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REGULAR ARTICLE

Gene cloning and gene expression characteristics of alcohol dehydrogenase in *Osmanthus Fragrans* var. *Semperflorens*

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ABSTRACT

Because of a lot of aroma components in the flowers, *Osmanthus fragrans* is widely used as ornamental tree species and its flowers is used as materials of spices and food additives. Alcohol dehydrogenase (ADH) plays an important regulation role in growth, development, stress resistance and aroma synthesis of plants. Using the flowers of *O fragrans* var. *semperflorens* as materials, the full-length cDNA of ADH gene (termed *OfADH*) was successfully cloned through rapid amplification of cDNA ends (RACE), which has the 1128 bp open reading frame (ORF) in length and codes 375 amino acids. The results of conserved domain analysis show that the *OfADH* belongs to the zinc-dependent medium-chain dehydrogenase/reductase (MDR) superfamily, contains 22 NAD(P) binding sites in the liver_alcohol_DH_like conserved domains, and also has the PLN02740 (Alcohol dehydrogenase-like) domain. The results of semi-quantitative RT-PCR indicate that the expression level of *OfADH* in *O. fragrans* flowers is consistent with the rule of aroma formation and release, while the expression of *OfADH* in leaves is positively correlated with high temperature stress, suggesting that *OfADH* has obvious functional diversity in regulation of aroma synthesis and response to high temperature stress.

Keywords: *Osmanthus fragrans* var. *semperflorens*; Alcohol Dehydrogenase; Aroma synthesis

INTRODUCTION

Alcohol dehydrogenase (ADH), a zinc metalloprotease, existing in all kinds of organs and cells of plants, animal and microorganisms widely (Jacquelyn LM et al., 2013; Kluver N et al., 2014), is one of the rates limiting enzyme of short chain alcohols metabolism in organism (Cheung et al., 2017; Naser and Lawrey, 2018). In the presence of NAD+ and NADH, ADH is able to catalyze a reversible reaction between primary alcohols and aldehydes, also between ethanol and aldehydes (Gottlieb et al., 1982). ADH plays an important role in the growth and development process of humans, animals and plants. It exhibits obvious biological functional diversity (Alpat et al., 2010). So there are many researchers have carried out researches on the functionality application and gene regulation mechanism of ethanol dehydrogenase (DiMeglio, et al., 2014), including the ethanol biosensor (Niculescu et al., 2002), drug therapy (Vanessa, et al., 2014), disease diagnosis (Charlton et al., 2002), fruit storage (Ning et al., 2009), fruit flavor improved (Ting et al., 2012), and stress and resistance in plants (Liang et al., 2012; Liu et al., 2007). In recent years, studies have indicated that alcohol dehydrogenase played an important role in the formation and diversity of plant aroma (Kmita et al., 2018; Liu and Liu, 2010; Shen et al., 2017). The main ingredients of plant fragrance are terpenoids (isoprene, monoterpene and sesquiterpene), fatty acid derivatives (volatile aldehydes and alcohols) and phenyl/phenylpropanoid substances, that are synthesized respectively by the terpenoid metabolism pathway, the fatty acid derivatives metabolic pathway and the phenylpropanoid pathway (Zhang Qiang, 2009). In the fatty acid derivatives metabolic pathway of apple aroma (Robert, et al., 2007), hexanal transformed by linoleic acid hydroperoxide could generate 2-methylbutanol which can produce 2-isoamyl acetate by the alcohol acyltransferase in presence of ADH (Dudareva et al., 2008). And also, in the mature process of fruits, the expression level of ADH has important influence on the diversity of aroma components, especially the content of aldehydes and alcohols (Iyit, 2018; Jiang et al., 2018; Khan et al., 2018). Studies have indicated that the increase of ethylene content would lead to lower expression of ADH (Defilippi et al., 2005), while

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the increase of alcohols would make genetically modified fruit more flavor (Speirs J et al., 1998).

