**Brief Communication**

**Enhanced vitamin E content in an Indica rice cultivar harbouring two transgenes from *Arabidopsis thaliana* involved in tocopherol biosynthesis pathway**

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Micronutrient deficiency results in malnutrition, which is prevalent all over the world and may lead to premature death in women and children (White and Broadley, 2009). Strategies formulated earlier including supplementation and fortified foods were not successful, owing to socio-economic and technical hurdles (Mayer et al., 2008). The subsequently evolved strategy of biofortification is a viable biotechnological tool to achieve desired results without compromising the agronomical values of crops. Conferring the genetic trait to improve vital nutrient accumulation in the edible parts of staple food crops, such as rice, through metabolic engineering is considered a fast, sustainable and cost-effective alternative to conventional breeding (Maestre et al., 2017). In earlier reports from our laboratory, enhanced \( \alpha \)-tocopherol levels in the stable transformants of *Nicotiana tabacum* (Harish et al., 2013a, 2013b) and in *Nicotiana benthamiana* adopting a transient expression system using *A. thaliana* tocopherol cyclase (*TC*) and homogentisate phytyl transferase (*HPT*) (Sathish et al., 2018) were shown. In the present study, *Agrobacterium*-mediated transformation of *Indica* rice *ASD16* with two genes involved in tocopherol biosynthesis, viz., *TC* and *HPT*, was carried out and the transgenic plants were analysed for the vitamin E (\( \alpha \)-tocopherol) content.

Mature seed-derived embryogenic calli were subjected to genetic transformation (Sundararajan et al., 2020) by employing two gene constructs, viz., pCAMBIA 1305.1 harbouring *TC* and pNutKan harbouring *HPT* (Fig 1a), individually, and in a co-transformation system, the calli were infected with both gene constructs. Putative transgenic plants recovered from all three independent experiments were established in a containment facility and no detectable morphological variations were found between the acclimatized NT and the transgenic plants (Fig 1b). Ten plants from all three experiments were taken for initial PCR analysis with the marker genes respective to each gene construct, that is *hpttl* in pCAMBIA 1305.1 *TC* and *npttl* in pNutKan *HPT*. Nine plants harbouring *TC*, 6 plants harbouring *HPT* and 7 *TC* + *HPT* plants showed positive PCR amplification of the marker genes (data not shown).

A preliminary GUS histochemical assay for the transgenic lines generated with *TC* and *TC* + *HPT* revealed the formation of blue colour in the leaves and roots of transgenic plants confirming the presence of the *GUS* gene, whereas no blue coloration was detected in the NT control. PCR analysis of the transgenic plants harbouring individual *TC* and *HPT* showed the expected band sizes (1.2 Kb for *HPT* and 1.4 Kb for *TC*). With the *TC* + *HPT* plants, amplification of both selectable marker genes during the initial PCR analysis was observed. However, in the PCR using gene-specific primers, among the seven plants, only five showed the presence of both the transgenes (plants subsequently renamed as TH1 to TH5). Southern blot analysis revealed the expected hybridization signals corresponding to *HPT* (1.2 kb) in all the six plants digested with *Pst*I and *Not*I, and *TC* (1.4 kb) in all the nine plants digested with *Kpn*I and *Bam*HI. For the *TC* + *HPT* lines, two individual blots were prepared using genomic DNA digested with *Kpn*I and *Bam*HI for *TC* and *Pst*I and *Not*I for *HPT*. The *HPT*-probed blot showed a corresponding hybridization signal of 1.2 kb, and a hybridization signal corresponding to 1.4 kb of the *TC* gene was observed in the *TC*-probed blot in all the five transgenic lines demonstrating the stable integration of the transgenes in the plants (data not shown).

Transcript analysis indicated that among the *TC* plants, *TC3*, *TC4* and *TC7* showed significantly higher levels of transgene expression (19.7-fold, 21-fold and 20.2-fold, respectively) as compared to the control. The relative gene expression of *HPT* lines was found to be higher as compared to *TC* lines. The quantum increase ranged from 9.5 to 17.0 among *HPT* lines with *HPT4* showing the highest gene expression (26.3-fold) followed by *HPT5* (23.6-fold increase). Among the *TC* + *HPT* lines, TH4 showed maximum gene expression to the tune of 26.7-fold (*HPT*) and 32.5-fold (*TC*) (data not shown).

