5. Korea

Molecular characterization of high level of VitE accumulating rice mutant induced by *in vitro* mutagenesis

J.E. Hwang, D.S. Kim, S.-Y. Kang

Radiation Breeding Research Team, Advanced Radiation Research Institute,
Korea Atomic Energy Research Institute

5.1 Introduction

Vitamin E is an important constituent of the human diet (Evans and Bishop, 1922). Vitamin E is an essential lipid-soluble nutrient that consists of eight tocochromanols in nature, which can be separated into two groups, α-, β, -γ- and δ- tocopherols and four corresponding unsaturated derivatives, α-, β-, γ- and δ-tocotrienols. Tocopherols help maintain membrane structure and integrity (Falk and Munne Bosch, 2010), act as antioxidants and free radical scavengers (Tappel, 1962; Behl and Moosmann, 2002), and perform other nonantioxidant functions related to signaling and transcriptional regulation (Ricciarelli et al., 2002). Both tocopherols and tocotrienols occur in plants, as well as in photosynthetic microbes such as *Synechocystis*, with the highest concentration in seeds. The seeds of most plants have significant amounts of γ- tocopherol, whereas leaves have predominately α- tocopherol. Tocotrienols, however, are not as prevalent in plants as are tocopherols, but they are the primary form of vitamin E in the seeds of most monocot species (Padley et al., 1994) and fruits of some dicots (Aitzetmüller, 1997).

In the case of tocochromanol biosynthesis pathway, seven genes coding for tocochromanol biosynthesis enzymes have been identified and characterized in Arabidopsis and rice (Mene-Saffrane and DellaPenna, 2010). This pathway diverges at the step where polyprenyl side chain is attached to homogentisic acid (HGA) by homogentisic acid phytyl transferase (VTE2) or homogentisic acid solanesyl transferase (HST) (Mene-Saffrane and DellaPenna, 2010). The first committed step in the biosynthetic pathway is the prenylation of homogentisic acid with phytyldiphosphate to form 2-methyl-6-phytylbenzoquinol (MPBQ). The overall tocopherol composition is determined by the combined activities and substrate specificities of the homogentisate prenyltransferase (VTE2), tocopherol cyclase (VTE1), and two methyltransferase enzymes (VTE3 and VTE4) present in a given tissue (Jo and Hyun, 2011). The action of the cyclase directly on MPBQ leads to the formation of δ-tocopherol. By contrast, ring methylation at position R_2 by the first methyltransferase, MPBQ methyltransferase (VTE3), leads to 2,3-dimethyl-5-phytylbenzoquinol and the subsequent action of tocopherol cyclase leads to the formation of γ-tocopherol. The second methyltransferase, γ -tocopherol methyltransferase (VTE4), performs ring methylation at R_3, converting the γ – and δ isoforms to the α-and β-isoforms, respectively. Tocotrienols are formed when geranylgeranyldiphosphate is used in place of phytyldiphosphate as the side chain precursor.

In this study, T1001-1 was isolated from *in vitro* mutagenized population by ionizing radiation and shown to have increased VitE contents. To study the molecular mechanism of VitE biosynthesis, we identified rice genome encodes seven VitE biosynthetic enzyme and we analyzed its expression patterns. The results of DNA sequencing analysis demonstrate that the *OsVTE2* promoter and

genomic sequences contain mutated region. In addition, we showed that the mutant confers retarded seedling growth during the early seedling growth stage in rice. These observations suggest that the mutation of the *OsVTE2* might affect a Vitamin E biosynthesis.

5.2 Materials and methods

5.2.1 Plant materials and growth conditions

The wild-type rice cv. Dongan embryo culture had been irradiated with gamma rays of 30–120 Gy generated from a ^{60}Co gamma irradiator (150 TBq of capacity; ACEL, Nordion, Ottawa, ON, Canada) at the Korea Atomic Energy Research Institute. The mutant cell lines were regenerated on medium containing 0.5 mM 5MT. Regenerated plants from 5MT-resistant calli were genetically stabilized through consecutive generations of self-pollination and the homozygous M10 progenies were obtained as reported elsewhere (Kim et al., 2004).