Osmanthus fragrans Lour is a traditional flower in China. Because of its unique scent, commercial extracts are in high demand for use in the production perfumes and cosmetics, such as flavouring tea, wine, and foods. *O. fragrans* var. *semperflorens*, that is a special variety of *O. Fragrans* with more longer flowering period than others, could be flowering in all four seasons (Khan, 2018; Sultana et al., 2018; Kibria et al., 2018). The flowers of *O. fragrans* var. *semperflorens* are collected as materials for *OfADH* full-length cDNA cloning and expression analysis. This study will provide the basis for exploring the regulation mechanism and biological mechanism of ADH gene in aroma synthesis of *O. fragrans* var. *semperflorens*.

MATERIALS AND METHODS

Materials

The tissues of *O. fragrans* var. *semperflorens* (Fig.1) were collected from the plant garden of Central South University of Forestry and Technology in 2014 and 2015.

Flowers and leaves are collected separately in March, June, September and December. The flowers in different flowering stages and the blooming flowers in 18:00, 22:00, next 2:00, 6:00, 10:00, 14:00 also have been collected. All of materials have been stored in -80°C low temperature refrigerator.

Methods

RNA extraction and First-strand cDNA synthesis

Total RNA was extracted by modified CTAB combined with plant RNA Kit (Omega) and detected by 1% agarose gel electrophoresis. First-strand cDNA synthesis was performed using the Invitrogen SuperScript™ III Reverse Transcriptase.

O. fragrans ADH gene cloning

The main fragment of *OfADH* was amplified with the primers (Table. 1). The PCR product was purified and inserted into pMD-18T vector (TaKaRa) and sequenced. To clone the full-length *OfADH*, the 5'-end and 3'-end were obtained by RACE-PCR (Invitrogen 3' RACE/5' RACE System for Rapid Amplification of cDNA Ends). The full-length cDNA of *OfADH* was finally amplified using the primers Fl (Table. 1). The PCR products were cloned into pMD-18T vector for squencing.

Bioinformatics analysis

The full-length *OfADH* sequence was analyzed by BioEdit. The DNAMAN soft had been used to carry out homology sequence alignment between *O. fragrans* and other plants. All of plant ADH genes were searched in the NCBI Genbank. Phylogenetic tree was built by MEGA4 in NJ component, and tested 1000 times by bootstrap analysis. The protein secondary structure was analyzed online (http://web.expasy.org/protscale/). The protein conserved domain of ADH was also analyzed and forecasted by online software (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Searching similar sequence in PBD (http://www.rcsb.org/pdb/home.do) according to the OfADH amino acid sequence. Through homology modeling by Swiss-model (http://swissmodel.expasy.org/) in alignment mode, the three-level structure of OfADH protein was predicted.

Gene expression pattern by Semi-quantitative RT-PCR

OfADH mRNA expression in different seasons, different flowering stages and different times a day were evaluated by semi-quantitative RT-PCR analysis. The *OfADH* mRNA transcript was amplified with specific primers (BDADH-F:5 '-CTCTGTTTCCTCGGATTCCC-3 BDADH-R:5'; '-CCCCCAGTCCACCTGTAGTC-3') designed from the conserved region of *OfADH* cDNA. A parallel amplification with specific primers (18s-F: 5'-TCTAAATCCCTTAACGAGGATC-3'; 18s-R: 5'-CTATGAAATACGAATGCCCC-3') for 18s gene employed as an internal control to show that equal amount of cDNA was used in each PCR (Susanne B. et al, 2010). The Quantity One had been used to carry out the gray level analysis for semi-quantitative RT-PCR results.

RESULTS AND ANALYSIS

Cloning and sequence analyses of *OfADH*

The main fragment of *OfADH* was 828bp, 3'-end fragments was 475bp, and 5'-end fragrans was 393bp. The full-length of *OfADH* containing a whole open reading frame was 1295bp (Fig. 2).

Fig 1. Flowering of *Osmanthus fragrans* Var. Semperflorens. 1. Flower buds; 2. Full flowering; 3. Flowering drop-off.

