RESEARCH PAPER

**CitAP2.10** activation of the terpene synthase **CsTPS1** is associated with the synthesis of (±)-valencene in ‘Newhall’ orange

Shu-ling Shen1,2,†, Xue-ren Yin1,2,†, Bo Zhang1,2,3, Xiu-lan Xie1, Qian Jiang1, Donald Grierson1,4 and Kun-song Chen1,2,3,*

1 College of Agriculture & Biotechnology, Zhejiang University, Zijingang Campus, Hangzhou 310058, PR China
2 Zhejiang Provincial Key Laboratory of Horticultural Plant Integrative Biology, Zhejiang University, Zijingang Campus, Hangzhou 310058, PR China
3 The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Zijingang Campus, Hangzhou 310058, PR China
4 School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK

* Correspondence: akun@zju.edu.cn
† These authors contributed equally to this manuscript.

Received 2 December 2015; Accepted 19 April 2016

Editor: Qiao Zhao, Tsinghua University

**Abstract**

Aroma is a vital characteristic that determines the quality and commercial value of citrus fruits, and characteristic volatiles have been analyzed in different citrus species. In sweet orange, *Citrus sinensis*, the sesquiterpene (±)-valencene is a key volatile compound in the fruit peel. Valencene synthesis is catalyzed by the terpene synthase CsTPS1, but the transcriptional mechanisms controlling its gene expression are unknown. Here, the AP2/ERF (APETALA2/ethylene response factor) transcription factor, **CitAP2.10**, is characterized as a regulator of (±)-valencene synthesis. The expression pattern of **CitAP2.10** was positively correlated with (±)-valencene content and CsTPS1 expression. Dual-luciferase assays indicated that **CitAP2.10** could trans-activate the **CsTPS1** promoter. Ethylene enhanced expression of **CitAP2.10** and this effect was abolished by the ethylene antagonist 1-methylcyclopropene. The role and function of **CitAP2.10** in (±)-valencene biosynthesis were confirmed using the Arabidopsis homolog (**AtWRI1**), which also transiently activated the **CsTPS1** promoter. Furthermore, transient over-expression of **CitAP2.10** triggered (±)-valencene biosynthesis in sweet orange fruit. These results indicate that **CitAP2.10** regulates (±)-valencene synthesis via induction of **CsTPS1** mRNA accumulation.

**Key words:** CitAP2/ERF, *Citrus sinensis*, citrus volatile, ethylene, sweet orange, terpene synthase, TPS, transcriptional regulation, valencene.

**Introduction**

Over 1000 individual volatile organic compounds have been identified from plants (Qualley and Dudareva, 2009). These volatiles affect plant growth, development, and environmental adaptation (Kessler and Baldwin, 2001). For fruit, aroma is one of the most significant quality traits, potentially influencing consumer acceptance. Most fruit, such as mango (Singh...
and Saini, 2014), strawberry (Van de Poel et al., 2014), pear (Moya-Leon et al., 2006), melon (Gonda et al., 2013), and citrus (Shi et al., 2007) produce large amounts of volatiles. Fruits are particularly useful for aroma research, as specific compounds are unique to the fruit and are not found in other plant tissues, e.g., esters can be detected in mango fruit but are absent from leaves (da Silva et al., 2012; Lalel et al., 2003), and linalool and C10 lactones appear in ripe peach but are absent from leaves (Horvat and Chapman, 1990). Citrus fruit are also excellent materials for terpenoid research. The volatile terpenoids, a large and structurally diverse group of secondary metabolites, are represented by isoprenes (C5), monoterpenes (C10), and sesquiterpenes (C15). Volatile terpenoids are all derived from two C5-isoprene building units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthesized from the mevalonate (MVA) and non-mevalonate (MEP) pathways (Megarvey and Croteau, 1995). Most of the terpenes are synthesized from their corresponding substrates by terpene synthases (TPS). In citrus species, several TPS genes have been characterized, including CitMTSE1 (AB110636.1) from satsuma mandarin (Citrus unshiu) (Marcow, Shimada et al., 2004), RfMT1 (AB691531.1, AB266584.1, AB266585.1) from citrus jambhiri (Yamasaki and Akimitsu, 2007; Shishido et al., 2012), and (+)-valencene biosynthesis-related CsTPS1 (AF514289.1) from sweet orange (Citrus sinensis, Sharon-Asa et al., 2003).

The mechanisms and identities of transcription factors regulating terpenoid biosynthesis have been characterized in model plants, where members of the ARF (auxin response factors), MYC (myelocytomatosis related proteins), MYB (myeloblastosis related proteins), and WRKY (amino-acid sequence WRKYGQK) families have been implicated. It has been reported that the Arabidopsis auxin responsive factors AtARF6 and AtARF8, as well as AtMYB21 and AtMYB24, can induce the production of sesquiterpenes (Mandaokar et al., 2006; Reeves et al., 2012), and AtMYC2 can regulate synthesis of sesquiterpenes by binding to the promoter of AtTPS11 and AtTPS21 (Hong et al., 2012). PtMYB14 is involved in regulating the MVA pathway, as well as jasmonate metabolism, resulting in the release of volatile terpenoids in coniferous trees (Bedon et al., 2010). Similarly, NaWRKY3 and NaWRKY6 participate in defense against herbivores in Nicotiana attenuata by producing terpenoids to attract predators and reduce the number of herbivorous larvae (Skibbe et al., 2008). Despite the increasing number of investigations of the transcription factors regulating aroma in the plant kingdom, there have been few previous reports on the transcriptional regulation of fruit volatiles.