For seedling growth test, seeds were sown on 1/2 MS medium containing Murashige and Skoog salts (Duchefa, Haarlem, Netherlands), 3% Sucrose and 0.7% phyta-agar (Duchefa, Haarlem, Netherlands) under 16 h light/8 h dark cycle at 24^{o}C.

5.2.2 HPLC analysis

Tocopherols and tocotrienols were determined using HPLC (Sykam system S1211). Resolution of vitamin E species was achieved using an Agilent Eclipse LC-Si column (4.6 mm length, 5 μm) and a solvent system consisting of methanol:water (95:5, v/v) with a flow rate of 1.5 ml min^{-1}. Sample components were detected and quantified by fluorescence with excitation at 292 nm and emission at 330 nm. A sample volume of 10 μl was injected for the chromatographic analysis. Peaks of α-tocopherol, β-tocopherol, γ-tocopherol, α-tocotrienol, β-tocotrienol and δ γ-tocotrienol, δ-tocotrienol were identified by comparing their retention times with commercially available authentic standards. Pure tocopherols and tocotrienols were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. The content of each form was calculated based on standard curves of external standards.

5.2.3 Semi quantitative RT-PCR analysis

Total RNA was isolated using Trizol reagents according to the manufacturer's recommended protocols (GibcoBRL, Cleveland, OH). 1 μg of total RNA was reverse transcribed in Power cDNA Synthesis Kit (Intron Biotech Inc., Sungnam, Korea) for 60min at 42°C using 1 μg oligo(dT)15 primers. 1 μl of cDNA was used for PCR amplification with each gene specific primer (Table 1). The resultant RT-PCR products were electrophoresed and analyzed on a 2.0% (w/v) agarose gel after staining with ethidium bromide.

5.2.4 Western-blot analysis

The OsVTE2 expression pattern was determined by western blot analysis using the anti-OsVTE2 polyclonal antibody (1:5,000 dilution), followed by the addition of peroxidase- conjugated goat anti-rabbit IgG (1:5,000 dilution) according to the manufacturer's guidelines. Hybridization to protein bands was detected using the SuperSignal West Pico Trial Kit (Thermo, USA)

5.3 Results and Discussion

5.3.1 HPLC detection of tocopherols and tocotrienols in rice grains

Rice mutant lines in the M_{10} generation and the control (Dongan byeo) were grown in the same filed under normal cultural conditions, and mature seeds were harvested for determination of vitamin E composition. Normally, the β- and δ-isomers usually appear in trace amounts, while α-tocopherol and γ-tocotrienol are the most abundant isomers. In our experiments, we also found that it was difficult to detect δ-tocopherol. Therefore, this isomer was not included. In rice seeds, the major tocopherol found is α-tocopherol, and amount of β-tocopherol and γ-tocopherol are relatively low (Fig. 1). As a result, the contents of α-tocopherol increased to 42% in T1001-1 mutant seeds than that of the controls. HPLC analysis showed that α-tocotrienol and γ-tocotrienol were the major tocotrienols present in seeds of mutants and control. However, we found that there was no significant change in the content of each tocotrienol (Fig. 1). In the T1001-1 C mutant line, the γ-tocotrienol content was increased to 14% than in the control. The content of VitE (total of tocopherol and tocotrienol) was increased to 26% in T1001-1 c mutant seeds. Tocotrienols, like tocopherols, are also potent antioxidants (Serbinova et al., 1991), but they are less readily absorbed than α-tocopherol (Kamal-Eldin and Appelqvist, 1996). Thus, high accumulation of α-tocopherolin T1001-1 mutants provides effective protection against oxidative damage.

