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Research article

Molecular characterization of tocopherol biosynthetic genes in sweetpotato that respond to stress and activate the tocopherol production in tobacco

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ABSTRACT

Tocopherol (vitamin E) is a chloroplast lipid that is presumed to be involved in the plant response to oxidative stress. In this study, we isolated and characterized five tocopherol biosynthetic genes from sweetpotato (*Ipomoea batatas [L.] Lam*) plants, including genes encoding 4-hydroxyphenylpyruvate dioxygenase (IbHPPD), homogentisate phytyltransferase (IbHPT), 2-methyl-6-phytylbenzoquinol methyltransferase (IbMPBQ MT), tocopherol cyclase (IbTC) and γ-tocopherol methyltransferase (IbTMT). Fluorescence microscope analysis indicated that four proteins localized into the chloroplast, whereas IbHPPD observed in the nuclear. Quantitative RT-PCR analysis revealed that the expression patterns of the five tocopherol biosynthetic genes varied in different plant tissues and under different stress conditions. All five genes were highly expressed in leaf tissues, whereas *IbHPPD* and *IbHPT* were highly expressed in the thick roots. The expression patterns of these five genes significantly differed in response to PEG, NaCl and H_2O_2-mediated oxidative stress. *IbHPPD* was strongly induced following PEG and H_2O_2 treatment and *IbHPT* was strongly induced following PEG treatment, whereas *IbMPBQ MT* and *IbTC* were highly expressed following NaCl treatment. Upon infection of the bacterial pathogen *Pectobacterium chrysanthemi*, the expression of *IbHPPD* increased sharply in sweetpotato leaves, whereas the expression of the other genes was reduced or unchanged. Additionally, transient expression of the five tocopherol biosynthetic genes in tobacco (*Nicotiana bentamiana)* leaves resulted in increased transcript levels of the transgenes expressions and tocopherol production. Therefore, our results suggested that the five tocopherol biosynthetic genes of sweetpotato play roles in the stress defense response as transcriptional regulators of the tocopherol production.

1. Introduction

Environmental stresses trigger various plant responses, ranging from differential expression of genes and altered cellular metabolism to changes in growth rate and agricultural output. Excessive plant responses exist to circumvent the potentially harmful effects of a wide range of both abiotic and biotic stresses, including high salinity, drought and pathogen infection. Oxidative stress derived from reactive oxygen species (ROS) is one of the major factors affecting plant productivity when plants are exposed to various environmental stresses (Inzé and Montagu, 1995). Plants have

Abbreviations: DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; HGA, homogentisic acid; HPP, p-hydroxy phenylpyruvate; HPPD, 4-hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyltransferase; MPBQ MT, 2-methyl-6-phytylbenzoquinol methyltransferase; ROS, reactive oxygen species; TC, tocopherol cyclase; TMT, γ-tocopherol methyltransferase.

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developed many powerful antioxidant defense systems to quench and scavenge ROS triggered by environmental stress such as antioxidant enzymes and low molecular weight antioxidant compounds. Tocopherol is one such antioxidant compound. Tocopherols are a group of amphiphilic lipid soluble antioxidants (collectively known as vitamin E) that are synthesized exclusively in photosynthetic organisms (Fryer, 1992; Wang and Quinn, 2000; Munné-Bosch and Alegre, 2002). These compounds act as antioxidants in chloroplasts and play an important role in protecting plants from singlet oxygen and the propagation of lipid peroxidation, thus helping protect photosystem II (PSII) from photoinactivation and membrane lipids from oxidation (Trebst et al., 2002; Havaux et al., 2005). In addition to having antioxidant activity, tocopherols also help prevent lipid peroxidation during seed germination (Sattler et al., 2004), increase plant tolerance to metal-induced oxidative stress (Collin et al., 2008) and improve cold adaptation in plants (Maeda et al., 2006).

Tocopherols usually occur in one of four forms, namely, α-, β-, γ- and δ-tocopherol, differing only in the number and position of methylation on the chromanol ring. Among tocopherols, α-tocopherol is usually the predominant form in leaves and has the highest vitamin E activity, whereas seeds are rich in γ-tocopherol (Grusak and DellaPenna, 1999; Desel et al., 2007). β- and δ-tocopherol are rare in most plant species. Although the biosynthetic pathway of tocopherol in plants was elucidated three decades ago (Schultz et al., 1985; DellaPenna, 2005), the enzymes that are involved in this pathway were only recently identified. At least five types of enzymes are involved in the tocopherol biosynthetic pathway, excluding the phytyl-tail synthesis and utilization pathway. Homogentisic acid (HGA) is produced from the tyrosine aromatic amino acid catabolite p-hydroxy phenylpyruvate (HPP) by 4-hydroxyphenylpyruvate dioxygenase (HPPD) (Norris et al., 1995). Prenylation of HGA with phytyl diphosphate (PDP) is catalyzed by homogentisate phytyltransferase (HPT, VTE2) (Collakova and DellaPenna, 2001). The product of this reaction, 2-methyl-6-phytylbenzoquinol (MPBQ), is the first phytylquinol intermediate in the pathway and can be methylated to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) by MPBQ methyltransferase (MPBQ MT, VTE3) (Shintani et al., 2002). Both MPBQ and DMPBQ are substrates of tocopherol cyclase (TC, VTE1), yielding the first tocopherols of the pathway, δ-tocopherol and γ-tocopherol, respectively (Porfirova et al., 2002). Both δ- and γ-tocopherol can be methylated by γ-tocopherol methyltransferase (TMT, VTE4) to yield β- and α-tocopherol, respectively (Shintani, 1998).

