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Functional biotechnology

Engineered drought-induced biosynthesis of α-tocopherol alleviates stress-induced leaf damage in tobacco

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ABSTRACT

Tocopherols are members of the vitamin E complex and essential antioxidant compounds synthesized in chloroplasts that protect photosynthetic membranes against oxidative damage triggered by most environmental stresses. Tocopherol deficiency has been shown to affect germination, retard growth and change responses to abiotic stress, suggesting that tocopherols may be involved in a number of diverse physiological processes in plants. Instead of seeking constitutive synthesis of tocopherols to improve stress tolerance, we followed an inducible approach of enhancing α-tocopherol accumulation under dehydration conditions in tobacco. Two uncharacterized stress inducible promoters isolated from *Arabidopsis* and the *VTE2.1* gene from *Solanum chilense* were used in this work. *VTE2.1* encodes the enzyme homogentisate phytyltransferase (HPT), which catalyzes the prenylation step in tocopherol biosynthesis. Transgenic tobacco plants expressing *ScVTE2.1* under the control of stress-inducible promoters showed increased levels of α-tocopherol when exposed to drought conditions. The accumulation of α-tocopherol correlated with higher water content and increased photosynthetic performance and less oxidative stress damage as evidenced by reduced lipid peroxidation and delayed leaf senescence. Our results indicate that stress-induced expression of *VTE2.1* can be used to increase the vitamin E content and to diminish detrimental effects of environmental stress in plants. The stress-inducible promoters introduced in this work may prove valuable to future biotechnological approaches in improving abiotic stress resistance in plants.

Introduction

Tocopherols are lipid-soluble molecules and members of the vitamin E compounds that are synthesized exclusively in oxygenic photosynthetic organisms. Natural vitamin E includes tocopherols and tocotrienols that function as potent lipid-soluble antioxidants (Schneider, 2005). Despite the similar antioxidant properties of these vitamers (Munne-Bosch and Alegre, 2002), α-tocopherol is the most active in animals because of its high retention in their bodies (Schneider, 2005). In plants, α-tocopherol usually accounts for more than 90% of the foliar content of vitamin E, whereas γ-tocopherol is more abundant in seeds. β- and δ-tocopherol are scarce forms in most plant species. Hence, it has been proposed that α-tocopherol plays a major function as an antioxidant soluble compound along with ascorbate and glutathione (Foyer and Noctor, 2005). Tocopherols have two main antioxidant functions. First, in the quenching of singlet oxygen generated mostly by triplet chlorophyll in photosystem II (Krieger-Liszkay, 2005) and second, in the scavenging of harmful radicals by donating an electron from the chromanol ring to, for example, a lipid peroxyl radical to prevent membrane lipid peroxidation reactions (Sattler et al., 2004a).

Roles for tocopherols and tocotrienols in plants have been reviewed in recent years (Li et al., 2008; Falk and Munné-Bosch, 2010; DellaPenna and Mène-Saffrané, 2011; Lushchak and Semchuk, 2012). For some time, the function of tocopherols has been controversial. Initial analyses of tocopherol-deficient mutants (*vte1* and *vte2*) of the cyanobacterium *Synechocystis* and of *Arabidopsis* showed limited contribution of tocopherols to the protection against photo-oxidative stress under normal growth or high light stress conditions (Maeda et al., 2005). Other studies have determined that tocopherols have an antioxidant function in the photo-protection and reduction of lipid peroxidation (Maeda et al., 2005; Falk and Munné-Bosch, 2010) as well as a positive effect on seed longevity and prevention of fatty acid oxidation

Abbreviations: ABA, abscisic acid; GUS, β-glucuronidase; HPT/VTE2.1, homogentisate phytyltransferase; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ROS, reactive oxygen species; WT, wild type.

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during germination (Sattler et al., 2004b). Abbasi et al. (2007) showed that tobacco plants with low total tocopherol content are more susceptible to oxidative and abiotic stress conditions; however, plants that accumulate only γ-tocopherol can withstand osmotic stress, suggesting specific roles for the α and γ forms. In another study, Sirikhachornkit et al. (2009) found that accumulation of β-tocopherol but not α-tocopherol improves tolerance to photo-oxidative stress in a *Chlamydomonas reinhardtii* strain lacking xanthophylls. Consequently, it is still unclear why some forms of tocopherols and tocotrienols are found in certain organs or plant groups and why there is a preference to accumulate α-tocopherol in chloroplasts.

Genetic engineering strategies have been used to increase vitamin E content in *Arabidopsis*, tobacco and soybean. The outcomes depended on the gene used to manipulate the biosynthetic pathway and varied among species. The strategies have considered constitutive over-expression of *HPPD* (p-hydroxyphenylpyruvate dioxygenase), *HGGT* (homogentisic acid geranylgeranyl transferase), *VTE1* (tocopherol cyclase) and *VTE4*/γ-*TMT* (γ-tocopherol methyltransferase) as well as down-regulation of *VTE1* and *VTE2*/*HPT* (homogentisate phytyltransferase) through RNAi technology (Tsegaye et al., 2002; Cahoon et al., 2003; Collakova and DellaPenna, 2003; Li et al., 2010, 2011). Evidences of the broad effects of tocopherols on plant growth and responses to stress have been shown through manipulation of the biosynthetic pathway and analyses of biosynthesis mutants. It has been postulated that tocopherols play an important role in the ROS-scavenging network along with other antioxidants such as ascorbate and glutathione (Miller et al., 2010). In addition, α-tocopherol also seems to have effects on carbohydrate metabolism and photoassimilate transport (Maeda et al., 2006) and on the crosstalk with hormone signals, which suggest a non-antioxidant function in plants (Falk and Munné-Bosch, 2010).