The formation of plant volatiles is regulated by both internal (developmental, tissue-specific) and external (environmental) factors. For instance, some volatile terpenoids can be induced or inhibited by environmental stresses such as wounding, insect and herbivore attack, and by hormones such as gibberellins and jasmonates (Faldt et al., 2003; Feng et al., 2008; Dicke et al., 2009). Ethylene is an important hormone that regulates fruit ripening and quality, including aroma, and ethylene treatment induces ripening and volatile synthesis (Alexander and Grierson, 2002), while inhibition of ethylene production by antisense ACC (aminocyclopropane-carboxylate) oxidase RNA in melon prevents volatile production (Pech et al., 2008, 2012). Ethylene response factors (ERFs) have recently been identified as targets for fruit ripening and quality research, and SIERRFs have been demonstrated to be ripening-related transcription factors (Liu et al., 2016). MaERFs have been shown to be involved in banana fruit ripening (Xiao et al., 2013); PsERFs determine the rate of ripening in Japanese plum (El-Sharkawy et al., 2009); apple MdCBF and kiwifruit AdCBF9 participate in fruit softening (Tacken et al., 2010; Yin et al., 2010); and persimmon DkERF9/10/19/22 contributes to fruit deastringency (Min et al., 2012, 2014). Although an increase in fruit aroma production is usually associated with fruit ripening, the potential role of AP2/ERF (APETALA2/ethylene response factor) transcription factors in fruit aroma has not previously been explored.

Using a citrus genome database, 126 CitAP2/ERF genes were isolated and most of them were found to be differentially expressed during citrus fruit development, which indicates their potential involvement in the ripening of citrus fruit (Xie et al., 2014). In the present study, the transcriptional regulatory roles of CitAP2.10, a CitAP2/ERF family member, in regulating (+)-valencene production in citrus sweet orange ‘Newhall’ fruit were investigated during different stages of fruit development, and in response to treatment with ethylene and its action inhibitor 1-methylcyclopropene. A potential role for CitAP2.10 in regulating citrus (+)-valencene production was examined by transient overexpression in citrus peels and the action on the promoter of the target gene CsTPS1 was studied using a tobacco dual-luciferase assay.

Materials and methods

Plant materials and treatments

Fruit of eleven citrus cultivars from six species were collected at the commercial ripened stages (Table 1; related physiological data are indicated in Supplementary Table S1 at JXB online).

Fruits of the sweet orange (Citrus sinensis) cultivar ‘Newhall’ were obtained from a commercial orchard in Songyang (Zhejiang, China) at seven different developmental stages, namely at 120, 135, 150, 165, 180, 195 and 210 d after full bloom (DAFB) in 2011. In order to study the effect of ethylene on the biosynthesis of citrus sesquiterpenes, ‘Newhall’ sweet orange fruit were collected at 150 DAFB from the same orchard in 2012, treated separately with 40 μl 1-MCP and stored at 20 °C for 5 d after harvest. For treatments, the harvested fruit were randomly divided into three groups for each treatment, as three biological replicates. Peel samples taken from the equatorial portion of fruits were cut into small pieces, immediately frozen in liquid nitrogen and stored at –80 °C for subsequent study.

Volatile compounds analysis

The frozen tissues were ground in liquid nitrogen and 1 g was weighed into a 15-ml headspace vial containing 5 ml saturated sodium chloride solution. Before capping of the vial, 50 μl of 1-Hexanol (0.1%, v/v) was added as an internal standard. After vigorous vortexing, the samples were incubated at 40 °C for 30 min with continuous agitation (600 rpm). Following this, a SPME fiber coated with 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)
Table 1. Commercial ripened stages of 11 citrus cultivars

| Common name         | Cultivar and species                  | Days after full bloom (DAFB) |
|---------------------|---------------------------------------|-----------------------------|
| Bitter Orange       | ‘Goutoucheng’ (Citrus aurantium)      | 215                         |
| Citron              | Lemon (Citrus limon)                  | 210                         |
|                     | ‘Bergamot’ (Citrus medica)            | 170                         |
| Hybrids             | ‘Huyou’ (Citrus changshanhensis)      | 205                         |
|                     | ‘Hongshigan’ (Citrus reticulata× Citrus sinensis) | 215 |
| Mandarin            | Satsuma (Citrus unshiu)               | 180                         |
|                     | ‘Ponkan’ (Citrus reticulata)          | 225                         |
| Pummelo             | ‘Yuhuan’ (Citrus grandis)             | 210                         |
|                     | ‘Zaoxian’ (Citrus grandis)            | 200                         |
| Sweet Orange        | ‘Newhall’ (Citrus sinensis)           | 210                         |
|                     | ‘Fengjie’ (Citrus sinensis)           | 210                         |

