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Molecular Cloning and Expression Analysis of a Terpene Synthase Gene, *HcTPS2*, in *Hedychium coronarium*

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Abstract The *Hedychium coronarium* can emit a strong scent which is mainly composed of monoterpenes. A cDNA clone, *HcTPS2* (*H. coronarium* terpene synthases), was cloned from *H. coronarium* flower. The gene has an open reading frame of 1,788 bp which encodes a protein of 596 amino acids with a calculated molecular mass of 66.7 kDa. The deduced amino acid sequence shows 35–38% identity with known monoterpene synthases in other angiosperm species. *HcTPS2* was appreciably expressed in the petals, sepals, and stamens of *H. coronarium*, whereas no expression signal was detected in those of nonscented species. To the best of our knowledge, this is for the first time to clone the terpene synthase gene from *H. coronarium*, which provides the basis for biotechnological manipulation of scent composition in *H. coronarium*.

Keywords Terpene synthase · Expression · *HcTPS2* · *Hedychium coronarium*

Abbreviations

HcTPS2	*Hedychium coronarium* terpene synthases 2
RACE	Rapid amplification of cDNAs ends
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
SSC	Sodium chloride/sodium citrate (buffer)
TPS	Terpene synthase

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Introduction

In addition to color and shape, scent is an important factor for insects, birds, and mammals to help assure pollination (Andersson 2006; Ayasse 2006; Dobson 2006; Roeder et al. 2007). Many plants emit variable floral scents with respect to quality (composition) and quantity (Knudsen et al. 2006). Benzenoids, fatty acid derivatives, and terpenoids are the major components of floral scents (Chen et al. 2003; Tholl et al. 2004; Knudsen et al. 2006). In particular, monoterpenes, the C10 members of the terpenoid of natural products, are common constituents of floral scents, they are important in the reproductive biology of certain plants giving flowers and fruit scents that attract pollinating insects and dispersal agents (Pichersky et al. 2002; Aharoni et al. 2005). More recently, monoterpenes have been demonstrated to have other functions, such as attraction of herbivore enemies (Kessler and Baldwin 2001; Schnee et al. 2006; Tholl 2006; Köllner et al. 2008, 2009), induction of defense against insets (Keeling and Bohlmann 2006; Köpke et al. 2008; Yuan et al. 2008), and protection from oxidative damage (Dudareva et al. 2004; Loreto et al. 2004). These floral odors may have evolved from protective functions of monoterpenes in vegetative and reproductive organs of angiosperms and gymnosperms (Bohlmann et al. 2000).

Various terpene synthase (TPS) genes and enzymes have been isolated and characterized from several angiosperms and gymnosperms. Although terpenoids comprise compound classes that are emitted by many flowers, only a few terpene synthase genes for floral scent biosynthesis have been reported to date. Linalool synthase, a monoterpene synthase, was first isolated and characterized in *Clarkia breweri* flowers (Pichersky et al. 1995; Dudareva et al. 1996). Another two monoterpene synthases' genes,

(E)-beta-ocimene and myrcene synthase, were isolated in snapdragon (Dudareva et al. 2003). Two flower-specific terpene synthases have been isolated from satsuma mandarins (*Citrus unshiu* Marc), producing 1, 8-cineole and (E)-beta-ocimene, respectively (Shimada et al. 2005). The germacrene D synthase from *Rosa hybrida* is a sesquiterpene synthase, which uses farnesyl pyrophosphate as substrate to synthesize germacrene D (Guterman et al. 2002). A humulene synthase gene was cloned from the rhizome of shampoo ginger, and its expression can be induced by methyl jasmonate (Yu et al. 2008). Recently, two terpene synthase genes were isolated from the flowers of kiwi fruit, which mainly produce sesquiterpene, and are localized in the cytoplasm, the site for sesquiterpene production (Nieuwenhuizen et al. 2009). A new monoterpene synthase gene was recently identified from tomato that uses neryl diphosphate as the precursor for monoterpenes synthesis (Schilmiller et al. 2009).

