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Brief Communication

Vitamin B_1 enhancement in the endosperm of rice through thiamine sequestration

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A core tenet of food security is ensuring people have access to sufficient nutritious food. Vitamin B_1 (thiamine) is an essential micronutrient for humans, deficiency in which causes numerous diseases of the nervous and cardiovascular systems (Dhir *et al*., 2019). Such ailments are particularly associated with populations that have a high carbohydrate intake, and especially those that have sustenance diets comprised largely of cereals such as rice. Thiamine insufficiency is a major public health concern in Asian countries, for example, in Cambodia 27%–100% of infants and women are deficient and account for up to 45% of deaths in under 5-year-olds (Johnson *et al*., 2019). Rice is a staple crop for half of the global population, but seeds are low in thiamine content, and polishing (i.e. removal of the embryo and bran layers) further aggravates chronic deficiencies as up to 90% of the thiamine content is in the removed tissues (Strobbe *et al*., 2021). Previous biofortification attempts increased thiamine content in leaves and unpolished seeds, but the trait failed to be retained in polished grains, sometimes negatively impacting yield (Dong *et al*., 2016; Strobbe *et al*., 2021), and no field trials have been reported that are necessary to confirm achievements. Natural variation studies revealed no substantial differences in thiamine content in rice seeds and minimal correlation with the expression of known biosynthesis genes (Mangel *et al*., 2022), discouraging exploitation via this route. Furthermore, the molecular form of vitamin B_1 is an important consideration because the biosynthesis of the coenzyme thiamine diphosphate (TDP) is tightly regulated and necessary for cellular homeostasis, whereas supplementing with thiamine suggests that this form is innocuous (Pourcel *et al*., 2013). Indeed, mature cereal seeds are a natural sink for thiamine (rather than TDP, Figure S1), which is associated with certain members of the large diverse class of albumin proteins (Watanabe *et al*., 2001). Significantly, a sequence has been reported from this class encoding a thiamine binding protein (TBP) from *Sesamum indicum* (*Si*, sesame) (Watanabe *et al*., 2001).

Here, we address previous bottlenecks related to enhancing thiamine content in rice endosperm and field performance in a proof-of-concept study. We expressed *Si*TBP under the control of the rice endosperm-specific *glutelin D-1* promoter in the rice *japonica* model variety TP309 (wild type) (Figure 1a), after confirmation of thiamine binding to a codon-optimized *Si*TBP expressed in *Escherichia coli* (Figure S2a–c). Among 45 transformants, 3 with independent single T-DNA insertion events were selected to homozygosity and were morphologically similar to wild type, but had increased thiamine content in the polished grain (L14, 20, 26). The lines were sown in an experimental field in Taiwan (GPS coordinates 24°04′41.3″N 120°42′53.8″E) initially for bulking and then monitored for their agricultural performance during season 1 in years 2022 and 2023. Each line was grown on three randomized plots of 6 × 8 plants each. The transgenic plants expressing *Si*TBP could not be distinguished morphologically from the wild type (Figure 1b). Various agronomic traits were recorded in each year such as plant height, tiller and panicle number, single-plant yield, 100-grain weight and fertility. No statistically significant differences were observed between the lines (Figure 1c–i; Figure S3a–f). The vitamin B_1 contents of leaf and unpolished seed material indicated no differences in vitamer levels of thiamine (Figure 1j,k; Figure S3g). In contrast, thiamine levels in the polished grains of the transgenic lines were increased 3 to 4-fold in each line compared to wild-type polished grains (Figure 1l; Figure S3h). Thus, *Si*TBP boosts the accumulation of thiamine in the endosperm without impacting yield. A proportion of this increase appears to be derived from the bran and/or embryo (Figure 1m; Figure S3i). Expression of the transgene was stable in the field, and expression of the biosynthesis genes was not changed with the exception of a small but significant decrease in the kinase *OsTPKc* in some of the transgenic lines (Figure S4). This represents a key step forward in vitamin B_1 biofortification by potentially achieving 35%–27% of the RDI (1.2–1.4 mg/day) for adults and lactating women with a bowl of rice (ca. 315 g), respectively.

