Brief Communication

The rice PALE1 homolog is involved in the biosynthesis of vitamin B1

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Vitamin B1 is an essential cofactor for central metabolism in all organisms. In humans, vitamin B1 deficiency is often associated with cardiovascular diseases and neurological disorders. Polished white rice (Oryza sativa) is a poor source of vitamin B1. Biofortification of rice with vitamin B1 has the potential to improve human health and nutrition, but the implementation of this strategy requires detailed knowledge about the biosynthesis of vitamin B1, which is poorly studied in rice.

Most, if not all, genes involved in vitamin B1 biosynthesis have been uncovered in the reference plant Arabidopsis thaliana (Strobbe and Van Der Staeten, 2018). Vitamin B1 is composed of thiazole and pyrimidine moieties. The thiazole moiety, hydroxethyl thiazole phosphate (HET-P), is synthesized from glycine, nicotinamide adenine dinucleotide (NAD) and a sulphide group from HET-P synthase (TH1) that catalyses the reaction (Chatterjee et al., 2011; Fitzpatrick and Thore, 2014). The pyrimidine moiety, hydroxymethylpyrimidine phosphate (HMP-P), is derived from a complex rearrangement of aminoisimidazole ribonucleotide catalysed by HMP-P synthase (THIC) (Raschke et al., 2007). HMP-P is phosphorylated to HMP-PP by HMP-P kinase/thiamin monophosphatase (TMP) pyrophosphorylase (TH1), which also catalyses the condensation of HMP-PP and HET-P to TMP (Goyer, 2017). TMP synthesizes in the chloroplast is dephosphorylated to thiamin, which is then converted to the bioactive thiamin diphosphate (TDP) by thiamin pyrophosphokinase (TPK) in the cytosol (Ajawwi et al., 2007).

Recently, a TMP phosphatase encoded by the PALE1/TH2 gene has been shown to be involved in the dephosphorylation of TMP to thiamin in Arabidopsis (Hsieh et al., 2017; Mimura et al., 2016). While Mimura et al. (2016) proposed that the TMP phosphatase is mainly localized in the cytosol, the data derived from 35S:PALE1-GFP complemented pale1 mutants indicate that Arabidopsis PALE1 is localized to the mitochondrion (Hsieh et al., 2017). The Arabidopsis pale1/th2 mutants are not lethal, indicating that additional TMP phosphatases or nonspecific phosphatases capable of dephosphorylating TMP to thiamin are present in the mutants. Nonetheless, the localization of Arabidopsis TMP phosphatase in the mitochondrion suggests that the conversions among TMP, thiamin and TDP are more complicated than originally thought.

In contrast with Arabidopsis, very few genes involved in vitamin B1 biosynthesis have been characterized in rice. The OsDR8 gene encodes a TH1 homolog that has dual function in disease resistance and thiamin accumulation (Wang et al., 2006). The ROX1 gene, encoding a positive regulator of XA21 in innate immune response, corresponds to OsTPK1 (Lee et al., 2011). Here, we provide experimental evidence to show that OsPALE1 (Os08g0566000) is involved in vitamin B1 biosynthesis in rice.

To study the function of OsPALE1, we used CRISPR/Cas9 genome editing to create knockout mutants. The single-guide RNA (5’-CGAGGAGGGCCGCGCTGCGC-3’) was cloned into a CRISPR/Cas9 vector and transformed into rice. We successfully obtained 10 transgenic plants showing similar phenotypes. Initially, the mutant leaves were pale green, but they gradually turned yellowish brown and eventually died. A representative Ospale1 mutant plant is shown in Figure 1a.

DNA sequence analyses revealed that all 10 transgenic plants have the same biallelic mutation: an A or G single-nucleotide insertion at the same site (Figure 1b). Thus, these plants may be derived from the same OsPALE1 line during callus regeneration. OsPALE1 has a targeting peptide followed by the TenA and HAD domains (Figure 1c). The single-nucleotide ‘A’ insertion results in a frame shift starting from the 68th amino acid residue and creates a premature stop codon (Figures 1b and c). The ‘G’ insertion does not change the 68th AGG codon, and the frame shift occurs from the 69th to the 110th amino acids (Figures 1b and c). We also sequenced the reverse transcription PCR products from the Ospale1 mutant and confirmed that both ‘A’ and ‘G’ insertions are present in the Ospale1 mutant transcripts.