Monoterpene biosynthesis occurs in plastids (Pérez et al. 1990; Wise et al. 1998), and all known monoterpene synthases are encoded by nuclear genes and possess an N-terminal transit peptide that directs their import into the plastid (Turner et al. 1999). The 30–80 amino-terminal residues are characterized with a low degree of similarity, typical of targeting sequences, yet those sequences are serine- and threonine-rich but with few acidic residues (Keegstra et al. 1989). The DDxxD motif was found in virtually all deduced sequences for enzymes that utilize prenyl diphosphate substrates (Starks et al. 1997; Whittington et al. 2002), is responsible for the coordination of divalent cations and is essential for substrate binding and ionization (Starks et al. 1997; Whittington et al. 2002; Christianson 2006; Zhou and Peters 2009). In addition to DDxxD, the monoterpene synthases possess characteristic motifs, including RR(x)8W and LYEASY,GTLXEL. The RR(x)8W motif is essential for the enzymatic activity of many monoterpene synthases (Bohlmann et al. 1998; Williams et al. 1998), and LYEASY.GTLXEL motif was also thought to be part of the active site (Wise et al. 1998). As a result, the characterization of enzymes and genes involved in flowers scent production and emission is still in its initial stages. Most monoterpene synthase genes were characterized in dicotyledon plants, and that from monocotyledon plants is especially limited.

Hedychium coronarium is a herbaceous perennial that reaches up to 2 m in height. The white ginger lily (*H. coronarium*) originated from the Himalayas region of Nepal and India, it has been introduced to many countries as an ornamental. *H. coronarium* is a monocotyledon plant, its flowers have a delicious perfume, particularly pronounced in the evening (Nakamura et al. 2009). The flowers emit a strong scent consisting of a relatively simple mixture of monoterpeniod and benzoid compounds, and its main components in petals is L-linalool,1,8-cineole and1,3, 7-octatriene-3,7-dimethyl, as have been reported in our previous studies. (Li and Fan 2007). So this species appeared to be an ideal source for the isolation of the related monoterpene synthase genes. In this study, a terpene synthase gene was isolated from *H. coronarium* flower using a functional genomics approach, and its expression was further analyzed at different flower development stages.

Materials and Methods

Plant Materials

Eight species from a local commercial plantation are used, including *Hedychium coccineum*, *Hedychium gardnerianum*, *Hedychium flavum*, *Hedychium chrysoleucum*, *H. gardnerianum* var. pink, *H. coccineum*, *Hedychium forrestii* and *Hedychium* spp.

RNA Extraction and Isolation of *HcTPS2*

Total RNA was isolated from frozen plant tissues using the hot borate method of Wan and Wilkins (1994). And that extracted from *H. coronarium* petals were used as templates for reverse transcription polymerase chain reaction (RT-PCR). The product (the first-strand cDNA) was subjected to PCR amplification by forward primer LR1 (5′-ACATCGCTGCTCTTCAGGCT-3′) and reverse primer LR2 (5′-GCCCAGCCCACTGTCCACAA-3′), which were designed with the conserved motifs of Zingiber officinale TPS gene (DY352211).

The 3′ and 5′ end of *HcTPS2* was isolated with a 3′ and 5′ rapid amplification of cDNAs ends (RACE) cDNA Amplification Kit (TaKaRa. Shiga, Japan), respectively. Gene-specific primers of LR3 (5′-CAGAGATGAGAA AGGCCAGT-3′), and LR4 (5′-TTGGAGGATGGAGAGA CTGC-3′) were used for 3′ RACE. And the following specific primer: LR5 (5′-GCCCAGCCCACTGTCCACAA-3′) and LR6 (5′-TCTCCATCCTCCAATTCAA-3′) were used for 5′ RACE.

Northern Blotting

Approximately 20 μg of total RNA was denatured, separated on 1.2% denaturing formaldehyde agarose gels, and transferred onto a Polyvinylidene difluoride (PVDF) membrane (Amersham Bioscience, Buckinghamshire, UK). The membrane was blot dried and cross-linked with UV at 280 nm. Digoxin (Dig)-labeled specific probes of 3′-untranslated regions of *HcTPS2* was made using a PCR DIG probe synthesis kit (Roche Applied Science, Mannheim, Germany)

with the following specific primers: LR7(5′-ACTACG-GATCCGAT TACGAACTATGAAGGA-3′) and LR8(5′-CTGTAAGCTT GTCTTTGAAGTGGTATGCCA-3′). The membrane was hybridized with DIG-labeled probe for 16 h at 45°C in high sodium dodecyl sulfate (SDS) buffer [7% SDS, 5× sodium chloride/sodium citrate (SSC), 50 mmol/L sodium-phosphate (pH 7.0), 2% blocking reagent, and 0.1% *N*-lauroylsarcosinel containing 50% deionized formamide (*v*/*v*)]. Blots were washed twice at 37°C in 2× SSC and 0.1% SDS for 10 min, followed by washing twice at 62°C in 0.1× SSC and 0.1% SDS for 30 min. The membranes were then subjected to immunological detection following the manufacturer′s instructions.