Comparison of two *Solanum* species (*S. chilense* and *S. lycopersicum*) differing in their tolerance to drought revealed that, in addition to lower stomatal conductance, *S. chilense* presented higher levels of α-tocopherol than *S. lycopersicum* under water-limiting conditions (Loyola et al., 2012). The expression of the gene encoding VTE2/HPT1 that catalyzes the condensation of homogentisate and phytyl diphosphate (the committed step for the synthesis of tocopherol), was higher in *S. chilense* than in *S. lycopersicum*. In addition, a lower degree of lipoperoxidation was detected in *S. chilense* leaves than in cultivated tomato (Loyola et al., 2012), suggesting that α-tocopherol biosynthesis may be part of an adaptative mechanism of *S. chilense* to adverse environmental conditions. In this work, tocopherol biosynthesis was engineered in tobacco plants by expressing the *VTE2.1* gene from *S. chilense* under the control of drought-inducible promoters as an alternative to a constitutive expression approach. A significant increase in α-tocopherol synthesis was observed only in transgenic lines under water stress. Among stress-related physiological parameters, low lipoperoxidation and delayed leaf senescence were evident in transgenic plants compared to wild type.

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh ecotype Columbia, *Solanum chilense* (Dunal) Reiche and *Nicotiana tabacum* (L.) cv. Xanthi seeds were germinated and grown under standard greenhouse conditions (16 h light at 18 °C, 8 h dark at 23 °C and 60–70% relative humidity) and watered with 0.5X Murashige and Skoog (MS) nutrient solution (Murashige and Skoog, 1962).

Cloning of the stress-inducible promoters and VTE2.1 cDNA

Selection of drought-inducible promoters was performed from transcriptomic data published for *Arabidopsis* (Seki et al., 2001). Uncharacterized genes that are highly expressed under drought and osmotic stress conditions in vegetative tissues were identified. Their promoter regions (P47 and P80) were identified by analyzing the genomic sequence using the TAIR's Seqviewer tool (http://www.arabidopsis.org/servlets/sv) and were defined from the border of the preceding gene to the ORF's start codon. The specific primers design for PCR amplification of the promoter regions were: P47-Fwd, 5′-AAGCTTCTTGTCTTAGTCGGTGTAGAGCC-3′ and P47-Rev, 5′-TCTAGAATCGAAGGAACAAGATTACTGAGATTTGAG-3′ for At2g47770 and P80-Fwd, 5′-AATGAGTTATGTTGTAA-GCTTCATCTAGCC-3′ and P80-Rev, 5′-TCTAGAGAGACGTACAGA-AACAGAACGC-3′ for At1g80160.

A full length cDNA for *VTE2.1* from *S. chilense* (*ScVTE2.1*) was synthesized using specific primers (ScVTE2.1-Fwd, 5′-ATGGAATCTTTGCTTATTGGG-3′ and ScVTE2.1-Rev, 5′-CACCTCACGAGCGGTATG-3′) that were designed according to the homologous sequence for tomato found in the DFCI (TIGR) database (http://compbio.dfci.harvard.edu/tgi/plant.html) and total mRNA extracted from *S. chilense* using the SV total RNA isolation system (Promega, Madison, WI, USA). The mRNA sample was treated with DNaseI (Ambion, Austin, TX, USA) and reverse transcribed using the ThermoScript RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA, USA). The PCR reaction was performed as follows: one cycle of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1.5 min at 72 °C and finally 5 min at 72 °C. PCR products were separated by agarose gel electrophoresis and bands were extracted and purified using the E.Z.N.A® Gel extraction kit (Omega Bio-Tek, Inc., Norcross, USA). Both promoters and cDNA were cloned into the pGEM-T vector (Promega) and sequenced (Macrogen Inc., Chongro-ku, Seoul, South Korea).

Transient expression assays

Promoter-*GUS* constructs (P47-GUS and P80-GUS) were generated to evaluate the activation of promoters in tobacco leaves upon dehydration. These constructs were made by replacing the CaMV 35S promoter from the binary plasmid vector pBI121 (Clontech, Palo Alto, CA, USA) by the P47 or P80 promoters to drive transcription of the *GUS* reporter gene. All constructs were introduced into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation and a liquid bacterial culture was grown for each construct for 24 h at 28 °C. Cells where then spun down (4500 × *g* for 10 min) and resuspended in 10 mM $MgSO_4$, 10 mM MES and pH adjusted to 5.6, to reach a final OD_{600} of 0.6. Young leaves of 6-week-old tobacco plants were manually infiltrated with each bacterial suspension through the abaxial surface using a needleless plastic syringe. Transient *GUS* expression was evaluated by histochemical staining according to the method of Mohamed et al. (2004) and by quantification of the GUS activity using the fluorimetric method described by Jefferson et al. (1987). Enzyme activity was recorded as concentration of the MU product over total protein content in each reaction. Protein concentration was estimated by the Bradford method (Bradford, 1976).

Stable transformation of tobacco

The *ScVTE2.1* cDNA was inserted between the *Sma*I-*Sst*I sites in each promoter-*GUS* constructs replacing the *GUS* gene to generate the P47-ScVTE2.1 and P80-ScVTE2.1 genetic constructs. Both constructs were introduced into the *A. tumefaciens* strain LBA4404 and used to transfect tobacco by the standard leaf disc method (Horsch et al., 1985). T1 seeds were collected from T0 plants that were