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Specific roles of tocopherols and tocotrienols in seed longevity and germination tolerance to abiotic stress in transgenic rice

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ABSTRACT

Tocopherols and tocotrienols are lipophilic antioxidants that are abundant in plant seeds. Although their roles have been extensively studied, our understanding of their functions in rice seeds is still limited. In this study, on the basis of available RNAi rice plants constitutively silenced for homogentisate phytyltransferase (HPT) and tocopherol cyclase (TC), we developed transgenic plants that silenced homogentisate geranylgeranyl transferase (HGGT). All the RNAi plants showed significantly reduced germination percentages and a higher proportion of abnormal seedlings than the control plants, with HGGT transgenics showing the most severe phenotype. The accelerated aging phenotype corresponded well with the amount of H_2O_2 accumulated in the embryo, glucose level, and ion leakage, but not with the amount of O^{2-} accumulated in the embryo and lipid hydroperoxides levels in these genotypes. Under abiotic stress conditions, HPT and TC transgenics showed lower germination percentage and seedling growth than HGGT transgenics, while HGGT transgenics showed almost the same status as the wild type. Therefore, we proposed that tocopherols in the germ may protect the embryo from reactive oxygen species under both accelerated aging and stress conditions, whereas tocotrienols in the pericarp may exclusively help in reducing the metabolic activity of the seed during accelerated aging.

1. Introduction

Orthodox seeds on mother plants are desiccated during maturation and dispersed in a dehydrated state. Therefore, dry seeds reduce their metabolic activity to a drastically low level (quiescence) and can be stored for long periods [1]. However, seeds inevitably undergo aging or deterioration and progressively lose germination vigor during storage. Therefore, seed longevity is an important trait for both ecological and agronomical values. Indeed, seeds provide the genetic information needed for plant species and constitute the main vector for plant propagation. Seeds are also the most basic means for agriculture, and their quality is very crucial for production. Although there is little information on seed longevity, accumulation of reactive oxygen species (ROS) and free radicals is proposed as one of the most important factors that affect seed aging [2,3]. Therefore, a large number of studies on the issue have concentrated on the mechanisms of protection, detoxification, and repair evolved by seeds during the evolutionary process [4].

The seed coat or testa is the only barrier of the seed against the outer environment. In most seeds, the testa is brown as it contains phenolic compounds that serve as antioxidants to protect embryos from oxidative damage. Therefore, seeds from both structural and pigmentation mutants, such as transparent testa (*tt*) and banyuls (*ban*) mutants, showed reduced seed longevity during storage [5–7]. The detoxification mechanism in seeds includes enzymatic and non-enzymatic antioxidant systems. Owing to the low water content in seeds, lipophilic antioxidants such as tocopherols may have a more important role in seed longevity. The antioxidant enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase, and glutathione peroxidase. If these enzymes are destroyed during storage, seeds will show decreased viability during germination. This was demonstrated using function gain or loss in transgenic plants such as overexpressing SOD plants and APX6 mutants [8,9]. Since the attack of DNA, proteins, and membranes by ROS will result in damage, mechanisms related to the

Abbreviations: APX, ascorbate peroxidase; DAB, 3,3′-Diaminobenzidine; DAI, days after imbibition; DMPBQ, 2,3-Dimethy-6-phytyl-1,4-benzoquinol; HGGT, homogentisate geranylgeranyl transferase; HPT, homogentisate phytyltransferase; LOOH, lipid hydroperoxides; MDA, malondialdehyde; NBT, nitrotetrazolium blue chloride; NT, non-transformed plants; PC-8, plastochromanol-8; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SOD, superoxide dismutase; TC, tocopherol cyclase; WT, wild type.

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repair of these cellular components, such as Met sulfoxide reductase [10], DNA glycosylase/apurinic/apyrimidinic lyase [11], and L-isoaspartyl methyltransferase [12], also affect seed longevity.

As mentioned above, tocopherols and tocotrienols, collectively known as tocochromanols, are members of a group of lipophilic antioxidants that are exclusively synthesized by photosynthetic organisms, including all plants. Tocotrienols differ structurally from tocopherols by the presence of three trans-double bonds in the hydrocarbon tail. These substances are accumulated to the greatest extent in plant seeds. Tocopherols are usually found in the seeds of most dicots and embryos of monocots, while tocotrienols are primarily limited to the seed endosperm of monocots and some dicots [13,14]. The first committed steps in tocopherol and tocotrienol biosynthesis are the condensation of homogentisic acid and either phytyl diphosphate or geranylgeranyl diphosphate, which are catalyzed by homogentisate phytyltransferase (HPT) and homogentisate geranylgeranyl transferase (HGGT), respectively. The subsequent methylation and cyclization steps are in common for tocopherol and tocotrienol biosynthesis, among which, the cyclization of 2,3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ), which is catalyzed by a common tocopherol/tocotrienol cyclase (TC), is also a crucial step in the biosynthesis [14,15].