(Supelco Co., Bellefonte, PA, USA) was used to extract the volatiles under the same conditions (40 °C, 600 rpm). Volatile analysis was carried out with an Agilent 7890A gas chromatograph (GC) coupled to an Agilent 5975C Network Mass Selective Detector (MS, inert XL MSD with triple-axis detector). After extraction, the fiber was exposed to the GC injection port at 250 °C for 5 min in splitless mode. Samples were separated using a HP-5MS column (5% phenyl methyl siloxane, 30 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA, USA). Helium was used as a carrier gas at 1.0 mL min⁻¹. For the peel samples, the oven temperature was programmed to start at 40 °C for 3 min, and then ramped to 70 °C at a rate of 3 °C min⁻¹, followed by a second ramp to 130 °C at a rate of 1 °C min⁻¹, and a third ramp to 230 °C at a rate of 15 °C min⁻¹. MS conditions were as follows: ion source, 230 °C; electron energy, 70 eV; GC-MS interface zone, 250 °C, and a scan range of 35–350 mass units. Volatiles were identified based on the database of the NIST/EPA/NIH Mass Spectral Library (http://chemdata.nist.gov/) and the Wiley Registry of Mass Spectral Data (http://onlinelibrary.wiley.com/book/10.1002/9780470175217). The identities of most of the volatiles were then confirmed by comparison with authentic standards. The internal standards were used for compensating for differences between samples, and the abundance of each volatile was calculated as its peak area. The identification of (+)-valencene was made by comparison with the mass spectrum of the standard compound, retention time, and its retention index. The results are also displayed as a heatmap in order to provide an overview of differences in volatile compounds among the citrus fruit samples. ANOVA was used for identification of characteristic volatiles. The differences indicated in Supplementary Table S4 are based on Tukey’s test at the 5% level (SAS version 8.0, SAS Institute, Cary, NC, USA).

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen tissues (0.3 g) of citrus peel according to the CTAB (cetyltri-methylammonium bromide) method developed from Chang et al. (1993). After removing the gDNA contamination by TURBO DNase (Ambion), 1.0 μg RNA-free RNA was used to synthesize the cDNA by means of an iScript cDNA Synthesis Kit (Bio-Rad). RNA extractions were carried out in parallel on three biological treatment replicates and samplings.

Gene isolation and sequence analysis

CitAP2/ERF genes and the promoter of CsTPS1 were isolated based on the sequences in the online citrus genome database (http://citrus.hzau.edu.cn/, Cs5g12900), and full-length AtWRIs (Arabidopsis AP2/ERF) genes were obtained based on the sequences in TAIR (The Arabidopsis Information Resource, https://www.arabidopsis.org/) with the primers listed in Supplementary Table S2. The gene sequences were translated by Primer Premier 5.0. Alignment and phylogenetic analysis was carried out by ClustalX (V.1.81) and Mega 4.0.2.

Real-time quantitative PCR

The oligonucleotide primers of CitAP2/ERF genes used for real-time quantitative PCR were as described in Xie et al. (2014). The CsTPS1 primers were designed by primer3 (http://frodo.wi.mit.edu/primer3) and are described in Supplementary Table S3. The specificity of the primers was tested with melting curves and resequencing of PCR products.

The real-time PCR was carried out with a Ssofast Eva Green Supermix Kit using a CFX96 instrument (Bio-Rad). The PCR mixture and reactions were as described in our previous report (Yin et al., 2012). Abundance of cDNA templates was monitored with citrus β-actin (Pilletteri et al., 2004). ΔΔCt was used to calculate the relative expression levels of genes.

Dual-luciferase assay

Transactivation activities of AP2/ERF on the target promoter were measured with dual-luciferase assays according to our previous reports (Yin et al., 2010; Xu et al., 2014). The full-length CitAP2.10 and AtWR1-4 (the homolog of CitAP2.10 in Arabidopsis) sequences were amplified with the primers described in Supplementary Table S2 and were inserted into a pGreenII 0029 62-SK vector. The promoter of CsTPS1 (573 bp) was constructed in the pGreenII 0800-LUC vector. All constructs were individually electroporated into Agrobacterium tumefaciens GV3101 and stored as glycerol stock at –80 °C. Agrobacterium cultures were prepared with infiltration buffer (10 mM MES, 10 mM MgCl₂, 150 mM acetosyringone, pH 5.6) to an OD₆₀₀ of 0.75. The mixtures of transcription factors 1 ml) and promoters (100 μl) were infiltrated into tobacco leaves by needleless syringes. Tobacco plants were grown in a growth chamber with a light/dark cycle of 16 : 8 h, at 24 °C.

Four-week-old plants were prepared for injection. Enzyme activities of firefly luciferase and renilla luciferase were assayed using dual-luciferase assay reagents (Promega), at 3 d after infiltration. For each transcription factor–promoter interaction, at least three independent experiments were performed, with four replicates in each experiment.

Transient overexpression in citrus peel

In order to determine the role of the CitAP2.10 gene in the regulation of (+)-valencene synthesis in citrus, transient overexpression analysis was performed on ‘Newhall’ fruit peel, as used previously for various other fruits, such as apple (Li et al., 2012), persimmon (Min et al., 2012), and strawberry (Hoffmann et al., 2006; Salvatierra et al., 2013). The Agrobacterium cultures carrying empty vector (SK) and constructs containing the target gene (CitAP2.10) were infiltrated into the same fruit, on opposite sides of the equatorial portion. Five days after infiltration, the peel near (<12 mm) the infiltration point (without including the infiltration spot) was
collected and immediately frozen in liquid nitrogen. These samples were stored at −80 °C for analysis of volatiles (1 g for volatile measurements for each replicate) and endogenous CsTPS1 expression (0.3 g for RNA extractions for each replicate).

Statistical analysis
The statistical significance of differences was calculated by single-factor ANOVA using Microsoft Excel (2013 version). Least-significant difference (LSD) at the 5% level was calculated using DPS7.05 (Zhejiang University, Hangzhou, China). Figures were drawn using Origin 8.0 (Microcal Software Inc.).