We measured the amounts of TMP, thiamin and TDP in the wild-type (WT) and Ospale1 leaves with a modified method described previously (Hsieh et al., 2017). The level of TMP in the Ospale1 mutant was about fivefold of the WT (Figure 1d). By contrast, the levels of thiamin and TDP in the Ospale1 mutant were 11% and 37% of the WT, respectively (Figure 1d). In addition to the characterization of the Ospale1 mutant, we also confirmed that 35S:OsPALE1 was able to complement the Arabidopsis pale1 mutant (Figure 1e). The phenotypes of the pale1 mutant (Hsieh et al., 2017) were fully restored in the complementation lines. Taken together, these results support the notion that OsPALE1 is involved in the conversion of TMP to thiamin in vitamin B1 biosynthesis.

The 35S:OsPALE1-GFP construct was transformed into rice protoplasts for subcellular localization assays. The OsPALE1-GFP is localized to the mitochondrion (Figure 1f), which is consistent...
with Arabidopsis PALE1-GFP (Hsieh et al., 2017). We could not exclude the possibility that OsPALE1 is dually localized in the cytosol and mitochondria. The usage of the 35S promoter may disproportionately enrich the fusion protein in the mitochondrion. Nevertheless, an OsPALE1 mutant complemented by the OsPALE1p:OsPALE1-GFP construct driven by a native promoter may provide a better answer for the subcellular localization of OsPALE1 in planta.

We used quantitative RT-PCR analysis to compare the expression levels of vitamin B1 biosynthesis genes in WT and OsPALE1 leaves. While the expression of TH1 (Os07g0529600), THIC (Os03g0679700), TH1 (Os12g0192500) and TP1K (Os01g0931400) was down-regulated, the expression of TP2K (Os01g0356500) and TPK3 (Os05g0367400) was not affected in the mutant (Figure 1g). The abundance of the OsPALE1 mutant transcripts in the mutant was lower than that of the OsPALE1 transcript in the WT (Figure 1g), which is consistent with the notion that transcripts with a premature termination codon will be selectively degraded by the nonsense-mediated mRNA decay pathway.

The pale-green to yellowish-brown phenotype of the OsPALE1 knock-out mutant is reminiscent of the Arabidopsis thiamin-deficient mutant pale1 (Hsieh et al., 2017). Still, there are at least two distinct aspects of OsPALE1. First, OsPALE1 is an essential gene in rice. The TDP levels in the OsPALE1 leaves are approximately 40% of the WT (Figure 1d), and the mutant is lethal. Similarly, the TDP levels in the Arabidopsis pale1 mutant seedlings are also about 40% of the WT, but the mutant is not lethal (Mimura et al., 2016; Hsieh et al., 2017). These results suggest that rice may have a stricter demand for TDP to complete its life cycle. Second, Arabidopsis and rice may have distinct mechanisms to regulate the expression of vitamin B1 biosynthesis genes. The expression of vitamin B1 biosynthesis genes was up-regulated in the Arabidopsis pale1 mutant (Hsieh et al., 2017). By contrast, a blockage in the conversion of TMP to thiamin results in down-regulation of vitamin B1 biosynthesis genes in rice (Figure 1g). Further studies on vitamin B1 biosynthesis genes and their regulation in rice may have practical application in agriculture and nutrition in the future.

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Conflicts of interest
The authors declare no conflicts of interest.

Author contributions
M.H. Hsieh conceived and designed the experiments and wrote the paper. P.H. Hsieh, Y.H. Chung and K.T. Lee performed the experiments and analysed the data. S.Y. Wang and C.A. Lu generated the transgenic plants.

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