The next step in realizing the goal of vitamin B_1-biofortified plants will be to pursue this approach in customer-preferred elite varieties. This could be combined with the appropriate expression of the dual thiamine/POLYAMINE UPTAKE TRANSPORTER identified in Arabidopsis (Martinis *et al*., 2016) to further facilitate enhanced endosperm thiamine content. Notably, exogenous

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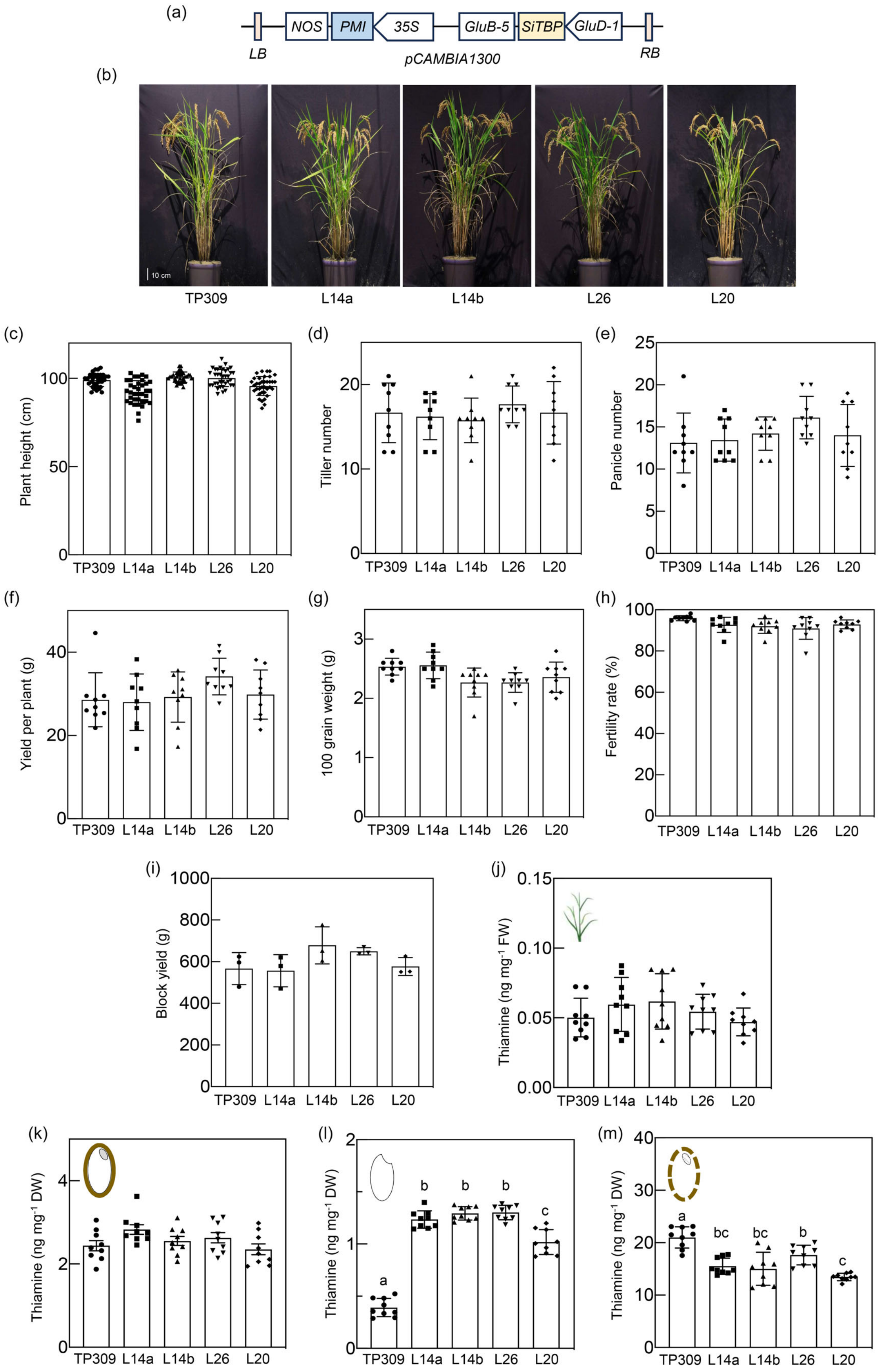
(a)
LB
NOS
PMI
35S
GluB-5
SiTBP
GluD-1
RB
pCAMBIA1300
(b)
10 cm
TP309
L14a
L14b
L26
L20
(c)
Plant height (cm)
0
50
100
(d)
Tiller number
0
10
20
(e)
Panicle number
0
5
10
15
20
25
(f)
Yield per plant (g)
0
20
40
(g)
100 grain weight (g)
0
1
2
3
(h)
Fertility rate (%)
0
20
40
60
80
100
(i)
Block yield (g)
0
200
400
600
800
1000
(j)
Thiamine (ng mg^{-1} FW)
0.00
0.05
0.10
0.15
(k)
Thiamine (ng mg^{-1} DW)
0
2
4
(l)
Thiamine (ng mg^{-1} DW)
0
1
2
a
b
b
b
c
(m)
Thiamine (ng mg^{-1} DW)
0
10
20
30
40
a
bc
bc
b
c
TP309 L14a L14b L26 L20