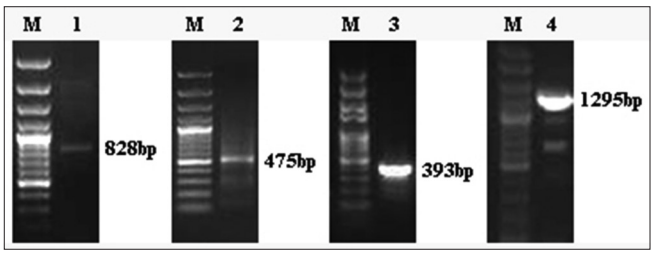

Fig 2. Full-length cDNA cloning of *OfADH*. M: DNA Marker; 1: Amplification product of conserved region; 2: Amplification product of 3' RACE; 3: Amplification product of 5' RACE; 4: Amplification production of DXPS ORF.

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1       CAGCCCACCCCGCACGGGACAATATTCTCCCATTTGCAGCCCAGTATCCACCATGACTAGCGGCAATCCAGGAGTAATTACATGCAAAGC
1                                                        M  T  S  G  N  P  G  V  I  T  C  K  A
91      TGCGGTGGTATGGAAATCAGGAGAGCCATTGAAAGTAGAGGAAATACAAGTGGATCCTCCCAAATCCTCTGAAGTTAGGATCAAAATGCT
14        A  V  V  W  K  S  G  E  P  L  K  V  E  E  I  Q  V  D  P  P  K  S  S  E  V  R  I  K  M  L
181     TTGTGCGAGTTTGTGTCACACTGATTTTCTGTGTTGCAATGGACTCCCTGTTCCTCTGTTTCCTCGGATTCCTGGACATGAAGGAGTTGG
44        C  A  S  L  C  H  T  D  F  L  C  C  N  G  L  P  V  P  L  F  P  R  I  P  G  H  E  G  V  G
271     CATGATAGAGAGTGTGGGAGAAGGAGTCACAAATCTGAAAGAAGGAGATATAGTGATGCCACTCTATCTGGGAGAATGTGGGGAATGTCT
74        M  I  E  S  V  G  E  G  V  T  N  L  K  E  G  D  I  V  M  P  L  Y  L  G  E  C  G  E  C  L
361     GAATTGCAAATCTGGGAAGACAAATTTATGCCACAAATATCCCTTAAATTTCACTGGCCTAATGCCAGATGGCACATCAAGAATGCACAT
104       N  C  K  S  G  K  T  N  L  C  H  K  Y  P  L  N  F  T  G  L  M  P  D  G  T  S  R  M  H  I
451     TGGAGATCAGATAATATATCACCATTTTAGCTGTGGTACATGGTCTGAATATGTTGTTATCGATGCGAATTTTGCAGTCAAGGTTGATCC
134       G  D  Q  I  I  Y  H  H  F  S  C  G  T  W  S  E  Y  V  V  I  D  A  N  F  A  V  K  V  D  P
541     CCGTGTTTCTCTTGCACATGCAAGTTTCCTTTGCTGTGGTTTCACCACAGGTTTTGGATCTGCCTGGAGGGAAGTCACTATTGAAAAGGG
164       R  V  S  L  A  H  A  S  F  L  C  C  G  F  T  T  G  F  G  S  A  W  R  E  V  T  I  E  K  G
631     CTCAACTATTGCTGTTATTGGTGTTGGTGCTGTTGGACTCGGAGTGATAGAGGCAGCGCGAATGAATGGAGCTTCTAGGATAATTGGGAT
194       S  T  I  A  V  I  G  V  G  A  V  G  L  G  V  I  E  A  A  R  M  N  G  A  S  R  I  I  G  I
721     TGACATAAACAACTTGAAGAAGAAGAAAGGAGAAGCCTTTGGAATGACTGAATTTATCAATCCAAAAGAATCTGATAAACCTGTATCAGA
224       D  I  N  N  L  K  K  K  K  G  E  A  F  G  M  T  E  F  I  N  P  K  E  S  D  K  P  V  S  E
811     ATTGATTAAAGATACTACAGGTGGACTGGGGGTGGATTACTGTTATGAGTGCACTGGAGTTCCAGAGCTGCTAAATGAAGCAATTGAGGG
254       L  I  K  D  T  T  G  G  L  G  V  D  Y  C  Y  E  C  T  G  V  P  E  L  L  N  E  A  I  E  G
901     ATCCAAAGTGGGACTTGGGACAATAGTTTTCATTGGTGCGGGACTTCATTTAAGTGGGGAGCTCAAATACATTCCCCTCTTGTGTGGTAG
284       S  K  V  G  L  G  T  I  V  F  I  G  A  G  L  H  L  S  G  E  L  K  Y  I  P  L  L  C  G  R
991     GACAATTAAGGGGTCCATTTATGGTGGAGTAAGACCTCAAACAGACCTCCCTAAAATAGTTGAGAAATGCATAAATAAGGAAATTCAGCT
314       T  I  K  G  S  I  Y  G  G  V  R  P  Q  T  D  L  P  K  I  V  E  K  C  I  N  K  E  I  Q  L
1081    GGATGAACTATTGACCCATGAAGTTTCACTTGAAGAAATTAACAAGGCATGGGAGTATCTGAAGCTTCCTAGCTGTGTTAAAGTTGTTAT
344       D  E  L  L  T  H  E  V  S  L  E  E  I  N  K  A  W  E  Y  L  K  L  P  S  C  V  K  V  V  I
1171    CAAATATTAGAGCGAATGATCTTCTTTTGTATTGAATGTTATTATGAAGCAGTACTCTTGTGTGCTTGTTTATTGAGGTGTCCTCGAAAA
374       K  Y  *
1261    CTACGTTTTCCTTTGGATTTAATAAAGAATGGAGAAAATTAATGAGAG
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Fig 3. cDNA and amino acid sequence of *O. fragrans* var. *semperflorens* ADH.

