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Full Length Research Paper

A DHHC-type zinc finger protein gene regulates shoot branching in *Arabidopsis*

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Formation of plant architecture is a complicated biological phenomenon and is influenced by a variety of factors such as genotype, hormone, environment and nutrition. In this study, an activation-tagging mutant, *scc10-D* (suppressor of *cry1cry2*) grown in long-day (16-h light/8-h dark) condition showed enhanced shoot branching. The mRNA expression of six genes adjacent to the T-DNA insertion locus were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR), and the transcript level of a DHHC-type zinc finger protein gene, *At5g04270*, was found to increase markedly in the *scc10-D* mutant. The *At5g04270* gene was then cloned and over-expressed in *Arabidopsis*. It was found that the *At5g04270* over-expression lines had the features of enhanced shoot branching, while the T-DNA mutant of *At5g04270* gene, *SALK_006515*, showed decreased shoot branching when compared to the wild type (WT). These results suggest that *At5g04270* plays an important role in regulating shoot branching in *Arabidopsis*.

Key words: *Arabidopsis,* DHHC-type zinc finger protein, *At5g04270*, shoot branching.

INTRODUCTION

The shoot system has an important role in generating a large variety of diverse plant forms (Sussex and Kerk, 2001). The diversified architecture of plants is determined primarily by the pattern of shoot branching. Plants modulate their shoot architecture by regulating activities of the shoot apical meristem (SAM) and axillary meristems (AM). Meristem activities are regulated by a network of environmental information, developmental stage and genetic makeup of the plant. The main axis of the plant body is provided by the SAM. Plant architecture is further modified by shoot branching that result from the activity of the AM. The complexity of the branching pattern lies with the temporal and spatial development of these branches (Wang and Li, 2008).

The shoot branching process generally involves two developmental stages: formation of AM in leaf axils and the growth of axillary buds (Gomez-Roldan et al., 2008; Umehara et al., 2008). The basic mechanism controlling the initiation of auxiliary meristem was indicated to be conserved among different plant species. The lateral suppressor (LS), the BL (blind) and their homologs were reported to regulate the initiation of auxiliary meristem in various plants. During the last century, the knowledge about the outgrowth of auxiliary buds was derived from the studies of auxin regulated pathways, which included the repression of cytokinin synthesis by auxin signaling and the regulation of auxin transportation by the more axillary branching (MAX) pathway as the two most characterized (Wang and Li, 2008). Recently, Gomez-Roldan et al. (2008) and Umehara et al. (2008) proposed that strigolactones act as the novel shoot branching inhibitor in regulating plant architecture. Up to date,

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Abbreviations: WT, Wild type; **SAM,** shoot apical meristem; **AM,** axillary meristems; **LS,** lateral suppressor; BL, blind; **MAX,** more axillary branching; **RT–PCR,** reverse transcriptase-polymerase chain reaction; **QPCR,** quantitative real time polymerase chain reaction.

Table 1. The primer sequences for RT-PCR.

Gene Name	Forward primer (5'→3')	Reverse primer (5'→3')
Actin2	5′ CACTGTGCCAATCTACGAGG GT 3′	5′ CACAAACGAGGGCTGGAACAAG 3′
At5g04240	5′ TAATGGCTCCAACTCAGGTAGTC 3′	5′ TTCCGCTTCTTCTTTGCTTCTC 3′
At5g04250	5′ GCGACTGAACCAAATGATACCT 3′	5′ TGTAATGAACCTCAGCCCAGA 3′
At5g04260	5′ ATCTCACCTTTGGTTCCTCCAT 3′	5′ CTATCATTTCCCTGACTTCGTT 3′
At5g04270	5′ TGTAGATCCAGGTCGTGTTCCT 3′	5′ GTCCCAAGTGTTATGCTTAGTCC 3′
At5g04280	5′ TTTGGTGATCGGGTCATCTC AG 3′	5′ TGCTTCTAAACCCTCTTCCCTC 3′
At5g04290	5′ CCAAAATCGCCGCTATCTCCTG 3′	5′ CTCCTATCGCCCAGTCTCCAGT 3′