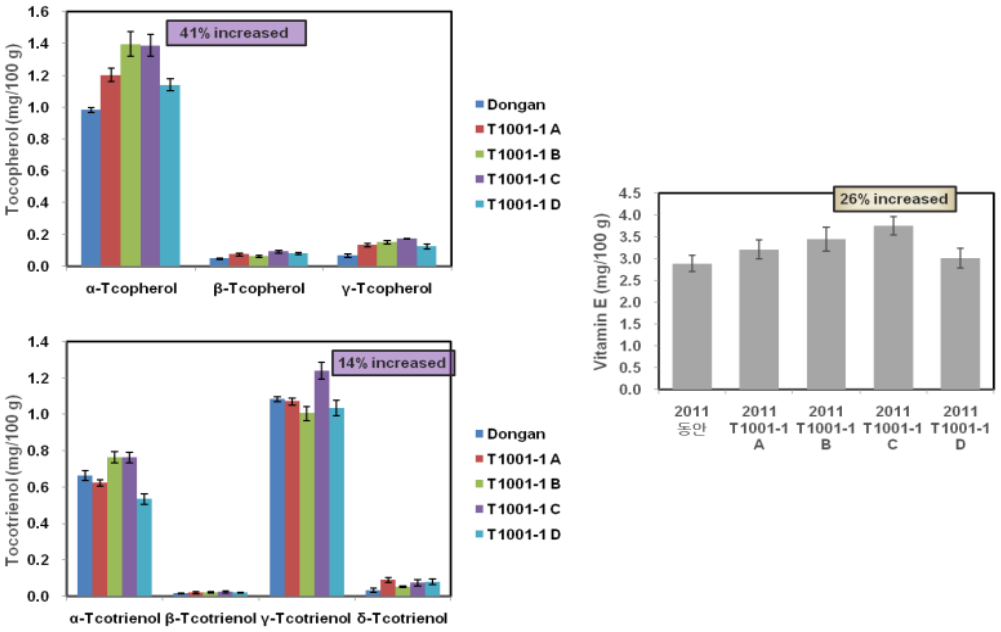

Figure 1. Total and individual tocochromanol accumulation in seed of the Dongan and T1001-1.

Tocopherol and tocotrienol composition of the seed of rice was determined by HPLC. The total VitE content was 26% increased in the T1001-1 mutant seeds compared with Dongan.

5.3.2 Phenotypic analysis of T1001-1 mutants

As T1001-1 mutants seedling grew slowly, we compared seedling growth rates in mutant and control plants (Fig. 2B). The primary root elongation pattern of T1001-1 mutant was not different from that of control (Fig. 2C). Sattler et al. (2004) reported that *vte2* mutants exhibited seedling growth in Arabidopsis. Thus, these T1001-1 mutant phenotypes have possibility by increasing of VTE2 expression.

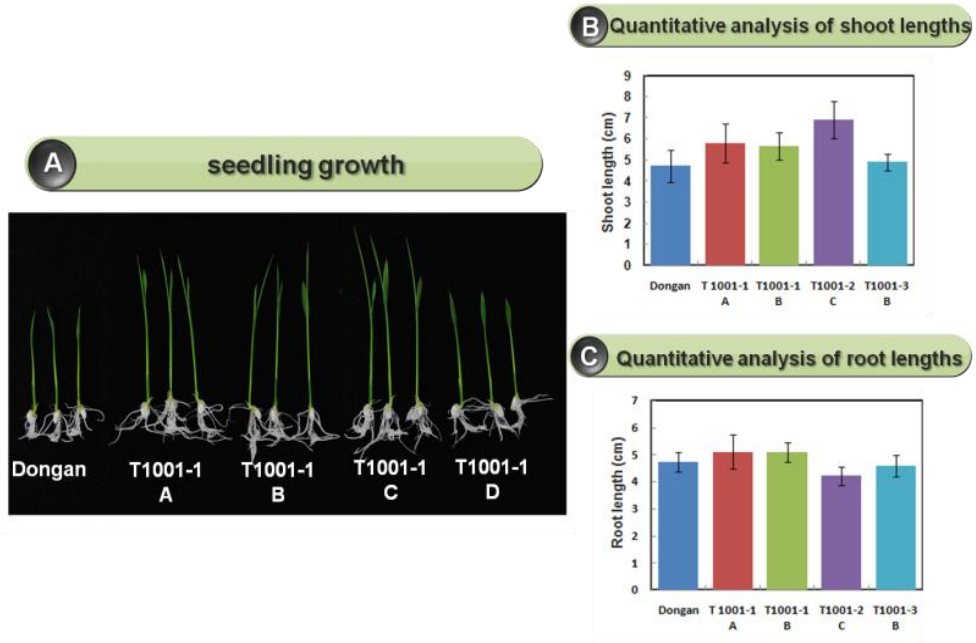

Figure 2. Comparison of seedling growth in mutant and control plants.