DNA Sequencing and Sequence Analysis

DNA fragments were sequenced at Invitrogen Biotechnology Company Ltd. (Shanghai, China). Coding sequences of the cDNAs were identified using DNASTAR (Burland 2000). HcTPS2 protein sequence was used as a query to search the protein database using NCBI resource. Alignments of protein sequences were performed using programs from DNAssist Package version 2 and CLUSTAL.W (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX). Sequence relatedness was analyzed using CLUSTAL.W and the neighbor-joining method (Kumar et al. 2001), the rooted tree was visualized using Treedraw (http://taxonomy.zoology.gla.ac.uk/rod/treeview.htm).

Results

Amino Acid Sequence Analysis of *HcTPS2*

The initial PCR primers were designed with conserved regions near the C-terminal Asp-rich motif (DDxxD) found in association with DY352211 fragment cloned from *Zingiber officinale*. The 560-bp cDNA fragment was amplified from mRNA in young flowers. The fragment exhibited sequence similarities to *TPS* genes (E≤e-19). To isolate full-length cDNA, 3′ and 5′ RACE approaches were used, and totally 2,145 nucleotides with a 174-bp 5′ untranslated region and an open reading frame of 1,788 nucleotides was obtained. The gene encodes a protein of 596 amino acids with a MW of 66.7 kDa and a calculated pI of 5.6. It was named as *HcTPS2*.

A BLAST search of GeneBank revealed that *HcTPS2* shared 38% identity with limonene synthase from *Citrus limon*, 36.4% identity with (3R)-linalool synthase from *Artemisia annua*, and 35.7% identity with myrecene synthase from *Arabidopsis thaliana* at the amino acids level (Fig. 1). There are several regions of conservation between *HcTPS2* and diverse original TPSs, including the active side DDxxD, RR(x)8W, and LYEASY.GTLXEL motifs.

Furthermore, *HcTPS2* contains an N-terminal transit peptide, approximately 26–52 amino acids, for plastid import, which is the characteristic terminal of monoterpene synhtase (Bohlmann et al. 1998; Williams et al. 1998; Lücker et al. 2002), when online analyzed with ChloroP1.1 software (http://www.cbs.dtu.dk/services/ChloroP/).

The monophyletic plant TPS family has been divided into seven subfamilies, from TPS-a to TPS-g, based on sequence relatedness as well as functional assessment (Bohlmann et al. 1998). The analysis of the phylogenetic relationship of *HcTPS2* by using Clustal.W and the neighbor-joining method has clustered *HcTPS2* into the TPS-b subfamily, which combines monoterpene genes of a variety of angiosperm species (Fig. 2).

Expression Pattern of *HcTPS2*

To determine tissues specificity of *HcTPS2* expression, total RNA was isolated from *H. coronarium* leaves, roots, tubers, stems, bracts, and floral tissues (floral shoots, petals, sepals, stamens, and pistils) of buds (1 day before opening) and used in RNA gel blot hybridizations (Fig. 3a). *HcTPS2* expression level was different of these floral tissues, its transcripts accumulated to high levels in stamens, petals, and sepals, the parts of the flowers that were shown previously to be primarily responsible for scent production and emission (Dudareva et al. 2000). A very low level of transcripts was detected in the pistils and floral shoots. No detectable signal was found in other tissues.

We also examined the expression of *HcTPS2* in buds of *H. gardnerianum*, *H. flavum*, *H. chrysoleucum*, and *H. gardnerianum* var. *pink*, which can emit scent, and *H. coccineum*, *H. forrestii*, and *Hedychium* spp that do not emit a detectable scent (data not shown). A hybridization signal was detected only in scented species, not in scentless species (Fig. 3b).