extensive research and great progress have been made on the molecular mechanisms of shoot branching. A number of genes relating to shoot branching have been found in plant, including the related genes controlling the formation of AM such as *LS* (Schumacher et al., 1999) and *BL* (Schmitz et al., 2002) in tomato, *LAS* (Greb et al., 2003), *RAX* (Keller et al., 2006), *REV* (Talbert et al., 1995), *SPS* (Tantikanjana et al., 2001) and *Bushy* (Reintanz et al., 2001) in *Arabidopsis*, *MOC1* (Li et al., 2003), *LAX* and *SPA* (Komatsu et al., 2003) in rice, *BIF2* (Skirpan et al., 2009) in corn and the related genes controlling the axillary bud outgrowth such as *RMSs* (Beveridge et al., 1996; Sorefan et al., 2003) in soybean, *MAXs* (Bennett et al., 2006), *AXR1* (Stirnberg et al., 1999), *BRC* (Aguilar-Martıínez et al., 2007) and *Hoc* (Catterou et al., 2002) in *Arabidopsis*, *D3* (Ishikawa et al., 2005), *D10* (Arite et al., 2007), *THD1* (Zou et al., 2006), *OsTB1* (Takeda et al., 2003) and *OsNAC2* (Mao et al., 2007) in rice, *DAD1* (Snowden et al., 2005) and *Sho* (Zubko et al., 2002) in Petunia, etc.

Proteins containing zinc finger domain(s) were found to play important roles in eukaryotic cells by regulating different signal transduction pathways and controlling processes, such as RNA binding, transcription, apoptosis, protein-protein interaction (Ciftci-Yilmaz and Mittlera, 2008). There are many types of zinc finger proteins, such as C_2H_2, C_2C_2, C_2HC, $C_2C_2C_2C_2$, $C_2HCC_2C_2$, and DHHC. The DHHC type zinc finger proteins are a class of highly conserved cysteine-rich zinc finger domain-containing proteins (Putilina et al., 1999). Studies indicated that the gene family is involved in palmitoyl-modification of protein (Roth et al., 2002; Gleason et al., 2006; Ducker et al., 2004; Jessica et al., 2005). Hemsley et al. (2005) found that *TIP1*, a DHHC-type zinc finger protein gene in *Arabidopsis thaliana*, encodes an S-acyl transferase and is involved in the regulation of plant cell growth. Particularly, it strongly affects the root hair growth. Two homologues of TIP1, AKR1p in yeast (*Saccharomyces cerevisiae*) (Roth et al., 2002) and HIP14 in human (*Homo sapiens*) (Ducker et al., 2004) have been reported to have S-acyl transferase (also known as palmitoyl transferase) activity.

In this study, we analyzed the expression of T-DNA flanking genes in the *scc10-D* mutant and preliminarily investigated the increased expression level of a DHHC-type zinc finger protein gene, *At5g04270*, which resulted in its enhanced shoot branching. Followed by analyzing the phenotype of *At5g04270* over-expression transgenic lines and the *SALK_006515* mutant, it was further confirmed that *At5g04270* plays a positive regulatory role in the *Arabidopsis* shoot branching.

MATERIALS AND METHODS

Plant materials

Ecotype of *A. thaliana* L. is Columbia (Col-4). The mutant *scc10-D* (suppressor of *cry1cry2*) was an activation-tagging dominant mutant in the *cry1cry2* double mutant containing a T-DNA insert in an intergenic region. The T-DNA insertion line *SALK_006515* was obtained from the *Arabidopsis* Information Resource (TAIR) seed stock (http://www.arabidopsis.org/).