In the model plant *Arabidopsis thaliana*, molecular genetic technology and biochemical genomics-based approaches have revealed all of the genes encoding tocopherol biosynthesis pathway enzymes (DellaPenna and Pogson, 2006). Functional studies of these genes using metabolic engineering have been conducted to increase the tocopherol contents in *Arabidopsis*, barley, tobacco and soybean (Tsegaye et al., 2002; Cahoon et al., 2003; Collakova and DellaPenna, 2003; Li et al., 2010, 2011). These strategies include constitutive over-expression of *HPPD*, *TC* and *TMT*, as well as down-regulation of *HPT* and *TC* through RNAi technology. The broad effects of tocopherols on plant growth and responses to stress have been revealed through manipulation of the tocopherol biosynthetic pathway and analysis 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). However, to our knowledge, the regulation, activities, integration and evolution of individual enzymes in root crop plants such as sweetpotato have not been clearly elucidated.

Sweetpotato (*Ipomoea batatas [L.] Lam*) is an economically valuable crop, providing food, animal feed and bioactive materials (Woolfe, 1992). In particular, sweetpotato is an important staple food in many developing countries ranging from tropical to temperate zones. Sweetpotato is not only a rich source of carbohydrates, potassium and dietary fiber, but it also contains various antioxidants such as carotenoids, anthocyanins, vitamin C and tocopherols (Yoshinaga et al., 1999; Ching and Mohamed, 2001; Teow et al., 2007). We previously carried out metabolic studies of carotenoids and anthocyanins in sweetpotato with a focus on biofortification and environmental stress tolerance (Kim et al., 2012, 2013; Park et al., 2015). Although sweetpotato is one of the richest sources of tocopherol among vegetables, molecular cloning and characterization of tocopherol biosynthetic genes have not yet been reported.

Therefore, in this study, to better understand the biosynthetic mechanisms of tocopherol underlying abiotic and biotic stress responses in sweetpotato, we isolated five full-length tocopherol biosynthetic genes (*IbHPPD*, *IbHPT*, *IbMPBQ MT*, *IbTC* and *IbTMT*) and characterized their expression profiles in response to various abiotic (PEG, NaCl and H_2O_2) and biotic (pathogen infection) stresses. The results of spatial and stress-responsive expression analysis of these genes suggest that some tocopherol synthetic genes play roles in specific tissues and in response to abiotic/biotic stresses in sweetpotato. Finally, we demonstrate that the five tocopherol biosynthetic genes can act as transcriptional regulators of tocopherol production by performing *Agrobacterium*-mediated transient expression of these genes in tobacco leaves.

2. Materials and methods

2.1. Plant materials

Sweetpotato (*Ipomoea batatas [L.] Lam* cv. Yulmi) plants were cultivated in a growth chamber at 25 ± 3 °C under a photoperiod of 16 h light/8 h dark for 3 months. Freshly harvested leaf, stem, fibrous root (<5 mm in diameter), thick pigmented root (<15 mm in diameter) and storage root (>15 mm in diameter) tissue were sampled 12 weeks after planting. The tissues were immediately frozen in liquid nitrogen and stored at −70 °C until further use.

2.2. Isolation of tocopherol biosynthetic genes

Total RNA was extracted from sweetpotato using TRIzol reagent (Invitrogen, USA), followed by treatment with RNase-free DNase I (Takara, Japan) to remove genomic DNA contamination. The RNA was used for rapid amplification of cDNA ends (RACE) after treatment with M-MLV reverse transcriptase (Clontech, USA). The sequence of a portion of the sweetpotato tocopherol cyclase (IbTC) gene was used to group the sequences of tocopherol biosynthetic genes obtained from the National Center for Biotechnology Information (NCBI) database. PCR was carried out to amplify the cDNA fragments for these genes with degenerate primers designed based on the conserved regions of the nucleotide sequences for cDNAs of *Ipomoea nil* and *Solanum tuberosum*. After sequencing the PCR products, 5′-RACE of the target cDNAs was performed using a SMART RACE cDNA amplification kit (Clontech) with Advantage 2 polymerase mix (Clontech). The RACE PCR gene-specific primer (GSP) sets 5′-ACATTGGACCCAGAACCCCTTTCTAGGG-3′ and 5′-AATGGTATCCGCTGTGAGGAGTTCG-3′ were used to isolate *IbTC* cDNA. Full-length cDNA for IbTC was amplified with the specific primers, and other tocopherol biosynthetic gene sequences were grouped based on the transcripts from the transcriptome library established in our laboratory.