# MODULATION OF V-PPASE EXPRESSION IN RICE BY CRISPR/CAS9-BASED MUTAGENESIS IMPROVES GRAIN QUALITY AND YIELD UNDER HIGH NIGHTTIME TEMPERATURE

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## Introduction

Chalkiness is the opaque area on the grain, and it is not just an appearance issue, it also impacts milling quality. Above the generally acceptable chalk values (6–10%), every 1% increase in chalkiness leads to 1% decline in head rice yield (HRY) (Zhao and Fitzgerald 2013).

Many studies have shown the effect high nighttime temperature (HNT) on grain quality and yield (Gann et al., 2023; Li et al., 2014). HNT disturbs key processes in reproductive development and grain filling that lead to reduced spikelet fertility and enhanced grain chalkiness (Srivastava et al. 2024). Therefore, breeding HNT tolerance is vital for safeguarding grain yields from heat waves in the future. However, breeding efforts have been impeded by the lack of reliable tolerance alleles in modern cultivars.

It is known that a gene encoding vacuolar H+ translocating pyrophosphatase (*V-PPase*), referred to as *VPP5* is a major quantitative trait locus in indica rice, controlling grain chalkiness. Higher transcriptional activity of this gene is associated with increased chalk content. Gann et al. (2023) found that the transcriptional modulation of VPP5 in japonica rice, Nipponbare, by CRISPR/Cas9-based mutagenesis of its promoter led to substantial reduction in grain chalkiness under HNT. In this study, we analyzed yield traits of the Nipponbare line harboring vpp5 allele (Nip_vpp5), and explored the underlying mechanism by RNA-seq.

## Materials and Methods

The Nip_*vpp5* line was developed by CRISPR/Cas9 based targeted mutagenesis of *VPP5* promoter in cv. Nipponbare. The lines contain a homozygous mutation consisting of 3 bp deletion in the AGATC element. The wildtype control line is a tissue-culture derived Nipponbare line. For phenotypic analysis, Nip_*vpp5* lines and Nip_WT plants were maintained in the greenhouse (average seasonal temperature: 27.8 °C daytime/25.8 °C nighttime), using 500 ml plastic pots with substrate soil and keeping 1 plant/pot, until booting stage. After then, half of them were transferred into HNT growth chamber (30 °C daytime/28 °C nighttime, 14 h photoperiod) until harvest. In the maturation stage, 15 plants were collected for phenotyping. The traits included spikelet fertility (SF), milling rice yield (MRY), head rice yield (HRY) and chalkiness, all of them was expressed in percentage, grain yield (GY grams) and grain length (GL in cm). The analyses were performed in the R environment (R Core Team 2021). Means were compared for significant differences using Tukey's multiple comparison test at $p<0.05$.

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RNA-seq analysis was carried out on developing caryopses to understand transcriptional changes associated with improved grain quality in Nip_*vpp5*. We selected caryopses at 10 days after flowering (10DAF) as VPP5 is highly expressed at this stage (Gann et al. 2023). The samples were sent to Novogene Inc. (https://www.novogene.com/us-en/) for quality check, library preparation, mRNA sequencing (150 bp paired-end), and bioinformatics analysis using their mRNAseq NovoSeq 6000 platform and the bioinformatics pipeline. Real time qPCR was performed on selected genes: heat shock protein (HSP), 11S globulin (Glob.), and 13 KDa prolamin (Prol.) . Relative expression was calculated against rice ubiquitin 2 gene.

## Results and Discussion

Although detrimental effect of HNT on SF was observed in both genotypes, Nip_vpp5 showed higher SF compared to Nip_WT (Fig. 1a). As observed in our previous study (Gann et al. 2023), Nip_vpp5 showed significantly lower (~ three fold lower) grain chalkiness compared to Nip_WT (Fig. 1b). Next, a negative effect of HNT on grain weight was observed in both genotypes, but significant differences between genotypes within a growing condition were not found (Fig. 1e). Finally, milled rice yield (MRY) and head rice yield (HRY) were significantly higher for Nip_vpp5 under HNT, but no significant difference was observed for grains ripened in the greenhouse (Fig. 1c–d). Interestingly, a small but significant increase in grain length was observed in Nip_vpp5 milled grains compared to Nip_WT (Fig. 1f). Overall, this analysis illuminated a positive effect of vpp5 mutation on major yield components: SF, MRY, and HRY.

Principal component analysis showed > 50% variance in the transcriptional profile of Nip_vpp5 and Nip_WT, and differential expression analysis found 615 differentially expressed genes (FDR < 0.05, |log2FCl>1). As expected, the key starch pathway genes were differentially expressed. However, gene enrichment analysis showed that heat response processes were downregulated. Specifically, gene ontology (GO) terms, ‘response to temperature stimulus’, ‘response to heat’, and ‘protein folding’ were highly significant (FDR <0.01). These pathways include heat shock protein (HSP) and heat shock transcription factor (HSF), 16 of which were downregulated (FDR < 0.05, |log2FCl>1) in Nip_vpp5 (Fig. 1g).

We validated these data by qPCR on a subset of these genes (Fig. 1h-i). HSPs and HSF play a major role in heat stress response, but they also exert developmental controls in non-stress condition, e.g., small HSP (sHSP) accumulate in embryo and endosperm during seed development (Waters and Vierling 2020). Further, since heat induces HSP expression in rapidly growing cells and heat is also a major inducer of grain chalkiness in most cultivars, a correlation of HSP expression and chalkiness is logical. This hypothesis is supported by studies that showed upregulation of HSP and HSF in rice caryopses under elevated temperature and accumulation of small HSP in chalky kernels (Yamakawa et al. 2007; Yamakawa and Hakata 2010). Thus, downregulation of sHSP genes in Nip_vpp5 arguably contributes to reduced chalkiness. Next, we found that a set of 13 kDa prolamin genes was upregulated in Nip_vpp5, while 11S globulin was downregulated (Fig. 1i). Lower abundance of 13 kDa prolamins and higher abundance of 11S globulin have been linked with chalky appearance of the grains (Lin et al. 2017; Yamakawa et al. 2007; Yamakawa and Hakata 2010). Prolamin is the major component of protein bodies in rice endosperm, and its deficiency could impact their morphology. Disturbance in protein body number and morphology was pointed out by Li et al. as the basis of chalky endosperm.

