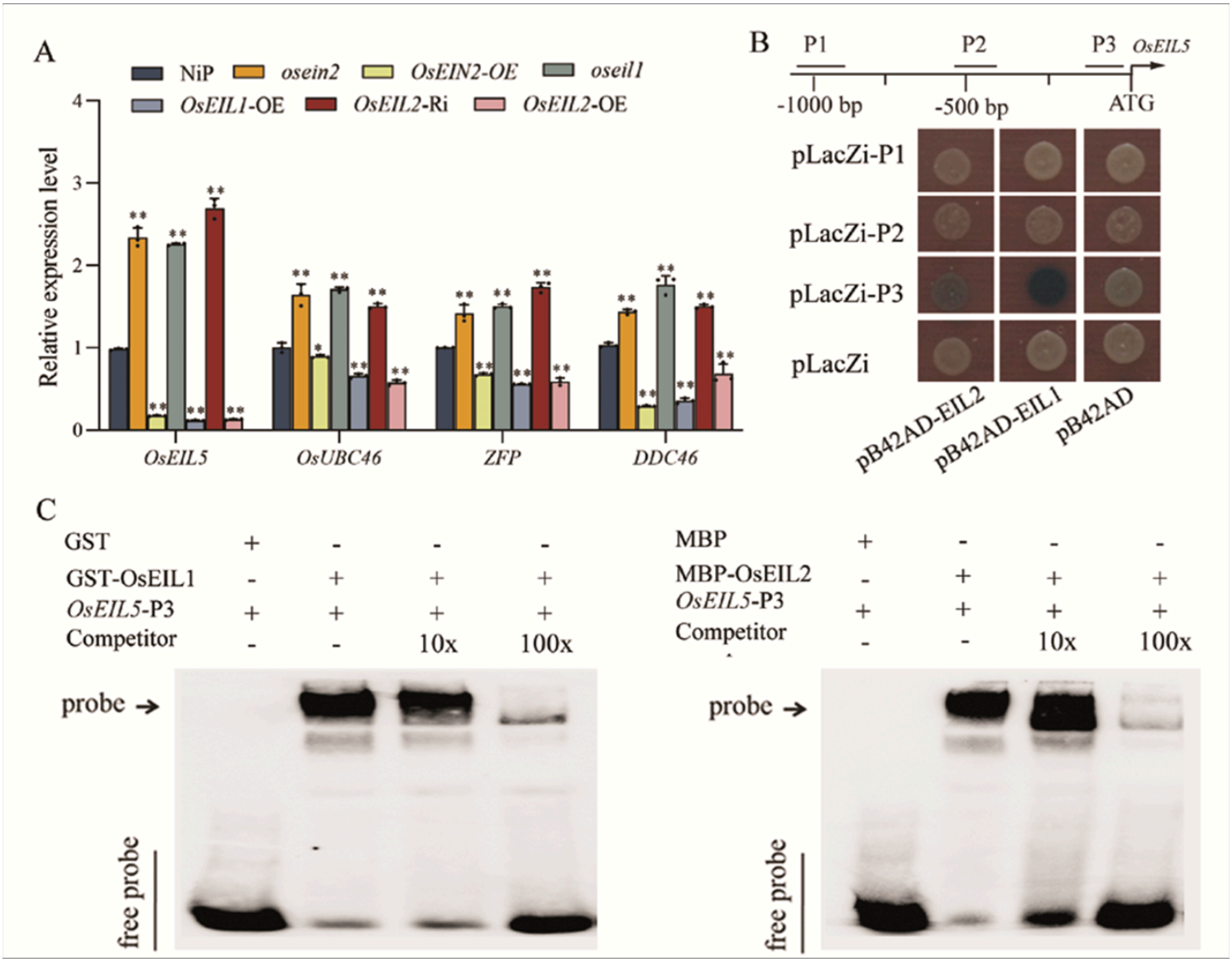


Figure 6

OsEIL1/2 antagonize OsEIL5 mediated rice thermotolerance.

(A) Expression of *OsEIL5*, *OsUbc46, ZFP and DDC* in different materials under normal condition. The leaves of 2-week-old plants were used for the RT-qPCR analysis. Asterisks indicate significant differences by the Student's *t*-test (*P < 0.05, **P < 0.01). (B) The interaction of OsEIL1 and OsEIL2 with the *OsEIL5* promoter revealed by yeast one-hybrid assay. The upper panel is a schematic diagram of the EBS-containing regions in the promoter. The putative EBS sequences are listed in Fig. S3B. (C) EMSA with GST-OsEIL1 or MBP-OsEIL2 protein was performed with P3 of *OsEIL5* promoter. Competition for the

labelled P3 was tested by adding an excess of unlabelled fragment (competitor) as indicated, and GST or MBP protein was used as negative control. The symbols + and − indicate the presence and absence of the corresponding proteins or probes, respectively.

