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Isolation and characterization of terpene synthases in cotton (*Gossypium hirsutum*)

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ABSTRACT

Cotton plants accumulate gossypol and related sesquiterpene aldehydes, which function as phytoalexins against pathogens and feeding deterrents to herbivorous insects. However, to date little is known about the biosynthesis of volatile terpenes in this crop. Herein is reported that 5 monoterpenes and 11 sesquiterpenes from extracts of a glanded cotton cultivar, *Gossypium hirsutum* cv. CCRI12, were detected by gas chromatography–mass spectrometry (GC–MS). By EST data mining combined with Rapid Amplification of cDNA Ends (RACE), full-length cDNAs of three terpene synthases (TPSs), *GhTPS1*, *GhTPS2* and *GhTPS3* were isolated. By *in vitro* assays of the recombinant proteins, it was found that GhTPS1 and GhTPS2 are sesquiterpene synthases: the former converted farnesyl pyrophosphate (FPP) into β-caryophyllene and α-humulene in a ratio of 2:1, whereas the latter produced several sesquiterpenes with guaia-1(10),11-diene as the major product. By contrast, GhTPS3 is a monoterpene synthase, which produced α-pinene, β-pinene, β-phellandrene and trace amounts of other monoterpenes from geranyl pyrophosphate (GPP). The TPS activities were also supported by Virus Induced Gene Silencing (VIGS) in the cotton plant. *GhTPS1* and *GhTPS3* were highly expressed in the cotton plant overall, whereas *GhTPS2* was expressed only in leaves. When stimulated by mechanical wounding, *Verticillium dahliae* (*Vde*) elicitor or methyl jasmonate (MeJA), production of terpenes and expression of the corresponding synthase genes were induced. These data demonstrate that the three genes account for the biosynthesis of volatile terpenes of cotton, at least of this Upland cotton.

1. Introduction

Terpenoids constitute the largest family of natural products with more than 30,000 structures (Degenhardt et al., 2009b), which are grouped into different classes on the basis of the number of 5-carbon building blocks (Aharoni et al., 2005; Haagen-Smit, 1953). In addition to their physiological roles as phytohormones (gibberellic acid, abscisic acid and strigolactone), photosynthesis pigments (carotenoids and chlorophylls), and membrane structural components (sterols), terpenoids also have ecological functions in mediating plant interactions with biotic and abiotic factors. Volatile terpenes may help plants to attract pollinators or predators of herbivores (Degenhardt et al., 2003; Pichersky and Gershenzon, 2002), and terpenoid phytoalexins can participate in defense against phytopathogens and herbivores (Balkema-Boomstra et al., 2003; Nagegowda, 2010; Tan et al., 2000; Wang et al., 2004).

In plant cells, precursors of terpenoids are synthesized via either the cytosolic mevalonate pathway (MVA pathway) or the plastidial 2-C-methyl-D-erythritol-4-phosphate pathway (MEP pathway), and are then condensed into structural diverse terpenoids by the family of terpene synthases (TPSs). The TPS family consists of isoprene synthases producing 5-carbon isoprene using dimethylallyl pyrophosphate (DMAPP), monoterpene synthases producing 10-carbon monoterpenes from geranyl pyrophosphate (GPP), sesquiterpene synthases producing 15-carbon sesquiterpenes from farnesyl pyrophosphate (FPP), and diterpene synthases producing 20-carbon diterpenes from geranylgeranyl pyrophosphate (GGPP) or copalyl pyrophosphate (CPP) (Nagegowda, 2010; Tholl, 2006). Most sesquiterpene synthases are localized in the cytosol, whereas monoterpene and diterpene synthases are usually in the plastid and have a N-terminal plastid transit peptide upstream of the "RRX_8W" motif (Williams et al., 1998). Almost all TPSs contain the "DDXXD" and the "NSE/DTE" motifs at the C-terminal region for the metal dependent (frequently Mg^{2+} or Mn^{2+}) ionization of the prenyl diphosphate substrate that are essential for their catalytic activities (Chen et al., 2011; Tholl,

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2006). They constitute families with various members in plant genomes, ranging from only one functional and three pseudogenes (or fragments) of TPSs in moss (*Physcomitrella patens*), to 152 TPSs in grapevine (*Vitis vinifera*) (Hayashi et al., 2006; Martin et al., 2010). Recent phylogenetic analysis of TPSs from gymnosperms and angiosperms established presence of 7 subfamilies of TPS-a, b, c, d, e/f, g and h, with most monoterpene and sesquiterpene synthases of angiosperms being distributed in TPS-a, b and g subfamilies (Chen et al., 2011).

Cotton (*Gossypium* spp.) is an important economic crop and a major source of natural fiber for the textile industry. Cotton plants with epidermal pigment glands accumulate the sesquiterpene aldehyde gossypol (**1**) and related sesquiterpene aldehydes (hemigossypol (**2**), hemigossypolone (**3**), heliocides H_1 (**4**), H_2 (**5**), H_3 (**6**) and H_4 (**7**)) as major toxins against herbivorous insects, such as *Helicoverpa armigera*, *Heliothis virescens* and *Spodoptera exigua*, of these, hemigossypol (**2**) is a major phytoalexin that protects the plant from pathogens such as *Verticillium dahliae*, *Rhizoctonia solani*, *Xanthomonas campestris* and *Fusarium oxysporum*. Together, these pests cause severe yield loss in cotton-producing areas of the world (Abraham et al., 1999; Bell, 1967; Liu et al., 1999a; Stipanovic et al., 2006, 2008; Turco et al., 2007; Wu and Baldwin, 2010). Progress has been made in characterizing enzymes and transcription factors for gossypol (**1**) biosynthesis. The enzymes, farnesyl diphosphate synthase (FPS) (Liu et al., 1999a), (+)-δ-cadinene synthase (CDN or CAD1) (Chen et al., 1995; Tan et al., 2000), and CYP706B1, a cytochrome P450 monooxygenase that hydroxylates (+)-δ-cadinene (**8**) at the 8-position (Luo et al., 2001), catalyze three consecutive steps of gossypol (**1**) biosynthesis. Other related enzymes and proteins include P450 reductases (Yang et al., 2010), a peroxidase (Benedict et al., 2006; Stipanovic et al., 1992), a laccase (Liu et al., 2008), a dirigent protein (Liu et al., 2008) and a methyltransferase (Liu et al., 1999b). The transcription factor GaWRKY1 was shown to regulate one of the (+)-δ-cadinene synthase gene, *CDN-A* (Xu et al., 2004).