Results

(+)-Valencene is one of the characteristic volatile compounds in mature sweet orange

Following earlier comparative studies (Del Rio et al., 1992; González-Mas et al., 2011; Miyazaki et al., 2011), the volatile compounds from six species of citrus fruit (mandarin, sweet orange, bitter orange, hybrids, pummelo, and citron) plus some new cultivars, (satsuma, ‘Newhall’ and ‘Fengjie’) were analysed. A total of 119 volatiles were identified from these citrus fruit, including 16 monoterpenes, 37 sesquiterpenes, 36 monoterpenoids, 10 sesquiterpenoids, 14 aliphatic aldehydes and alcohols, and six aliphatic esters (Supplementary Table S4). Differences in volatile profiles were observed between the samples and fifteen characteristic volatile compounds from citrus species were successfully identified (Fig. 1). Bitter orange was characterized by (+)-cycloisosativene and copaene. The volatile profile of citrus fruit was complex: cis-α-bisabolene, α-bisabolol, eлиxene, and β-bisabolene were exclusively found in citron but germacrine B, camphor, α-bergamotene, (E)-geranial, and neral were also found to be characteristic for citron due to their relative higher concentrations as compared to the other species. Hybrid cultivars had a higher concentration of octylacetate while the pummelo species were characterized by (−)-α-panasinsen, farnesol and (+)-valencene. Sweet orange was characterized by the volatile compound (+)-valencene, which was much more abundant than in other citrus species (Fig. 1).

Screening for CitAP2/ERF genes encoding proteins that activate the Terpene Synthase 1 (CsTPS1) promoter

CsTPS1 has previously been characterized as the key gene encoding the enzyme terpene synthase that catalyzes (+)-valencene biosynthesis (Sharon-Asa et al., 2003). Therefore, we screened a large set of citrus transcription factors for activation or inhibition using the promoter of CsTPS1 and a dual-luciferase assay (Supplementary Fig. S1). Previously, an AP2 domain transcription factor (CrORCA3) was reported as a regulator of terpenoid indole alkaloids (van der Fits and Memelink, 2000). However, a linkage between a CitAP2/ERF transcription factor and developmental accumulation of (+)-valencene in sweet orange fruit has not yet been demonstrated. Utilizing the promoter of CsTPS1 and a dual-luciferase assay, the CitAP2/ERF genes were screened to identify any that activated the CsTPS1 promoter (−573~−1 bp region). Among the CitAP2/ERFs, one transcription factor, CitAP2.10, showed a 2.1-fold trans-activation effect on the CsTPS1 promoter (Fig. 2).

Association of CitAP2.10 and CsTPS1 expression and (+)-valencene accumulation during ‘Newhall’ fruit development

The percentage content of five classes of aroma substances in citrus peel (monoterpenes, sesquiterpenes, sesquiterpenoids, monoterpenoids, others) was analysed and the relative change in content during fruit development was measured, using fruits harvested 120 DAFB as a reference point (set as 0). The most significant class of volatiles that accumulated was the sesquiterpenes (Fig. 3), which had doubled by 180 DAFB and increased 12-fold by 210 DAFB, while the other four classes of aroma substances remained relatively less abundant (Fig. 3). The main sesquiterpene that accumulated during fruit development was (+)-valencene, which increased by 9-fold and 93-fold at 180 DAFB and 210 DAFB, respectively, which was much higher than any of the other volatiles (Fig. 3).

The expressions of CitAP2.10 and CsTPS1 were analysed in parallel with volatile accumulation in sweet orange ‘Newhall’ (Fig. 3). The results indicated that mRNAs for CsTPS1 and CitAP2.10 accumulated during maturation of sweet orange fruit (120 DAFB to 210 DAFB). Thus, CitAP2.10 expression was positively correlated with CsTPS1 activity and (+)-valencene accumulation.

Modulation by ethylene and 1-MCP of (+)-valencene content and expression of CitAP2.10 and CsTPS1

The correlation between CitAP2.10 and (+)-valencene was further tested by treatment with exogenous ethylene and 1-MCP (an ethylene antagonist). The results indicated that ethylene enhanced and 1-MCP inhibited (+)-valencene biosynthesis. In control fruit (Fig. 4) (+)-valencene content increased by approximately 16.4% between 0 d and 4 d, whereas treatment with ethylene enhanced valencene content by 30.9% and treatment with 1-MCP reduced it to 6.89% over the same time period. Further analysis of gene expression indicated that CsTPS1 mRNA showed the same accumulation pattern as (+)-valencene in fruit in response to both ethylene and 1-MCP treatment. CitAP2.10 transcript abundance was significantly higher than in the control following ethylene treatment, and significantly lower in fruit treated with 1-MCP relative to the control (Fig. 5).

Trans-activation activity of AtWRI1, a homolog of CitAP2.10 on the CsTPS1 promoter

The results above indicate that CitAP2.10 is positively associated with CsTPS1 and also with the accumulation of the metabolite (+)-valencene. In order to verify the regulatory role of CitAP2/ERF, four Arabidopsis AtWRI genes showing
CitAP2.10 regulates citrus (+)-valencene synthesis

The closest similarity to CitAP2.10 (Supplementary Fig. S2) were isolated and their trans-activation activities were tested using the dual-luciferase assays. The results (Fig. 6) indicated that AtWRI1 functioned in a similar way to CitAP2.10 and was able to activate the CsTPS1 promoter, while the other three AtWRI genes had limited effects.