(A)The early seedling growth differences between the control (Dongan) and mutant lines (T1001-1) for 7-days were determined by measuring the shoot and root lengths. (B) Quantitative analysis of shoot lengths of 7-day-old seedlings from each mutant line. (C) Primary root length of 7-day-old control and mutant line seedlings.

5.3.3 Expression of tocochromanol biosynthesis genes

To study the molecular mechanism of VitE biosynthesis, we identified rice genome encodes seven VitE biosynthetic enzyme and we analyzed its expression patterns. Semi quantitative reverse transcription analysis revealed that seven VitE biosynthesis genes were expressed at different levels in four T1001-1 mutant lines. Interestingly, *VTE2* transcripts were highly accumulated in the T1001-1 C (Fig. 3), whereas the expression of *VTE3* was decreased in T1001-1 lines (Fig. 3). This result indicates that accumulation of VtiE in T1001-1 mutants is controlled by different expression of *VTE2* and *VTE3* transcript.

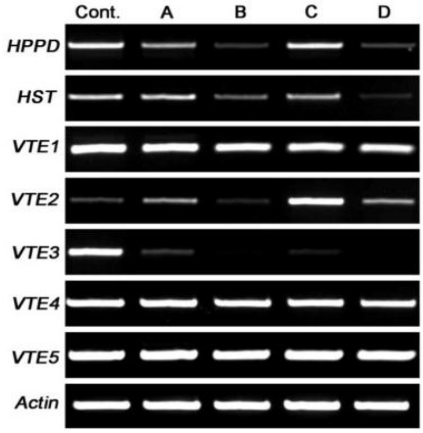

Figure 3. Transcript levels of VitE biosynthesis genes in rice plants.

Expression profiles of 7 rice VitE biosynthesis genes in Dongan (Cont.) and mutant lines (T1001-1). *Actin* gene was used as loading control.

5.3.4 Sequence analysis of VTE2 and VTE3

Fig. 2 showed dramatic change of *VTE2* and *VTE3* expression in T1001-1 C mutant. Therefore, *VTE2* and *VTE3* genes were sequenced in both the control and T1001-1 C mutant. The A nucleotide at position -245 is exchanged by a G nucleotide and the C nucleotide at position -106 is changed by T of the VTE2 promoter in the T1001-1C mutant compared to the wild-type sequence (Fig. 4). The position -245 mutation region was reported ZFP-TFs (zinc finger transcription factors) binding site and -106 mutation region existence-box-*cis* element. ZFP-TFs were reported to upregulate the expression of the endogenous *Arabidopsis* γ-tocopherol methyltansferage (GMT) gene (Van Eenenaam et al., 2004). The presence of these motifs indicates that *VTE2* may be regulated by ZFP-TFs binding *cis*-acting elements. Genomic sequence of *VTE2* was exchanged in only intron region, therefore, there was no effect at amino acid sequence. In the T1001-1 C mutant line, we observed a G to A base change at position 662 and an added C of VTE3 promoter but not found important *cis*-elements (Fig. 4). Sequencing of the *VTE3* genomic DNA sequence was not different. These results indicated that increasing of *VTE2* transcript expression was controlled by VTE2 promoter sequence mutation in accumulation of tocochromanol.