The role of tocopherols in seed has been studied using the model plant *Arabidopsis* [15]. Both *vte1* plants (defective in TC) and *vte2* plants (defective in HPT) lacked all 4 types of tocopherols, but *vte1* plants accumulated the pathway intermediate DMBPQ. Seeds of *vte1* and *vte2* showed reduced seed germination rates when subjected to accelerated aging [15], indicating the important role of tocopherols in seed longevity. However, only *vte2* exhibited severe seedling defects during germination, which corresponded with massively increased levels of major classes of non-enzymatic lipid peroxidation products such as hydroxyl fatty acids, malondialdehyde, and phytoprostanes [15,16]. Since *vte1* seeds accumulate the biosynthetic intermediate DMPBQ, which is thought to have some antioxidant activity, the role of tocopherols in seeds is probably not attributed exclusively to the scavenging ability of ROS. Recently, plastochromanol-8 (PC-8), a third type of tocochromanol with a longer side chain, was also found to be abolished in *Arabidopsis vte1* plants, but accumulated in *vte2* plants. *Vte1 vte2* double mutants that lack tocopherols, PC-8 and DMBPQ, exhibited the most severe physiological and biochemical phenotype for any tocochromanol-affected genotype, indicating that both PC-8 and tocopherols play essential roles in suppressing lipid oxidation initiated during seed desiccation and quiescence in *Arabidopsis* [17]. Contrary to tocopherols, the role of tocotrienol has only been investigated in human health and disease, while seldom investigated in plants.

Rice is both a model system for monocots and an important staple food crop worldwide, as it feeds over half of the world's population. Rice seeds undergo rapid deterioration in humid tropical regions [18] and under anaerobic conditions [19]. Therefore, rice breeders and physiologists are concerned about rice seed vigor and longevity traits. Rice has a completely different type of seed (endospermic and starchy seeds) when compared with the non-endospermic oleaginous seed of *Arabidopsis*. Rice seeds not only accumulate a high level of tocopherols in the embryo but they also accumulate tocotrienols, which occur exclusively in the pericarp and endosperm. There are few studies on the role of tocotrienols in monocots. Since rice grains can be dissected, they are a well-suited system for investigating the role of tocopherols and tocotrienols in starchy seeds. Previously, we generated RNAi rice plants with silenced HPT and TC activities in tocopherol biosynthesis [20]. In the current study, we generated RNAi rice plants that silenced HGGT activity and studied the impact of tocopherols and tocotrienols on seed longevity in rice. Our findings will further our understanding of the functions of tocochromanols, especially tocotrienols, in starchy seeds.

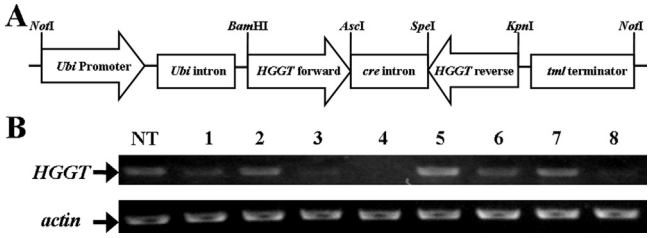

Fig. 1. Development of RNAi rice plants that disrupted HGGT activity by using RNAi-mediated silencing. A Representation of the T-DNA from the binary intron-spliced hairpin RNA (RNAi) expression construct used for transformation. The 444-bp rice HGGT cDNA (accession no. AY222862) was inserted into the pSTARLING vector in sense and antisense orientation separated by the cre intron and then cloned into the binary vector of pWec8 under the control of the *Ubi* promoter and *tml* terminator. B The transcript level of HGGT in the panicles of some HGGT:RNAi transgenics. β-actin (AB047313) was used as the internal control.

2. Materials and methods

2.1. Plant material

HPT:RNAi-4 and RNAi-10 and TC:RNAi-2 and RNAi-8 rice plants used in the study have Nipponbare background, and they were generated by constitutively silencing HPT and TC activities [20]. HGGT:RNAi-4 and RNAi-8 rice plants were generated by constitutively silencing HGGT activity by using the methods described below. Three types of RNAi plants were planted in Baodi Experimental Station, Tianjin, and self-pollinated for the homologous T_3 generation. Seeds were stored dry in paper bags at room temperature for at least one month before the experiments started.

2.2. Generation of HGGT:RNAi rice plants

A 443-bp fragment containing nucleotides 494–937 of the rice HGGT cDNA clone (accession no. AY222862) were amplified by reverse transcription PCR by using the primers RNAi-HGGT-F/RNAi-HGGT-R (Table S). Then, they were sequentially inserted in sense and antisense orientation into the *Bam*HI/*Asc*I site and *Kpn*I/*Spe*I site of pSTARLING (a kind gift from the Commonwealth Scientific and Industrial Research Organisation, CSIRO, Australia). Finally, the entire RNAi cassette (Fig. 1) was excised and inserted into pWec8 and then introduced into *Agrobacterium tumefaciens* EHA105. Transgenic plants were generated by *Agrobacterium*-mediated transformation of mature seed-derived calli, as described previously [20].

2.3. PCR and RT-PCR analysis

Genomic DNA was isolated from young leaves by using a modified CTAB method [21]. PCR was performed to preliminarily select transgenic T_0 plants by using the primers SL-Ubi-C-F/SL-Ubi-C-R (Table S). RT-PCR was performed to confirm the silencing effect of the target gene in PCR-positive plants. Total RNA was extracted from panicles by using the RNAultra Extraction Kit (Qiagen, Valencia, CA, USA). cDNAs were synthesized with oligo(dT) primers by using the SuperScript first-strand synthesis system, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The targeted fragment of HGGT (249 bp) was amplified using cDNAs as templates and HGGT-RT as primers. A 250-bp fragment of rice β-actin (AB047313) was also amplified as the quantitative control by using the primers described previously (Table S).

2.4. Tocochromanol analysis

Tocochromanols were extracted from whole kernels or kernel sub-fractions, according to the method described previously