Transient over-expression in ‘Newhall’ fruits

Citrus is a perennial woody plant and stable transformation is difficult and time-consuming. Thus, a more rapid and efficient transient overexpression assay was chosen to examine the function of CitAP2.10 in vivo. CitAP2.10 and the empty vector were separately injected on opposite sides of the equatorial portion in the same fruit and the volatiles of these parts were analysed 5 d after infiltration. The results indicated that introducing CitAP2.10 accelerated (+)-valencene synthesis (Fig. 7). The (+)-valencene content of the peels infiltrated with CitAP2.10 was 1.04 μg·g⁻¹, representing a significant
Fig. 4. Effects of ethylene and 1-MCP on production of aroma substances by fruit of sweet orange 'Newhall'. Fruits at 150 d after blossom were treated with ethylene (ETH, 40 μl l⁻¹, 12 h), 1-MCP (1 μl l⁻¹, 12 h), or air (CK, the control) separately and stored at 20 °C for 5 d. The quantity of aroma substances in treated fruits was calculated using the percentage content and compared to fruits at day 0 (fruits 120 d after full bloom). Error bars indicate SE from three replicates.
CitAP2.10 regulates citrus (+)-valencene synthesis

Discussion

(+)-Valencene is a characteristic terpene component in sweet orange

Aroma is a vital index of fruit quality, especially for citrus. Six citrus species covering eleven cultivars were examined, including some cultivars native to China, such as ‘Huyou’, ‘Goutoucheng’, and ‘Bergamot’. The results showed that volatiles were distributed differently in the various citrus samples. For mandarin, D-limonene, linalool, γ-terpinene, β-myrcene, α-pinene, (R)-(+)–citronellal, (-)-perillaldehyde, and (E, E)-2,4-decadienal were found to be abundant, which was similar to previous reports (Minh Tu et al., 2002; Njoroge et al., 2005; Miyazawa et al., 2010). For citron fruit, apart from (E)-geranial and neral that have been identified previously (Venturini et al., 2010), the characteristic volatile compounds consisted of germacrene B, (Z)-α-bisabolene, α-bisabolol, elexin, β-bisabolene, camphor, and α-bergamotene (Supplementary Table S4). In addition, some volatiles were unique to certain citrus fruit. Elexine was not detected in any of the species except those of citron, for which it is a unique volatile. In agreement with the report by Minh Tu et al. (2002), the detected peak area of δ-elemene was more than 0.005% but less than 0.05% in sweet orange and citron (Supplementary Table S4).

Valencene contributes to the powerful citrus aroma and is a characteristic component of ‘Sanguinello’ and ‘Moro’ juice (Citrus sinensis) (Maccarone et al., 1998). It traditionally acts as a marker due to the statistical correlation with the oil quality of citrus peel (Elston et al., 2005). We found that different species of citrus fruit had a great divergence in (+)-valencene content. The characteristic volatile compound in sweet orange, (+)-valencene, was much more abundant than in other citrus species (Fig. 1), which is consistent with the results of Maccarone et al. (1998). Based on a previous report (Sharon-Asa et al., 2003) and our results, (+)-valencene accumulation could also be considered as a characteristic for ripening sweet orange, as indicated by the burst of (+)-valencene production during late developmental stages (Fig. 3). Studies on citrus genomics indicate that sweet orange may be derived from pummelo (Xu et al., 2013), which is consistent with the detection of (+)-valencene in two pummelo cultivars (‘Yuhuan’ and ‘Zaoxiang’, Fig. 1).

Ethylene and 1-MCP modulated (+)-valencene biosynthesis in sweet orange fruit

Physiological analysis indicated that (+)-valencene is not only influenced by developmental stage, but also by stimuli such as...
the plant hormone ethylene. Ethylene regulation of the synthesis of volatile compounds has been widely studied in various fruits, including 'Charentais' melons (Flores et al., 2002), apple (Defilippi et al., 2005), and kiwifruit (Atkinson et al., 2011). The results of the current study demonstrated that ethylene enhanced (+)-valencene emission, which is similar to the findings of Sharon-Asa et al. (2003), whereas 1-MCP, an ethylene action inhibitor, reduced (+)-valencene emission in 'Newhall' fruit. Citrus is considered a non-climacteric fruit, yet according to a previous study, they are able to respond to ethylene (Katz et al., 2004). The results of ethylene treatment and especially 1-MCP treatment lead to the conclusion that activation of the ethylene signaling pathway in citrus stimulates (+)-valencene synthesis.

**CitAP2.10 modulates CsTPS1 transcription**

The impact of ethylene and 1-MCP on (+)-valencene synthesis led us to investigate the role of CitAP2/ERF genes in transactivation of the CsTPS1 promoter. The correlation between gene expression and (+)-valencene synthesis together with the dual-luciferase assays all support the conclusion that CitAP2.10 is involved in the regulation of (+)-valencene synthesis via CsTPS1. Previously, some AP2/ERF transcription factors have also been shown to be involved in the regulation of terpenoid synthesis in non-fruit systems. For example, CrORCA3 (an AP2 domain transcription factor) overexpression resulted in increased accumulation of terpenoid indole alkaloids in Catharanthus roseus MP183L cultures (van der Fits and Memelink, 2000), while AaERF1 and AaERF2 induced artemisinin synthesis by binding to the promoter of AaADS directly (Yu et al., 2012). However, none of these genes were reported to be involved in (+)-valencene biosynthesis. Thus, the results of the current study identify CitAP2.10 as a new regulator of terpenoid biosynthesis, and also as the first transcription factor involved in (+)-valencene production. However, the regulatory mechanisms of CitAP2.10 still require further investigation.