Developmental Modulation of *HcTPS2* Expression

We next analyzed the temporal and spatial accumulation patterns of *HcTPS2* mRNA in floral organs of *H. coronarium*. Total RNA was isolated from petals, sepals, stamens, and pistils of flowers at different times after anthesis (1 to 2 days) and also from buds at stage 0.5 (0.5 day before opening), stage 1 (1 day before opening), and stage 2 (2 days before opening).

Some *HcTPS2* mRNA was found in sepals and petals at stage 2 (2 days before opening; Fig. 3c), with trace amounts was found in stamens as well, but no detectable signals in pistils (Fig. 3d). *HcTPS2* expression was highly detected

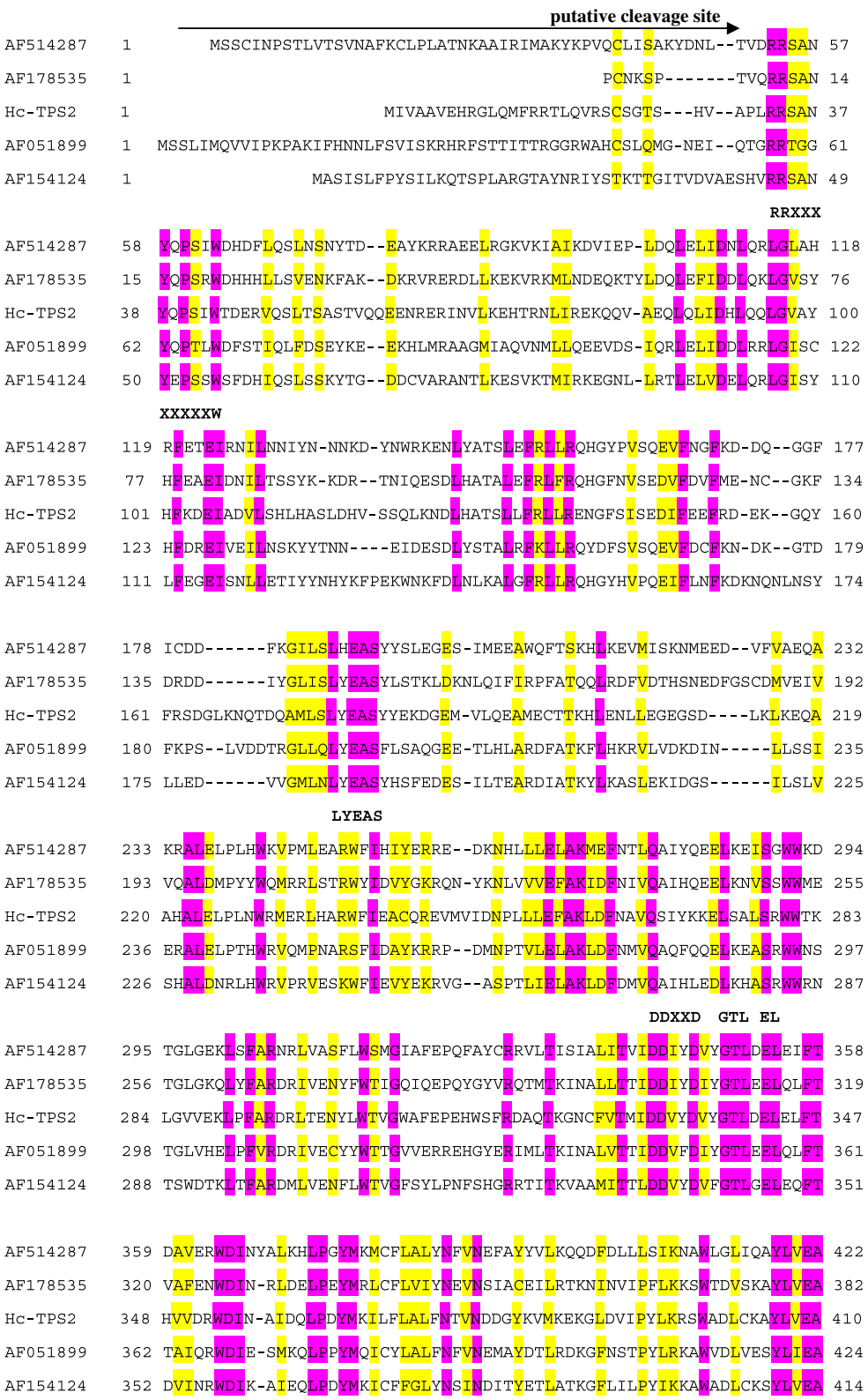

Fig. 1 Alignment of *HcTPS2* amino acid sequence with other closely related monoterpene synthase. *Shaded in purple* are amino acid positions identical in all five sequences. *Shaded in yellow* are amino acid positions identical in three or four sequences. The RRX8W, DDXXD, and LYEASY. GTLXEL motifs are *highlighted*. HcTPS2: *Hedychium coronarium* monoterpene synthase; AF514287: *Citrus limon* limonene synthase; AF178535: *Arabidopsis thaliana* myrecene synthase; AF051899: *Salvia officinalis* 1-8-cineole synthase; AF154124: *Artemisia annua* (3R)-linalool synthase