Two plants each harbouring *TC*, *HPT* and *TC* + *HPT* were analysed for \( \alpha \)-tocopherol content in the transgenic leaves by HPLC. The determination of \( \alpha \)-tocopherol in the samples was based on peaks observed at 295 nm with a UV detection lamp. The retention time of the metabolite was confirmed by injecting the authentic standard (Sigma-Aldrich, St. Louis, MO), and the quantification in transgenic lines was performed accordingly. Results showed that among the *TC* lines, *TC1* showed a 3.26-fold and *TC4* showed a 2.2-fold increase in \( \alpha \)-tocopherol. The *HPT* lines showed relatively higher \( \alpha \)-tocopherol content as compared to that of the *TC* lines, wherein *HPT3* showed a 3.5-fold and *HPT2*
showed a 2.8-fold increase in α-tocopherol. The TC + HPT plants exhibited the highest α-tocopherol content among all the transgenic lines. Accordingly, the line TH4 showed a 5.3-fold increase in α-tocopherol followed by TH3 (4.3-fold). Subsequently, three lines (TC1, HPT3 and TH4) were chosen for T1 analysis where transgenic plants germinated from T0 seeds showed a segregation ratio of 3:1 confirming the Mendelian pattern of inheritance. Ten samples for each progeny were checked by PCR, and the progenies showed respective positive amplicons for the TC (1.4 kb) and HPT (1.2 kb) and presence of both the genes in the TC + HPT progenies (data not shown).

Subsequently, Southern blot analysis revealed the stable integration of the transgenes in the T1 progenies. Accordingly, the hybridization signals corresponding to the expected sizes of transgenes were detected in all the samples and no hybridization signal was detected in the NT control. qRT-PCR analysis of the two randomly selected PCR-positive plants from each gene construct revealed comparable results as that of the T0 plants. The TC + HPT lines showed higher relative gene expression as compared to that of the lines that harbour only one gene. Both TC1 progenies showed a comparable increase to the tune of 8.36-fold (TC 1-1) and 11.6-fold (TC 1-2), and HPT4 progenies showed a significant increase of 11.26-fold (HPT 4-1) and 8.95-fold (HPT 4-2). The TC + HPT progeny TH4 showed the highest relative gene expression about 15.35 (TH 4-1) and 14.80 (TH 4-2) folds (data not shown). A sample each from T1 progeny that carried TC, HPT and TC + HPT were analysed for their α-tocopherol content. Results revealed that TC1-1 showed a 2.7-fold increase, HPT3-1 showed an increase of 3.3-fold and the TC + HPT progeny TH4-1 showed a 4.1-fold increase in α-tocopherol content in the transgenic leaves similar to that of the T0 progenies where the TC + HPT line showed the highest α-tocopherol as compared to the lines harbouring either TC or HPT (Fig. 1c, d).

The transgenic T1 seeds from the selected progenies were assessed for their α-tocopherol content and a 0.29-fold increase in the TC + HPT line (TH4-1) was observed as compared to TC1-1 (0.12-fold) and HPT3-1 (0.18-fold). Enhanced level of vitamin E in numerous plant species including A. thaliana leaves (4.4-fold) and seeds (40%), tobacco leaves (5.5-fold), lettuce (2-fold), Brassica napus (2.7-fold) and potato tuber

**Figure 1** 1(a) Maps of binary vectors harbouring TC and HPT mobilized into Agrobacterium strain LBA4404; (b) transgenic lines recovered from genetic transformation experiments hardened in plant containment facility; (c) representative chromatograms of HPLC analysis in T0 and T1 lines harbouring TC and HPT; and (d) quantification of α-tocopherol in T0 and T1 transgenic rice.
(106%) has been reported utilizing various enzymes of the vitamin E biosynthesis pathway (reviewed by Mène-Saffrané and Pellaud, 2017). Harish et al., (2013a) proposed that in higher plants, co-expression of TC and HPT might increase the overall rate of total pathway flux resulting in a higher content of the end products. In summary, successful enhancement of vitamin E in rice was achieved using overexpression of both TC and HPT. Though the accumulation is not significant in the seeds, improvement with a multi-gene strategy with appropriate endosperm-specific promoters might offer an attractive model for vitamin E biofortification in rice, which would immensely benefit human nutrition and health care.

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**Conflict of interest**

All authors read, approved the manuscript and declare that there is no conflict of interest.

**Author contributions**

SS did the experiments and prepared the manuscript. VR and HPS did the experimental analysis. SN contributed to manuscript preparation and data representations. HMC carried out the initial cloning and assisted in manuscript editing. AS contributed to experimental design, technical interpretation and manuscript editing, and SR conceptualized, supervised the research and critically evaluated the manuscript.

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