Reverse transcriptase-mediated PCR analysis

To analyze the expression of T-DNA flanking genes by reverse transcriptase-polymerase chain reaction (RT–PCR), total RNA was extracted from *Arabidopsis* tissues with EasyWay RNA PlantMini Kit (Ambiogen Life Science Technology Ltd., China). By use of an oligo (dT)18 as a common primer, total RNA (2 µg) was heated to 65°C for 7 min and then subjected to reverse transcription reaction using Moloney murine leukemia virus reverse transcriptase (promega, USA). PCR amplification was performed with initial denaturation at 95°C for 5 min followed by different (24 - 30) cycles of incubations at 95°C for 30 s, 58 - 63°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min using the specific primers (Table 1). The *Actin2* gene (*At3g18780*) was used as an internal control. PCR products were separated by 1.5% agarose gel electrophoresis. Each experiment was repeated at least three times in three independent trials.

Quantitative real time PCR analysis

Quantitative real time PCR (QPCR) was used to further confirm transcript levels of *At5g04270* in different organs of *Arabidopsis*. QPCR was performed on the MX3000P (Stratagene, USA) and carried out as described by Wang et al. (2009). Fluorescence intensities were normalized against reference dye, ROX. The *Actin7* gene (*At5g09810*) was used as an internal control. The specific-primers of *At5g04270* for QPCR were as follows: Forward sequence (F), 5'- CAACCGTGGCTTCCATCTACT -3'; and reverse sequence (R), 5'- GTCCCAAGTGTTATGCTTAGTCC -3'. The primers of *Actin7* for QPCR were as follows: Forward sequence (F),

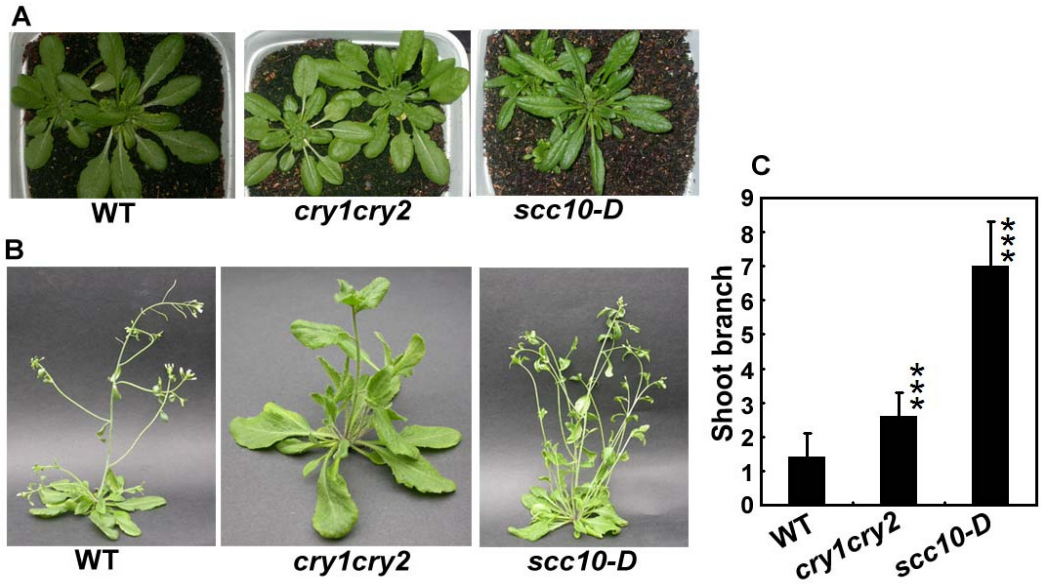

Figure 1. The phenotype and statistics of the shoot branch of WT, *cry1cry2* and *scc10-D* grown at 22℃ in long-day (16-h light/8-h dark) conditions. A, The phenotype of 30-days-old WT, *cry1cry2* and *scc10-D* plants; B, phenotype of 40-days-old WT, *cry1cry2* and *scc10-D* plants; C, statistics of the shoot branch of 40-days-old WT, *cry1cry2* and *scc10-D* plants. The data (Mean ± SD) were obtained from 25 to 30 samples each. An asterisk indicates significant difference between WT, transgenic plants and mutant; ***: significant at $P < 0.001$.

5'- ATCCCTCAGCACCTTCCAAC -3'; and reverse sequence (R), 5'- ACAAACTCACCACCACGAAC -3'. The data were obtained from three independent experiments with at least three repeated measurement each.