**Functional characterization of CitAP2.10 in (+)-valencene regulation**

Citrus is a perennial tree that is difficult to stably transform. Thus, transient overexpression experiments were conducted to test the role of CitAP2.10. Sweet orange peel infiltrated with CitAP2.10 exhibited a significant increase in the level of (+)-valencene. In parallel, CsTPS1 mRNA increased very significantly in response to CitAP2.10. Although further functional analysis needs to be performed with a stable transformation system, all the current results strongly indicate that CitAP2.10 can up-regulate synthesis of (+)-valencene via modulating transcription of CsTPS1. Recently, using a similar approach in tobacco leaves, apple MdoOMT1 and kiwifruit NAC and EIL transcription factors were shown to participate in biosynthesis of methylated phenylpropenes and terpenes, respectively (Nieuwenhuizen et al., 2015; Yauk et al., 2015), but the involvement of AP2/ERF members in fruit terpene synthesis has not been previously reported. The identification of CitAP2.10 means it could be used as target for genetic modification for manipulation of (+)-valencene content to meet marketing requirements.

**Activity of CitAP2.10-related genes in Arabidopsis**

In order to investigate the conservation of this function across species, a further experiment was conducted to isolate CitAP2.10 homologs from Arabidopsis (AtWRI1-4). CitAP2.10 has a tandem repeat of two AP2 domains and belongs to the AP2 subfamily, based on data from phylegetic analysis (Licausi et al., 2013). AtWRIs also belong to the AP2 subfamily (To et al., 2012). However, the possible relationship between AtWRIs and terpenoids has not yet been investigated.
been reported. In Arabidopsis, AtWRJs have been reported to be responsible for modulating the rate of acyl chain production and fatty acid synthesis (To et al., 2012). Here, of the four AtWRJs, AtWRRII was shown to interact with the CsTPSI promoter, producing a response of similar magnitude to the effect of CitAP2.10. These results suggest that AtWRRII might have conserved functions in regulating the production of terpenoids in Arabidopsis.

In conclusion, CitAP2.10 is a novel AP2/ERF transcription factor that is associated with (+)-valencene content in sweet orange fruit by modulating CsTPSI transcript abundance. The Arabidopsis transcription factor, AtWRRII, a CitAP2.10 homolog previously described as a key regulator of fatty acids (To et al., 2012), could also trans-activate the CsTPSI promoter, indicating that the function of CitAP2/ERF in (+)-valencene regulation may be conserved in various plants (Fig. 8). This result suggests an additional function for Arabidopsis AP2/ERF members. Despite the significant in vivo effects of CitAP2.10 on CsTPSI and (+)-valencene content, the underlying regulatory mechanisms will require further investigation.

Supplementary Data
Supplementary data are available at JXB online.

Table S1. Physiological data for mature citrus fruit from 11 commercial cultivars.

Table S2. Primers for full-length sequence amplification and promoter isolation.

Table S3. Primers for real-time PCR.

Table S4. Contents of 119 volatile compounds in citrus fruit from different species.

Figure S1. In vivo interaction of ethylene responsive factors and the promoter of CsTPSI.

Figure S2. Phylogenetic analysis of CitAP2.10 and Arabidopsis AP2 genes.

Acknowledgements
We would like to thank Dr Harry Klee (University of Florida) for editorial comments on the manuscript. This research was supported by the Program of International Science and Technology Cooperation (2011DFB31580), National Basic Research Program of China (2011CB100602), and National Natural Science Foundation of China (31372010).

References
Alexander L, Grierson D. 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. Journal of Experimental Botany 53, 2039–2055.

Atkinson RG, Gunaseelan K, Wang MY, Luo LK, Wang TC, Norling CL, Johnston SL, Maddumage R, Schroder R, Schaffer RJ. 2011. Dissecting the role of climacteric ethylene in kiwifruit (Actinidia chinensis) ripening using a 1-aminocyclopropane-1-carboxylic acid oxidase knockdown line. Journal of Experimental Botany 62, 3821–3835.

Bedon F, Bomal C, Caron S, et al. 2010. Subgroup 4 R2R3-MYB in conifer trees: gene family expansion and contribution to the isoprenoid- and flavonoid-oriented responses. Journal of Experimental Botany 61, 3847–3864.

Chang S, Puryear J, Cairney J. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11, 113–116.

da Silva RR, da Camara CAG, Almeida AV, Ramos CS. 2012. Biotic and abiotic stress-induced phenylpropanoids in leaves of the Mango (Mangifera indica L., Anacardiaceae). Journal of the Brazilian Chemical Society 23, 206–211.

Defilippí BG, Kader AA, Dandekar AM. 2005. Apple aroma: alcohol acetyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. Plant Science 168, 1199–1210.

Del Rio JA, Ortuno A, Garcia-Puig D, Porras I, Garcia-Lidon A, Sabater F. 1992. Variations of nootkatone and valencene levels during the development of grapefruit. Journal of Agricultural and Food Chemistry 40, 1488–1490.

Dicke M, van Loon JJA, Soler R. 2009. Chemical complexity of volatiles from plants induced by multiple attack. Nature Chemical Biology 5, 317–324.

El-Sharkawy I, Sherif S, Mela I, Bouzayan M, Jayasankar S. 2009. Molecular characterization of seven genes encoding ethylene-responsive transcriptional factors during plum fruit development and ripening. Journal of Experimental Botany 60, 907–922.

Elston A, Lin JM, Roseff R. 2005. Determination of the role of valencene in orange oil as a direct contributor to aroma quality. Flavour and Fragrance Journal 20, 381–386.

Fald J, Arimura G, Gershenson J, Takabayashi J, Bohlmann J. 2003. Functional identification of AtTPS03 as (E)-beta-ocimene synthase: a monoterpene synthase catalyzing jasmonate- and wound-induced volatile formation in Arabidopsis thaliana. Planta 216, 745–751.