first in mature flowers buds (1 day before opening) except pistils, and its level increased until it peaked on day 1 (1 day after anthesis). The higher level of *HcTPS2* expression was found in sepals, petals, and stamens, despite the gradual increase, this level was still relatively high in mature flower buds (1 day before opening). The amount of *HcTPS2* transcripts was significantly decreased in 2 days after anthesis in floral organs (Fig. 3).

Fig. 1 (continued)

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AF514287   423 KWYHSKYTPKLEEYLENGLVSITGPLIITISYLSGTNPIIKKELEFLESNPD-IVHWSSKIFRL 485
AF178535   383 KWYKSGHKPNLEEYMQNARISISSPTIFVHFYCVFSDQLSIQVLETLSQHQQNVVRCSSSVFRL 446
Hc-TPS2    411 KWYHRDYKPTINEYLDNTWISISGPAIFTNAYCMANN-LTKQDLERFSEYPA-IAKHSSMLGRL 472
AF051899   425 KWYYMGHKPSLEEYMKNSWISIGGIPILSHLFFRLTDSIEEEDAESMHKYHD-IVRASCTILRL 487
AF154124   415 QWYHRGHIPTLNEYLDNACVSISGPVALMHVHFLTSVSSTKEIHHCIERTQN-IVRYVSLIFRL 477

AF514287   486 QDDLGTSSDEIQRGDVPKSIQCYMHETGASEEVARQHIKDMMRQMWKKVN-AYTADKDSPLTGT 548
AF178535   447 ANDLVTSPDELARGDVCKSIQCYMSETGASEDKARSHVRQMINDLWDEMNYEKMAHSSSILHHD 510
Hc-TPS2    473 YNDLATSTAEIERGDVPKSIQCCMHERGVSEGVAREQVKQLIRGNWRCMN-GDRAAASS-FEEM 534
AF051899   488 ADDMGTSLDEVERGDVPKSVQCYMNEKNASEEEARERHVRSLIDQTWKMMN--KEMMTSS-FSKY 548
AF154124   478 TDDLGTSLGEMERGDTLKSIQLYMHETGATEPEARSYIKSLIDKTWKKLN-KERAIVSSESSRE 540