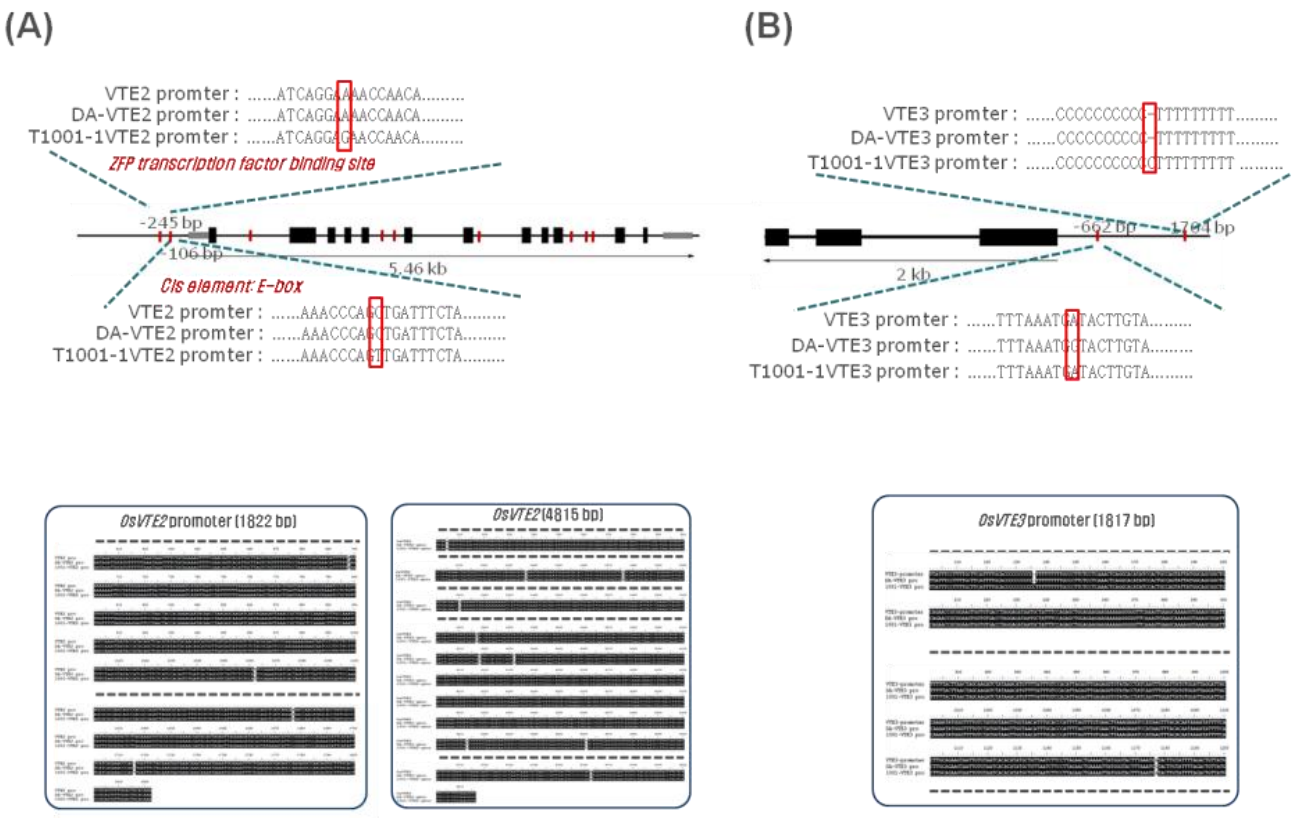

Figure 4. Comparison of the promoter and genomic DNA sequences from Dongan (DA) and T1001-1 mutant with the related region of *VTE2* (A) and *VTE3* (B).

Number of the DNA sequence is shown in the upper margin. The empty region at sequences indicates the different sequence in DA and T1001- 1C mutant.

5.3.5 Protein expression of VTE2

The VTE2 expression pattern was determined by western blot analysis using the prediction 4 of anti-VTE2 polyclonal antibody. To precisely define the expression pattern of VTE2, we studied the expression levels of VTE2 protein by western blot analysis (Fig. 5). These results are similar with the expression patterns of *VTE2* transcript that was accumulated more extensively in T1001-1 mutant than Dongan byoe in protein level.