Feng SH, Martinez C, Gusmaroli G, et al. 2008. Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. Nature 451, 475–479.
Flores F, El Yahyaoui F, de Billerbeck G, Romojaro F, Latche A, Bouzayen M, Pech JC, Ambid C. 2002. Role of ethylene in the biosynthetic pathway of aliphatic ester aroma volatiles in Charentais Cantaloupe melons. Journal of Experimental Botany 53, 201–206.

Gonda I, Lev S, Bar E, et al. 2013. Catabolism of L-methionine in the formation of sulfur and other volatiles in melon (Cucumis melo L.) fruit. The Plant Journal 74, 458–472.

González-Mas MC, Rambla JL, Alamar MC, Gutiérrez A, Granell A. 2011. Comparative analysis of the volatile fraction of fruit juice from different Citrus species. PLoS One 6, e22016.

Hoffmann T, Kalinowski G, Schwab W. 2006. RNA-induced silencing of gene expression in strawberry fruit (Fragaria × ananassa) by agroinfiltration: a rapid assay for gene function analysis. The Plant Journal 43, 818–826.

Hong GJ, Xue XY, Mao YB, Wang LJ, Chen XY. 2012. Arabidopsis MYC2 interacts with DELLA proteins in regulating sesquiterpene synthase gene expression. The Plant Cell 24, 2635–2648.

Horvat RJ, Chapman GW. 1990. Comparison of volatile compounds from peach fruit and leaves (Prunus persica) during maturation. Journal of Agricultural and Food Chemistry 38, 1442–1444.

Katz E, Lagunes PM, Riov J, Weiss D, Goldschmidt EE. 2004. Molecular and physiological evidence suggests the existence of a system II-like pathway of ethylene production in non-climacteric Citrus fruit. Plant Cell 219, 243–252.

Kessler A, Baldwin IT. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. Science 291, 2141–2144.

Lalé JHD, Singh Z, Tan SC. 2008. Aroma volatiles production during fruit ripening of ‘Kensington Pride’ mango. Postharvest Biology and Technology 27, 323–336.

Li YY, Mao K, Zhao C, Zhao XY, Zhang HL, Shu HR, Hao YJ. 2012. MdmCP1 ubiquitin E3 ligases interact with MdmYB1 to regulate light-induced anthocyanin biosynthesis and red fruit coloration in apple. Plant Physiology 160, 1011–1022.

Licausi F, Ohme-Takagi M, Perata P. 2013. APETALA/ethylene responsive factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. New Phytologist 199, 639–649.

Liu MC, Gomes BL, Milla I, et al. 2016. Comprehensive profiling of Ethylene Response Factors expression identifies ripening-associated ERF genes and their link to key regulators of fruit ripening in tomato. Plant Physiology 170, 1732–1744.

Maccarone E, Campisi S, Fallico B, Rapisarda P, Sgarlata R. 1998. Flavor components of Italian orange juices. Journal of Agricultural and Food Chemistry 46, 2293–2298.

Mandaokar A, Thines B, Shin B, Lange BM, Choi G, Koo YJ, Yoo YJ, Mandaokar A, Thines B, Shin B, Lange BM, Choi G, Koo YJ, Yoo YJ. 2011. Distribution of monoterpene volatiles in different Citrus kinokuni (Citrus × australasiaca) cultivars compared with Citrus reticulata and Citrus × limon. Chemistry and Biodiversity 8, 835–842.

Miyazaki T, Plotto A, Goodner K, Gmitter Jr FG. 2011. Distribution of aroma volatile compounds in tangerine hybrids and proposed inheritance. Journal of the Science of Food and Agriculture 91, 449–460.

Miyazawa N, Fujita A, Kubota K. 2010. Aroma character impact compounds in Kinokuni mandarin orange (Citrus kinokuni) compared with Satsuma mandarin orange (Citrus unshiu). Bioscience, Biotechnology, and Biochemistry 74, 835–842.

Moya-Leon MA, Vergara M, Bravo C, Montes ME, Moggia C. 2006. 1-MCP treatment preserves aroma quality of ‘Packham’s Triumph’ pears during long-term storage. Postharvest Biology and Technology 42, 185–197.

Nieuwenhuizen NJ, Chen X, Wang MY, Matich AJ, Perez RL, Allan AC, Green SA, Atkinson RG. 2015. Natural variation in monoterpene synthesis in kiwifruit: transcriptional regulation of terpene synthases by NAC and ETHYLENE-INSSENSITIVE3-like transcription factors. Plant Physiology 167, 1243–1258.

Njorge SM, Koaze H, Mwaniki M, Minh Tu NT, Sawamura M. 2005. Essential oils of Kenyan citrus fruits: volatile components of two varieties of mandarins (Citrus reticulata) and a tangelo (C. paradisi × C. tangerina). Flavour and Fragrance Journal 20, 74–79.

Pech JC, Bouzayen M, Latche A. 2008. Climacteric fruit ripening: ethylene-dependent and independent regulation of ripening pathways in melon fruit. Plant Science 175, 114–120.

Pech JC, Purgatto E, Bouzayen M, Latche A. 2012. Ethylene and fruit ripening. In: McManus M.T., ed. Annual Plant Reviews, volume 44, The Plant Hormone Ethylene. Oxford: Wiley-Blackwell, 275–304.

Pillitteri LJ, Lovatt CJ, Walling LL. 2004. Isolation and characterization of LEAFY and APETALA1 homologues from Citrus sinensis. Osbeck ‘Washington’. Journal of the American Society for Horticultural Science 129, 846–856.