AF514287   549 TTEFLLNLVRMSHFMYLH-GDGHGVQN-QETIDVGFTLLFQPIPLEDKHMAFTASPGTKG 606
AF178535   511 FMETVINLARMSQCMYQY-GDGHGSPEKAKIVDRVMSLLFNPIPLD              555
Hc-TPS2    535 LKTVAVDIARASQFFYHN-GDKYGKAD-GETMNQVRSLLINPII               576
AF051899   549 FVQVSANLARMAQWIYQHESDGFGMQH-SLVNKMLRGLLFDRYE                591
AF154124   541 FIDYATNLARMAHFMYGE-GDEDFRLD--VIKSHVSSLLFTPIQGI               583
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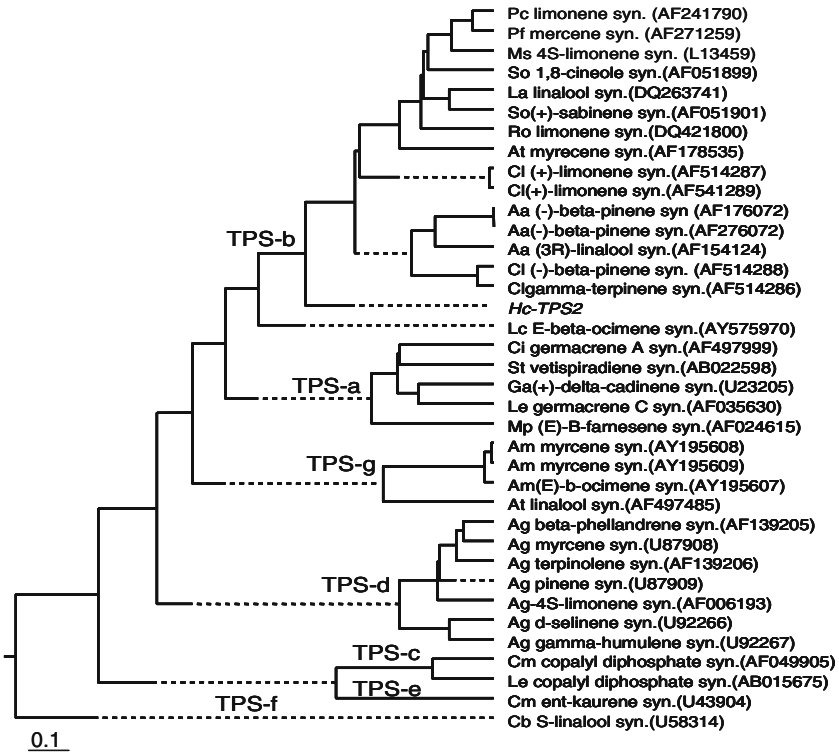

Fig. 2 Phylogenetic tree of plant terpene synthases from various species. The terpene synthases cloned from *H. coronarium* (*HcTPS2*) are part of the class TPS-b subfamily that contains predominantly monoterpene synthases of angiosperms. In the scale, the *bar* 0.1 is equal to10% sequence divergence. *Aa Artemisia annua*, *Ag Abies grandis*, *Am Antirrhinum majus*, *At Arabidopsis thaliana*, *Cb Clarkia breweri*, *Ci Cichorium intybus*, *Cl Citrus limon*, *Cm Cucurbita maxima*, *Hc Hedychium coronarium*, *Ga Gossypium arboretum*, *Lc Lotus corniculatus* var, *La Lavandula angustifolia*, *Le Lycopersicon esculentum*, *Mp Mentha piperita*, *Ms Mentha spicata*, *Nt Nicotiana tabcum*, *Pc Perilla citridora*, *Pf Periila frutescents*, *Ro Rosamarinus officinalis*, *Sc Solidago Canadensis*, *So Salvia officinalis*, *St Solanum tuberosum*. The GenBank accession numbers of all sequences are given in *parentheses*