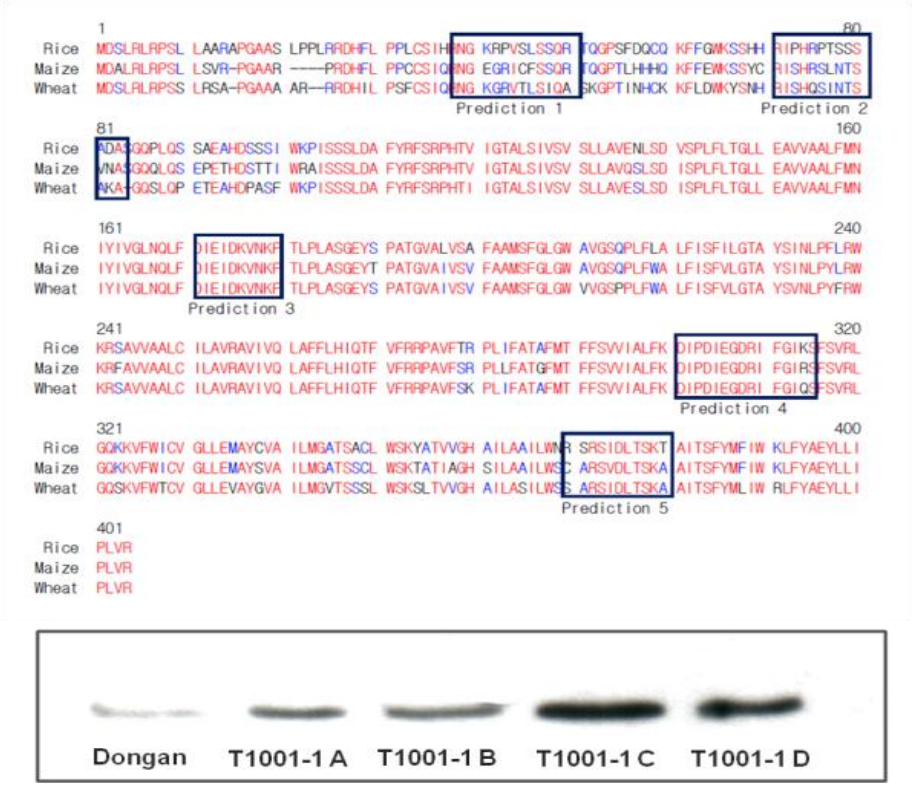

Figure 5. Western blot analysis of VTE2 in T1001-1 mutants and Dongan byeo.

Total proteins from seedling of Dongan or T1001-1 mutants were isolated and subjected to western blot analysis.

5.4 References

Aitzetmüller K (1997) Antioxidative effects of carum seeds. J Am Oil Chem Soc 74:185

Behl C and Moosmann B (2002) Oxidative nerve cell death in Alzheimer's disease and stroke: Antioxidants as neuroprotective compounds. Biol Chem 383: 521–536

Evans HM and Bishop KS (1922) On the existence of a hitherto unrecognized dietary factor essential for reproduction. Science 56: 650–651

Falk J, Munne-Bosch S 2010 Tocochromanol functions in plants: antioxidation and beyond. J Exp Bot 61: 1549–1566

Gunstone FD, HarwoodJL, Padley FB (eds) The lipid handbook, 2nd edn. Chapman & Hall, London, pp 127–130

Kamal-Eldin A, Appelqvist LA (1996) The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids 31:671–701.

Padley FB, Gunstone FD, Harwood JL (1994) Occurrence and characteristics of oils and fats. In: Sen CK, Khanna S, Roy S (2006) Tocotrienols: vitamin E beyond tocopherols. Life Sci 78:2088–2098

Ricciarelli R, Zingg JM, and Azzi A (2002) The 80th anniversary of vitamin E: Beyond its antioxidant properties. Biol Chem 383: 457–465

Tappel AL (1962) Vitamin E as the biological lipid antioxidant. Vitam Horm 20: 493–510