Qualley AV, Dudareva N. 2009. Metabolomics of plant volatiles. Methods in Molecular Biology 553, 329–343.

Reeves PH, Ellis CM, Ploense SE, et al. 2012. A regulatory network for coordinated flower maturation. PLoS Genetics 8, e1002506.

Salvatierra A, Pimentel F, Moya-Leon MA, Herrera R. 2013. Increased accumulation of anthocyanins in Fragaria chiloensis fruits by transient suppression of FcMYB1 gene. Phytochemistry 90, 25–36.

Sharon-Asa L, Shalit M, Frydman A, Bar E, Holland D, Or E, Lavi U, Lewinsohn E, Eyal Y. 2003. Citrus fruit flavor and aroma biosynthesis: isolation, functional characterization, and developmental regulation of C57PS1, a key gene in the production of the sesquiterpene aroma compound valencene. The Plant Journal 36, 664–674.

Shi JX, Goldschmidt EE, Goren R, Porat R. 2007. Molecular, biochemical and anatomical factors governing ethanal fermentation metabolism and accumulation of off-flavors in mandarins and grapefruit. Postharvest Biology and Technology 46, 242–251.

Shimada T, Endo T, Fujii H, Hara M, Ueda T, Kita M, Omura M. 2004. Molecular cloning and functional characterization of four monoterpene synthase genes from Citrus unshiu Marc. Plant Science 166, 49–58.

Shishido H, Miyamoto Y, Ozawa R, Taniguchi S, Takabayashi J, Akimitsu K, Gomi K. 2012. Geraniol synthase whose mRNA is induced by host-selective ACT-toxin in the ACT-toxin-insensitive rough lemon (Citrus japonica). Journal of Plant Physiology 169, 1401–1407.

Singh SP, Saini MK. 2014. Postharvest vapour heat treatment as a phytosanitary measure influences the aroma volatiles profile of mango fruit. Food Chemistry 164, 387–395.

Skibbe M, Qu N, Galis I, Baldwin IT. 2008. Induced plant defenses in the natural environment: Nicotiana attenuata WRKY3 and WRKY6 coordinate responses to herbivory. The Plant Cell 20, 1984–2000.

Tacken E, Ireland H, Gunaseelan K, et al. 2010. The role of ethylene and cold temperature in the regulation of the apple POLYGALECTURONASE1 gene and fruit softening. Plant Physiology 153, 294–305.

To A, Joubes J, Barthole G, Lecureuil A, Scagnelli A, Jasinski S, Gonda I, Lev S, Bar E, et al. 2008. Induced plant defenses in the natural environment: Nicotiana attenuata WRKY3 and WRKY6 coordinate responses to herbivory. The Plant Cell 20, 1984–2000.

van der Fits L, Memelink J. 2000. ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289, 295–297.

Venturini N, Curf F, Desjobert JM, Karp D, Costa J, Paolini J. 2010. Chemotaxonomic investigations of peel and petitgrain essential oils from 17 citron cultivars. Chemistry and Biodiversity 7, 736–751.

Xiao YY, Chen JY, Kang JF, Shan W, Xie H, Jiang YM, Lu WJ. 2013. Banana ethylene response factors are involved in fruit ripening through their interactions with ethylene biosynthesis genes. Journal of Experimental Botany 64, 2499–2510.
Xie XL, Shen SL, Yin XR, Xu Q, Sun CD, Grierson D, Ferguson I, Chen KS. 2014. Isolation, classification and transcription profiles of the AP2/ERF transcription factor superfamily in citrus. Molecular Biology Reports 41, 4261–4271.

Xu Q, Chen LL, Ruan XA, et al. 2013. The draft genome of sweet orange (Citrus sinensis). Nature Genetics 45, 59–66.

Xu Q, Yin XR, Zeng JK, Ge H, Song M, Xu CJ, Li X, Ferguson IB, Chen KS. 2014. Activator- and repressor-type MYB transcription factors are involved in chilling injury induced flesh lignification in loquat via their interactions with the phenylpropanoid pathway. Journal of Experimental Botany 65, 4349–4370.

Xu YH, Wang JW, Wang S, Wang JY, Chen XY. 2004. Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-δ-cadinene synthase-A. Plant Physiology 135, 507–515.

Yamasaki Y, Akimitsu K. 2007. In situ localization of gene transcriptions for monoterpene synthesis in irregular parenchymic cells surrounding the secretory cavities in rough lemon (Citrus jambhiri). Journal of Plant Physiology 164, 1436–1448.

Yauk YK, Chagné D, Tomes S, Maitch AJ, Wang MY, Chen XY, Maddumage R, Hunt MB, Rowan DD, Atkinson RG. 2015. The O-methyltranferase MdoOMT1 is required for biosynthesis of methylated phenylpropenes in ripe apple fruit. The Plant Journal 82, 937–950.

Yin XR, Allan AC, Chen KS, Ferguson IB. 2010. Kiwifruit EIL and ERF genes involved in regulating fruit ripening. Plant Biology 153, 1280–1292.

Yin XR, Allan AC, Xu Q, Burdon J, Dejnoprat S, Chen KS, Ferguson IB. 2012. Differential expression of kiwifruit ERF genes in response to postharvest abiotic stress. Postharvest Biology and Technology 66, 1–7.

Yu ZX, Li JX, Yang CQ, Hu WL, Wang LJ, Chen XY. 2012. The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in Artemisia annua L. Molecular Plant 5, 353–365.