Figure 1 Thiamine biofortification of rice endosperm. (a) Schematic representation of genes in the T-DNA region of the transformation construct (pCAMBIA1300 backbone). LB, left border; NOS, nopaline synthase terminator; PMI; *Escherichia coli* phosphomannose isomerase; 35S, *CaMV* 35S promoter; *GluB-5*, rice *Glutelin B-5* terminator; *Si*TBP, *Sesamum indicum* thiamine binding protein (Genbank accession AJ310131); *GluD-1*, rice *Glutelin D-1* promoter; RB, right border. (b) Representative photos of 120-days-old rice plants. TP309 is the wild type and L14, L20 and L26 are genetically independent single homozygous T-DNA insertion lines. L14a and L14b are progeny from the same T0 plant. (c)–(i) Agronomic traits, as indicated. (j)–(m) Thiamine content as determined by HPLC in leaves, unpolished seeds, as well as polished grains and polishings (bran and embryo) of the same seeds, respectively. The data are from the year 2022 season 1 and are the mean ± standard deviation of at least $n = 9$ biological replicates. Statistical relevance for thiamine content in (l) and (m) was determined by one-way ANOVA ($\alpha = 0.05$) with multiple comparisons and Tukey's *post hoc* test. Statistically significant differences are denoted by different letters.

thiamine application has been discussed as a strategy to improve content (Li *et al*., 2022), but this may switch off thiamine biosynthesis *de novo* through extant TDP riboswitches and moreover, affect plant microbiome communities. Thus, we consider it more advantageous to purposefully engineer endogenous thiamine increases in target plant tissues by expanding the strategy for increasing thiamine content in grain endosperm as we present here to enhance the nutritional quality of rice for human health.

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Conflict of interest

The authors declare no competing interests.

Author contributions

TBF conceived the project. TBF, JBF and WG contributed to the design of experiments. TBF, ID, JBF, KW, FHC, SHC, BM and WG performed the experiments and analysed the data. TBF wrote the manuscript. WG and JBF performed edits to the manuscript.

Data availability statement

Data available on request from the authors.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Vitamin B_1 profile of leaves and seeds of field grown rice plants.
Figure S2 *Si*TBP sequence and analysis of purified recombinant protein from *Escherichia coli*.
Figure S3 Agronomic traits and thiamine content for field grown rice lines in 2023.
Figure S4 Relative transcript levels in field grown wild type and transgenic rice lines in season 1 of year 2022.
Table S1 Fertilizer components.
Table S2 Primers used in this study. Forward (F), reverse (R).