Plasmid construction

The sequences of the *At5g04270* gene specific primers for PCR were as follows: Forward sequence (F), 5′ CGCGAATTC (*Eco*RI) ATGGGTTTCGTGTATTACGTCA 3′; reverse sequence (R), 5′ CCGCTCGAG (*Xho*I) CAATGCAATAACACAATTGGGC 3′. PCR products were cloned into pGEM-T vector (Promega, USA) for sequencing. The right sequence was then subcloned into the pEGAD vector (Lin et al., 2009). The resulted construct pEGAD-*At5g04270* contains a Basta resistant (*bar*) gene as the plant selective marker. Both *At5g04270* and *bar* were driven by the Cauliflower mosaic virus 35S promoter (CaMV 35S promoter).

Plant transformation and selection

The construct pEGAD-At5g04270 was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation for plant transformation. Floral-dip method was applied to transform *A. thaliana* according to the method of Clough (Mockler et al., 1999). Two weeks later, the seeds obtained were incubated at 4℃ for 4 days and sterilized before being sown on soil at 22℃ under long-day (16-h light/8-h dark) conditions. The putative transgenic lines were obtained through continuous three generations selection using herbicide Basta (1:1000, V : V).

Statistics of shoot branch

The seeds were incubated at 4℃ for 4 days and sterilized before being grown on soil at 22℃ under long-day conditions. The shoot branches of 40-days-old plants were counted according to the number of axillary inflorescence of rosette-leaves. Each value presents the mean ± SD of 25 to 30 samples each.

RESULTS

Characterization of *scc10-D* mutant

The *scc10-D* mutant was an activation-tagging dominant mutant in the *cry1cry2* double mutant. Compared with WT and *cry1cry2*, *scc10-D* showed increased axillary buds at vegetative stage and shoot branches at reproductive stage (Figure 1). Plasmid rescue method was used to identify the mutant *scc10-D* in Pro Lin's laboratory (Zhao et al., 2007). The T-DNA insertion locus was found to locate in the vicinity of *At5g04270* gene and its right border contained four 35S promoters (Figure 2A). The transcript levels of six T-DNA flanking genes were then analyzed by RT-PCR, and the *At5g04270* was found to be the only overexpressing gene (Figure 2B). These results indicated that the excessive expression of *At5g04270* might be associated with enhanced axillary buds and shoot branching.