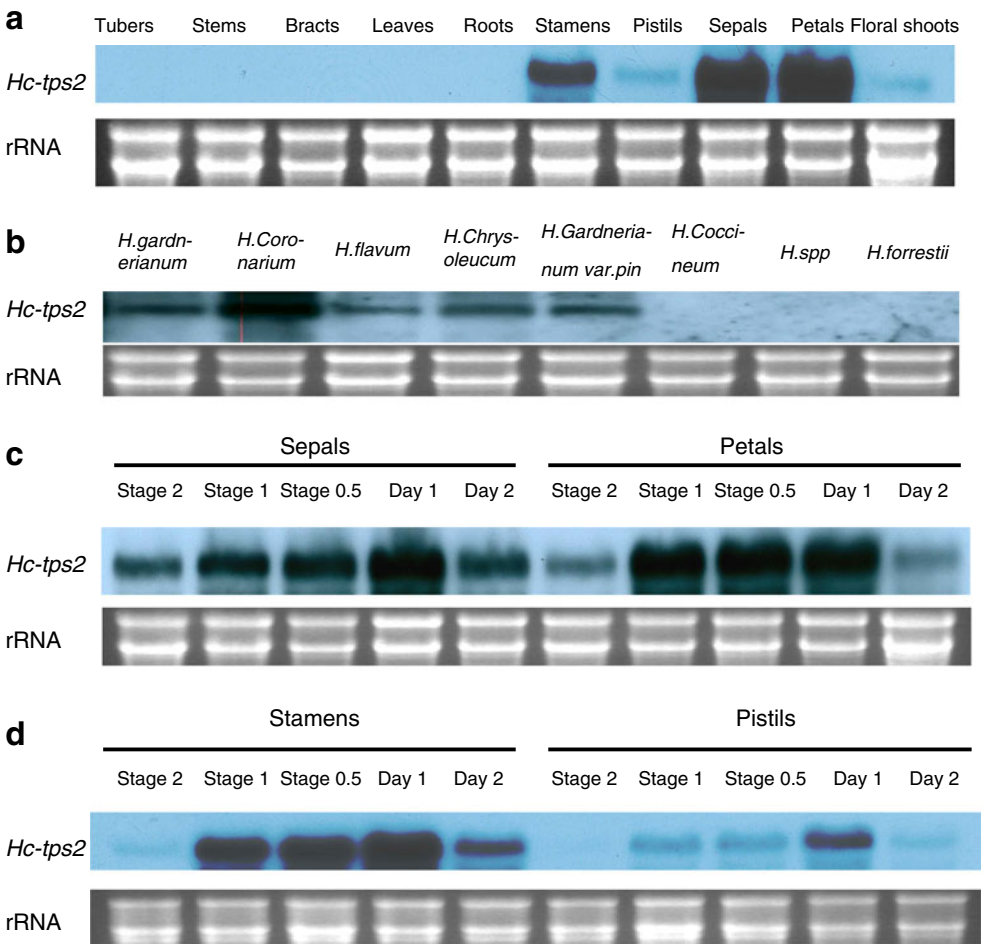

Fig. 3 Northern blot analysis of *HcTPS2* in different organs (**a**), different variety (**b**), sepals and petals (**c**), and stamens and pistils (**d**) during the lifespan of the flower in *H. coronarium*

Discussion

In this study, *HcTPS2* was isolated from *H. coronarium* flowers by using RT-PCR in combination with RACE technology. Northern blotting showed that *HcTPS2* was predominantly expressed in flower tissues, especially in stamens, petals, and sepals. Similar expression profiles of a monoterpene synthase were observed in *Nicotiana suaveolens* (Roeder et al. 2007). Moreover, the *HcTPS2* transcription was temporally and spatially regulated during flower opening and the steady state levels of *HcTPS2* in stamens, petals, sepals, and pistils are regulated developmentally, peaking on 1 day after anthesis and then beginning to decline. It is strongly up-regulated during scented species petals development rather than nonscented species. We found the scentless species also contain this gene in their genome (data not shown), but its expression level is too low to be detected by Northern blot analysis. In gray poplar, isoprene synthase promoter activity, which correlates with basal isoprene emission capacity, is not uniformly distributed within leaf tissue and can adapt rapidly towards internal as well as external environmental stimuli (Cinege et al. 2009). So it is likely because the differences in the *HcTPS2* promoters between these two types of species lead to the vastly different expression characteristics, but this remains to be verified experimentally.

Although changes in transcript level may not directly determine protein levels or enzyme activities due to possible post-transcriptional or enzyme-regulatory mechanisms, the positive correlation between transcript levels and volatile emission suggests that changes in transcript levels are an important determinant of scent production (Dudareva et al. 2004). The *HcTPS2* transcript was temporally and spatially regulated during flower opening, and its expression profiles, in this study, closely match the changes both quantitatively and qualitatively in monoterpenes emissions as observed in our previous studies (Li and Fan 2007), so there maybe a positive correlation between *HcTPS2* transcript levels and monoterpenes emission.

Conserved peptide sequence analysis has revealed that the *HcTPS2* protein has a typical monoterpene synthase structure. It contains a RR(x)8W motif, a LYEASY, GTLXEL motif, and an N-terminal transit peptide. And the sequence similarity of HcTPS2 to other monoterpene synthases is ranging from 35% to 38%. Further analysis of the phylogenetic relationship of terpene synthases has

clustered *HcTPS2* into the TPS-b subfamily, which predominantly contains monoterpene synthase from angiosperms (Fig. 2). So, *HcTPS2*, which possess all the important characters of monoterpene synthase, can be predicted as a monoterpene synthase, rather than a sesquiterpene synthase.

The *HcTPS2* cDNA present the first terpene synthase gene cloned from *H. coronarium* species that emit aroma and flavor bouquet composed of mainly monoterpenes and other volatiles. The isolation and expression analysis of *HcTPS2* will facilitate the cloning of additional genes of the TPS family in *H. coronarium*, and may be developed into molecular markers to aid in breeding and improvement of varieties with superior aroma and flavor traits.

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