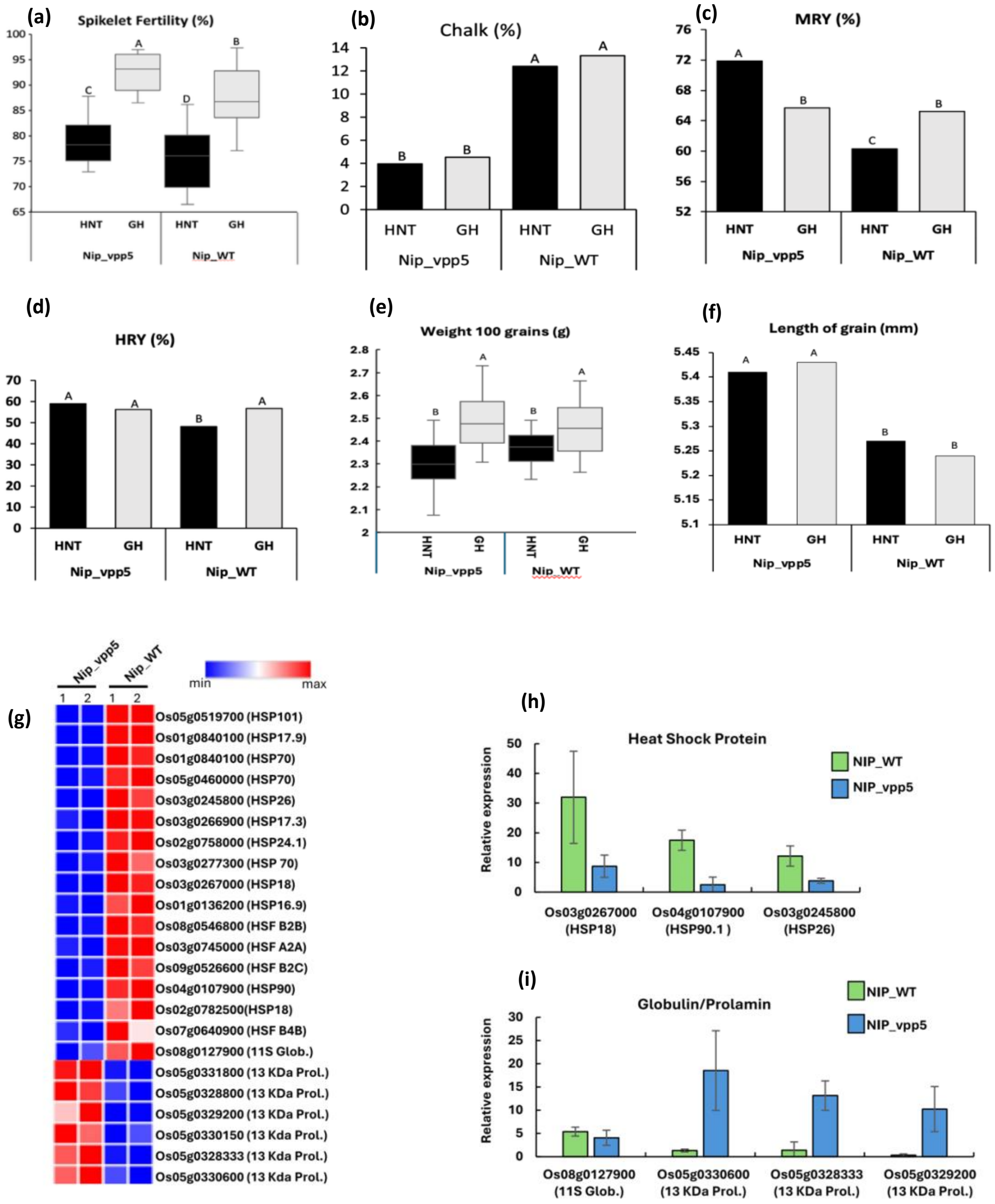

**Figure 1.** Grain quality and yield analysis of Nipponbare (Nip) rice lines harboring vpp5 mutation: Nip_vpp5 a–f (a: Spikelet fertility, b: percent chalk per grain, c: milled rice yield (MRY), d: head rice yield (HRY: number of unbroken kernels, e: Weight of 100 grains and f: grain length (n=100). Nip_vpp5: *vpp5* mutant in Nipponbare background, Nip_WT: wildtype Nipponbare. (g): expression pattern of heat-shock protein (HSP) and heat stress transcription factor (HSF) genes in Nip_vpp5 and Nip_WT. Expression values were obtained from RNA-seq analysis using two biological replicates of each genotype, h-i: gene expression analysis by real time qPCR on heat shock protein (HSP), 11S globulin (Glob.), and 13 KDa prolamin (Prol.) genes in the developing

caryopses. Error bars represent the standard deviation of data obtained from 15 plants in each genotype. Significance in Tukey's multiple comparison at $p<0.05$ is shown by capital letters.

## Conclusion

In conclusion, this study showed that CRISPR-based modulation of VPP5 generated a reliable allele for improved SF and reduced chalkiness under HNT. The underlying mechanism involves a range of metabolic changes that are not limited to starch biosynthesis and include differential accumulation of prolamin and sHSP.

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