Over-expression of *At5g04270* causes increased shoots branching

In order to further study the specific phenotype of *scc10-*

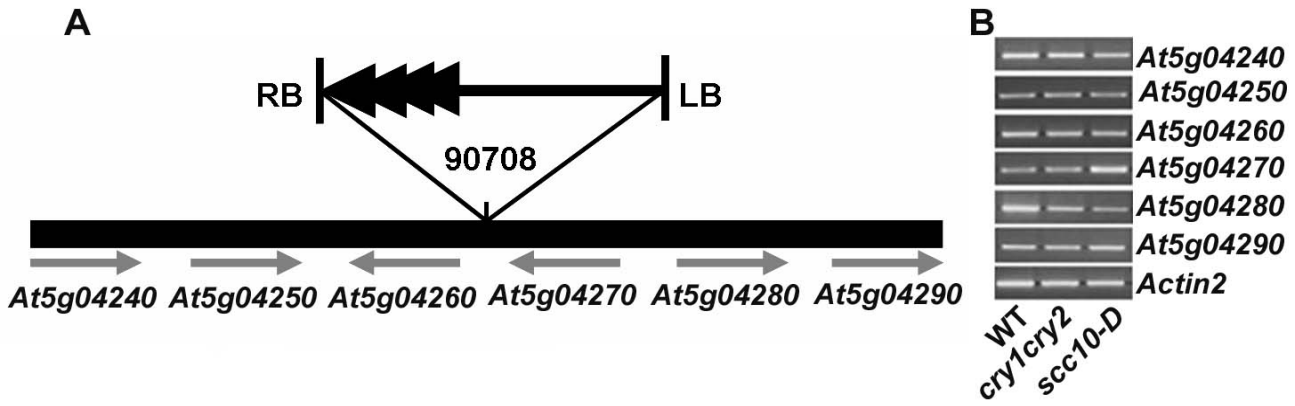

Figure 2. Characterization of the *scc10-D* mutant. A, Diagram depicting the *scc10-D* locus and the T-DNA insertion. LB and RB represent the left and right borders of T-DNA, and the number 90708 indicates the T-DNA insert site. B, RT-PCR analysis of mRNA expression of genes flanking the T-DNA insert of *scc10-D*. The transcript levels are shown as gel image and the *Actin2* was used as an internal control. Each experiment was repeated at least three times in three independent trials and similar results were obtained.

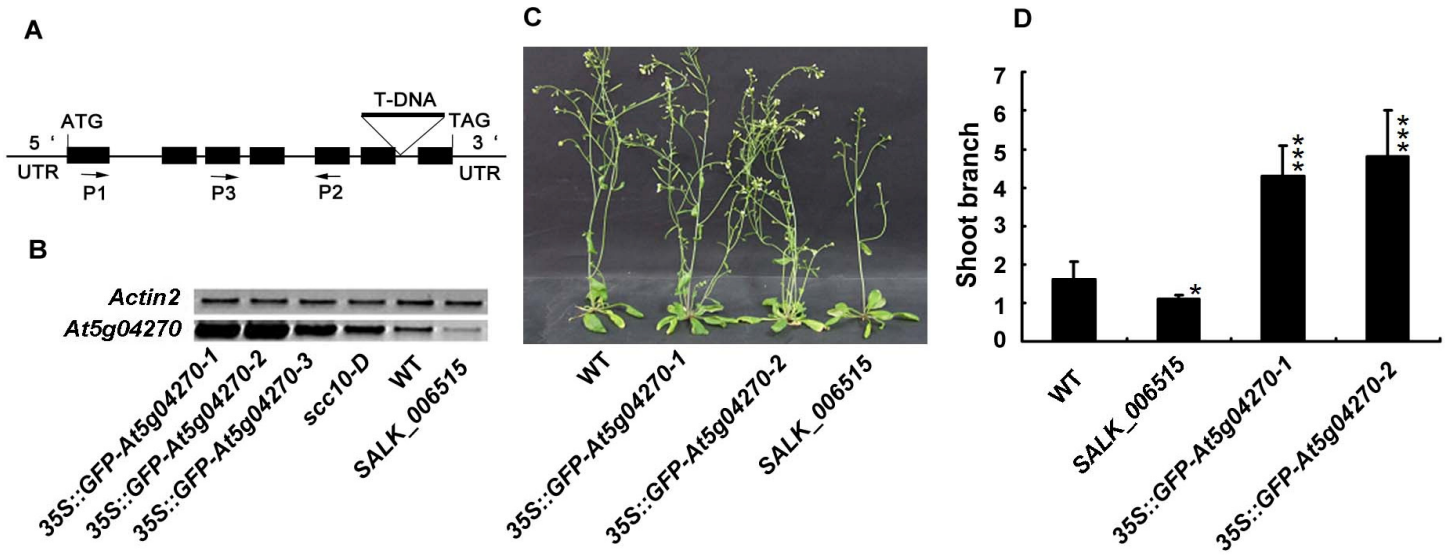

Figure 3. Characterization of the *At5g04270* transgenic plants and the *SALK_006515* mutant. A, T-DNA insertion site of *SALK_006515* mutant. Exons (filled boxes) and introns (lines between the filled boxes) of *At5g04270* are shown. T-DNA was inserted into the sixth intron of *SALK_006515*. UTR, untranslated region. Arrows indicate the primers used for analyzing the mRNA level of *At5g04270* by RT-PCR or QPCR. P1 and P2 were used for RT-PCR. P3 and P2 were used for QPCR. B, RT-PCR analysis of mRNA level of *At5g04270* gene. The transcript levels are shown as gel image, and the *Actin2* is used as an internal control. Each experiment was repeated at least three times in three independent trials and similar results were obtained. C, The phenotype of 40-days-old plants. D, The shoot branches of 40-days-old plants. The data (Mean ± SD) were obtained from 25 to 30 samples each. An asterisk indicates significant difference between WT, transgenic plants and mutant; *, ***: significant at $P < 0.05$ and $P < 0.001$, respectively.