Most plants produce and emit a large number of volatile compounds. For example, *Arabidopsis* flowers emit a mixture of volatiles consisting of over 20 sesquiterpenes (Tholl et al., 2005); rice (*Oriza sativa*) synthesizes 13 sesquiterpenes after methyl jasmonate (MeJA) treatment (Cheng et al., 2007); and *Selaginella moellendorffii*, a lycophyte, produces the monoterpene linalool (**9**) and the sesquiterpenes β-elemene (**10**), germacrene D (**11**), β-sesquiphellandrene (**12**) and nerolidol (**13**) after elicitation with alamethicin, a fungal antibiotic (Li et al., 2012). It has been reported that cotton

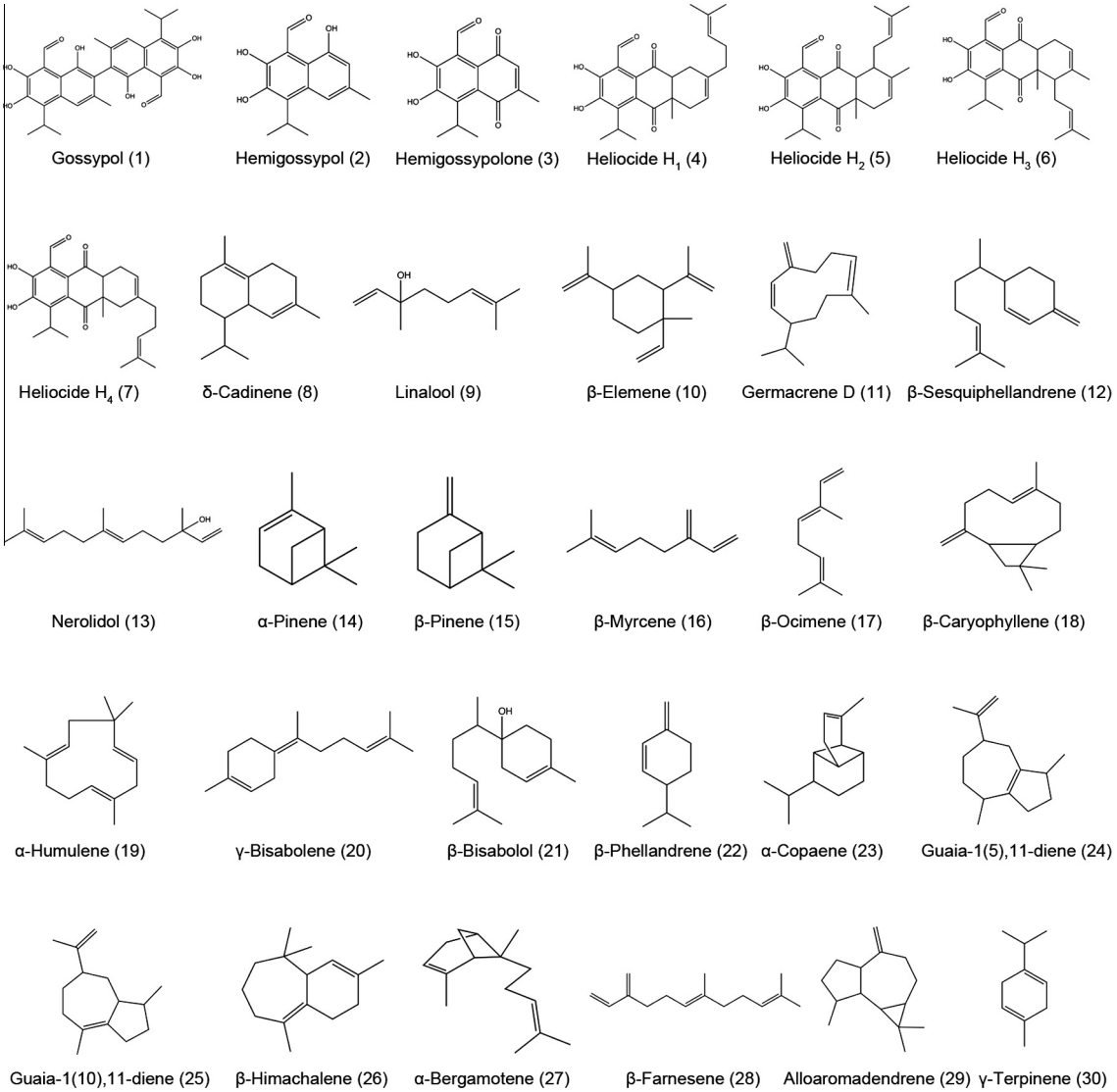

Fig. 1. Structures of monoterpenes and sesquiterpenes produced by cotton plants and *in vitro* activity assays of cotton terpene synthases.