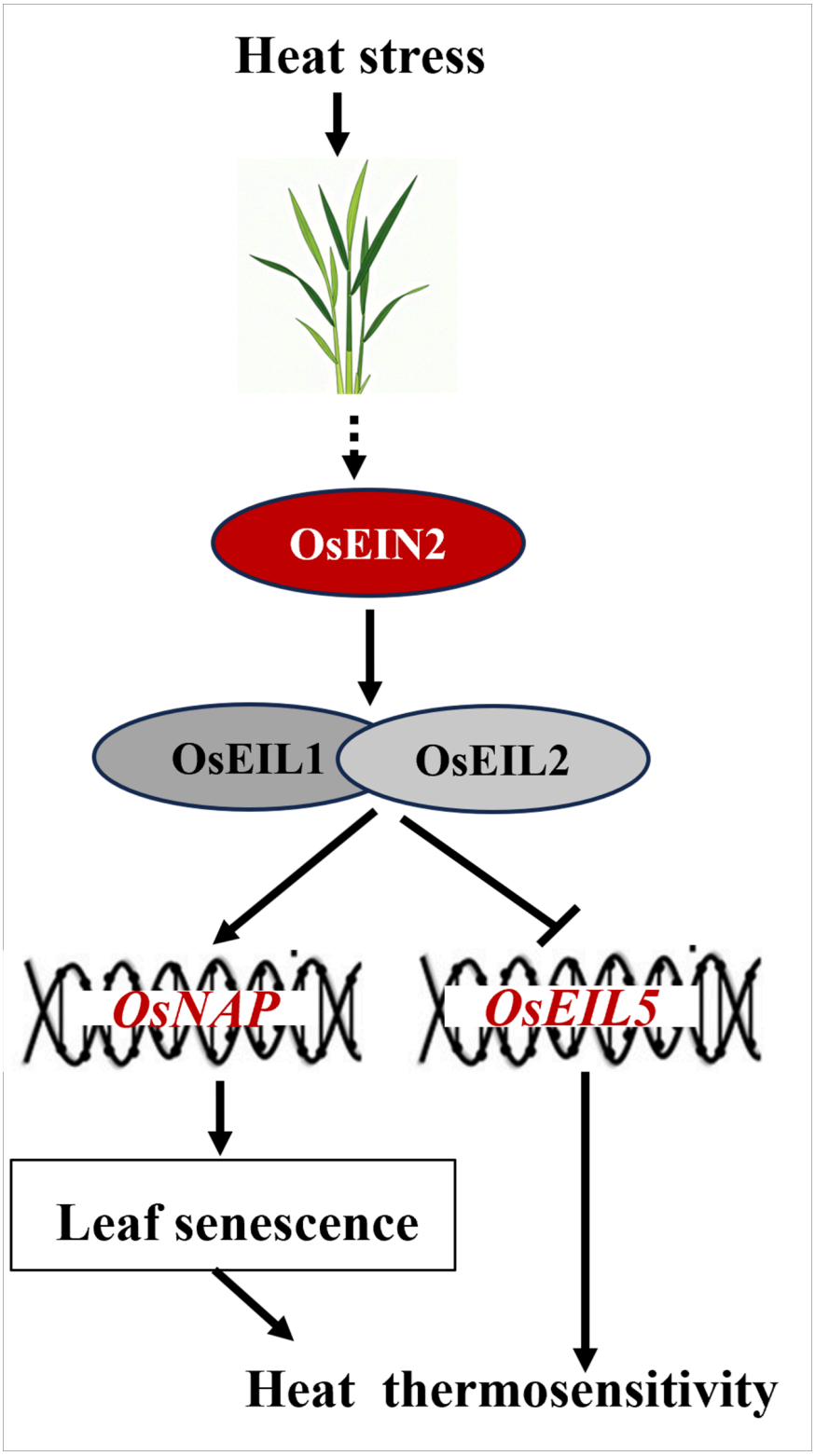


Figure 7

Proposed working model of OsEIN2-OsEIL1/2 pathway in regulating rice thermotolerance.

Under heat stress, the activated OsEIN2-OsEIL1/2 pathway induce the *OsNAP* and inhibited *OsEIL5* expression, resulted in accelerated leaf senescence and decreased thermotolerance.

Supplementary Files

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