D mutant, the cDNA sequence of *At5g04270* gene was cloned and found to be identical to that presenting in TAIR(http://www.arabidopsis.org/servlets/TairObject?type = sequence&id=2002 961981). Alignment of its cDNA sequence with genomic DNA sequence indicated that *At5g04270* contains seven exons and six introns (Figure 3A). Its coding region comprised a total of 765 bp, encoding 254 amino acids. As for the T-DNA insertion line *SALK_006515*, its insertion flanking sequence obtained from TAIR website (http://www.arabidopsis.org/servlets/TairObject?id=500221070&type=polyallele) indicated that the T-DNA was inserted into the sixth intron (Figure 3A).

The cDNA sequence of *At5g04270* gene was then subcloned into pEGAD vector and transformed into *Arabidopsis*. About 30 Basta resistant transgenic lines

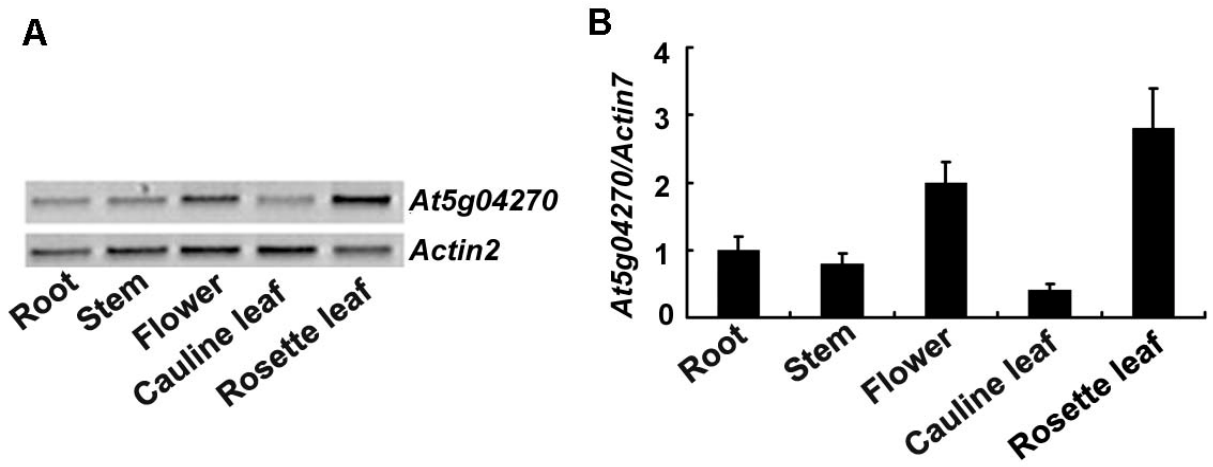

Figure 4. The mRNA expression of *At5g04270* gene in different organs of *Arabidopsis.* A, RT-PCR analysis. The transcript levels are shown as gel image and the *Actin2* is used as an internal control. Each experiment was repeated at least three times in three independent trials and similar results were obtained. B, Quantitative real time PCR analysis. *Actin7* was used as an internal control. The data were obtained from three independent experiments with at least three repeat

were obtained and three lines were randomly selected for further study. When the transcript level of *At5g04270* gene was analyzed by RT-PCR, it was found that the signals of *At5g04270* in the transgenic lines were stronger than that in WT, while the signal was weaker in the *SALK_006515* mutant than in WT (Figure 3B). These results indicated that the *At5g04270* over-expressed in the transgenic lines successfully, and the expression of *At5g04270* in *SALK_006515* mutant was negatively influenced to a large extent.

To investigate the effect of *At5g04270* gene expression on shoot branching, its over-expression lines, *SALK_006515* mutant and WT, were grown under long-day conditions. Compared with WT, the shoot branches of transgenic lines increased dramatically, which coincided with that of *scc10-D*, whereas the shoot branches decreased in the *SALK_006515* mutant when compared to the WT (Figure 3C and D), and the vast majority of mutant plants only had main stems (Figure 3C). These results indicated that the *At5g04270* gene might positively regulate shoot branching in *Arabidopsis.* In recent years, researches related with the molecular mechanism of plant development focus on AM initiation and axillary buds growth (Wang and Li 2008). In this study, the dormant axillary buds could be observed in the leaf axils of *SALK_006515* mutant (data not shown), indicating that the *At5g04270* gene positively regulates the growth of axillary buds instead of the initiation of AM in *Arabidopsis*.

At5g04270 gene expression in different organs of *Arabidopsis*

As mentioned previously, the coding region of *At5g04270* comprised of a total of 765 bp, encoding 254 amino acids. One of them contains a DHHC-type zinc finger domain, indicating that the protein encoded by this gene is a DHHC-type zinc finger protein. The amino acid sequence of the predicated protein was further analyzed by the TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0 /); it was found that the protein contains three transmembrane domains. The DHHC-type zinc finger domain is in the first and second membrane-spanning domains, and close to the second inter-membrane domain (results not listed). These results indicated that the protein encoded by *At5g04270* was a membrane-bound protein. To date, three DHHC-type zinc finger membrane-bound proteins, *Arabidopsis* TIP1 (Hemsley et al., 2005), yeast AKR1p (Roth et al., 2002) and human HIP14 (Ducker et al., 2004), were isolated and identified to have a protein acyltransferase activity. However, the specific features and functions of *At5g04270* gene encoding proteins still need further investigations.

To investigate the expression patterns of *At5g04270* in different organs in *Arabidopsis*, total RNA was extracted from roots, stems, flowers, cauline leaves and rosette leaves of *Arabidopsis*. RT-PCR results showed that *At5g04270* was expressed in all these organs and the transcript level in rosette leaves was the highest, and the cauline leaves had the lowest transcript level (Figure 4A). QPCR analysis also obtained the same results as that of RT-PCR analysis (Figure 4B). The transcript level of *At5g04270* was then interrogated using GENEVESTIGATOR expression profiling tool (https://www.genevestigator.com). In general, our results were consistent with the Microarray data from the genevestigator database that *At5g04270* was expressed stably in all organs (data not shown). The only difference was that the stems instead of rosette leaves had highest expression of

At5g04270 in the microarray data. At present, TIP1 is the only DHHC-type zinc finger protein whose functions have been known (Hemsley et al., 2005) in *Arabdopsis*. It is involved in the regulation of plant cell growth and has a particularly strong effect on root hair growth. *TIP1* is also expressed in various organs, but the expression level in the flowers is the lowest, which is different from the *At5g04270* gene. The differences in the expression patterns indicate that the two DHHC-type zinc finger proteins have different physiological functions in the development of *Arabidopsis*. Actually, the root hair were found to develop normally in the root tips of both *SALK_006515* mutant and *35S:: GFP-At5g04270* transgenic lines (results not shown). The only obvious difference between transgenic lines and wild-type plants is the number of shoot branches (Figure 3C and D).

DISCUSSION

Generally, the branching process involves two developmental stages: Formation of AM in leaf axils and the growth of axillary buds (Gomez-Roldan et al., 2008; Umehara et al., 2008). Therefore, the extent of branching depends not only on the formation of AM, but also on its subsequent viability and growth. Axillary meristem growth is inhibited by the main stems and main inflorescences, which is a phenomenon known as apical dominance. Through a long period of plant evolution, a fine mechanism on controlling axillary meristem growth and development has been established. After the formation of AM, axillary buds are generated and may branch or stay in a dormant state. The dormant buds can be re-activated to produce branches later, which depend on the development procedures or environmental conditions. At present, many mutants related to the AM growth have been found, including the *tb1* (Takeda et al., 2003), *axr1* (Stirnberg et al., 1999), *rms* (Beveridge et al., 1996; Sorefan et al., 2003), *max* (Bennett et al., 2006) and so on.

Physiological studies have shown that axillary bud growth and dormancy is determined by the ratio of auxin and cytokinin. When the auxin / cytokinin ratio is high, it will play an inhibitory effect on the growth of axillary buds, while if the ratio is low, it will promote the axillary bud growth (Shimizu-Sato and Mori, 2001). However, further study found that changing the ratio of these two hormones did not stop inhibition of the axillary bud growth in some mutants (Foo et al., 2001). It indicated that there were other factors regulating the growth of axillary buds beside auxin and cytokinin. Umehara et al. (2008) and Gomez-Roldan et al. (2008) found that strigolactone produced by the MAX pathway was a new hormone and controlled shoot branching. The discovery was different from the traditional theory which believed that branching was controlled by auxin and cytokinin, and it was very important for the research of plant hormones and their development. Brewer et al. (2009) found that strigolactone was a negative regulator which acted downstream of auxin to regulate axillary buds growth. In addition, other studies have shown that rice *TB1* gene and its homologous genes in *Arabidopsis BRC1* negatively regulated axillary bud growth in the MAX pathway (Takeda et al., 2003; Aguilar-Martıínez et al., 2007), while *OsNAC2* in rice up-regulated the growth of axillary buds (Mao et al., 2007).

In this study, the transcript level of *At5g04270* in the mutant *scc10-D* was higher than its parent *cry1cry2* and WT, which preliminarily indicated that the excessive expression of this gene might be associated with the enhanced shoot branching. The *At5g04270* over-expression transgenic lines were found to increase shoot branches dramatically, which coincided with that of *scc10-D*, whereas, the mutant *SALK_006515* showed decreased shoot branching. These results indicated that the *At5g04270* gene might positively regulate shoot branching in *Arabidopsis.* Nevertheless, the dormant axillary buds could be observed in the leaf axils of *SALK_006515* mutant (data not shown), indicating that AM could be formed normally but the axillary buds could not produce branches. It was demonstrated that the *At5g04270* gene did not positively regulate the initiation of AM but the growth of axillary buds in *Arabidopsis*. The mechanism of the *At5g04270* gene regulating the growth of axillary buds needs to be further studied.

The DHHC-type zinc finger proteins are a small class of zinc finger protein family and have DHHC (Asp-His-His-Cys) highly conserved zinc finger domain. A variety of DHHC proteins are both enzymes and substrate (Roth et al., 2002; Gleason et al., 2006; Ducker et al., 2004). To date, studies on DHHC type zinc finger proteins mainly focus on yeast and mammals, while there are few studies on plants. *Arabidopsis* contains 22 DHHC-type zinc finger protein genes, but their functions still remain unclear. Through map-based cloning, Hemsley et al. (2005) isolated and cloned a DHHC type zinc finger protein gene *TIP1* from *TIP1* (TIP GROWTH DEFECTIVE1) mutant. It was found that *Arabidopsis* DHHC type zinc finger protein TIP1 was similar with yeast AKR1p (Roth et al., 2002) and the Human HIP14 (Ducker et al., 2004). This protein is involved in the regulation of plant cell growth in *Arabidopsis*; the gene deletion leads to the hypoplasia of root hair on the tip. Thus, DHHC type zinc finger proteins also play a very important role in plant growth and development. In this study, amino acid sequence analysis of the predicated protein encoded by the *At5g04270* gene revealed that the protein is DHHC-type zinc finger protein and is homologous with *Arabidopsis* TIP1, yeast AKR1p and human HIP14. However, the function of the protein encoded by *At5g04270* is still unclear and needs further investigations. This study reveals a positive role of *At5g04270* gene in regulating *Arabidopsis* branching and suggests a new function of the DHHC-type zinc finger protein in the regulation of plant growth and development. This contribution shed light on the study of the function of

such proteins in the future.

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