Assembling all three fragments of *OfADH*, we get 1308bp full-length cDNA. *OfADH* has an open reading frame of 1128bp (53bp-1180bp), encoding a peptide of 375 animo acids (Fig. 3).

Analysis of *OfADH* protein structure

OfADH protein has an estimated molecular weight of 40497.11 Da with an isoelectric point of 5.9. Its formula is $C_{1806}H_{2886}N_{470}O_{532}S_{25}$. Analyzed in Hphob./Kyte & Doolittle model, this protein is a hydrophobic lipid soluble protein with grand average of hydropathicity 0.092 and aliphatic index 96.88 (Fig. 4). There are 21.07% alpha helix, 32.53% extended strand, 14.93% beta turn, and 31.47% random coil in *OfADH* protein structure.

As shown in Fig. 4, *OfADH* protein belongs to the zinc-dependent medium-chain dehydrogenase/reductase (MDR) superfamily, and contains 22 NAD(P) binding sites, 29 dimer interface, 4 catalytic Zn binding site, and 4 structural Zn binding site in the liver_alcohol_DH_like conserved domains, and it also had the PLN02740 (Alcohol dehydrogenase-like) domain (Fig.5).

Through the template of crystal structure and dynamic structure of Arabidopsis ADH protein (4jji.1), homo-dimer model is constructed by Swiss-Model (Fig. 6). The protein model GMQE score is 0.76, and the similarity of template protein sequence is 45.73%.

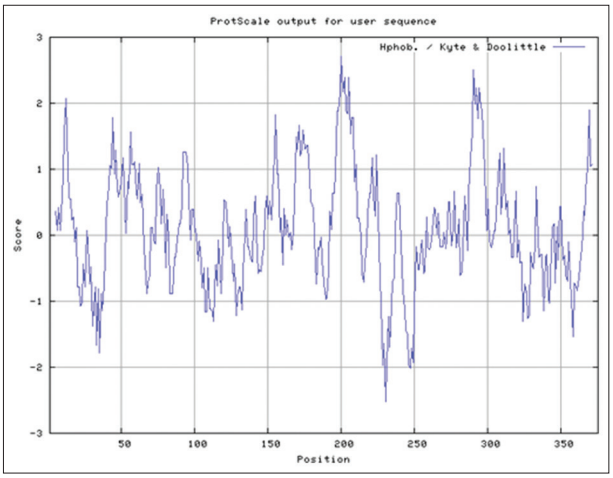

Fig 4. Profiling of hydrophobicity and hydrophilicity of *OfADH* protein.

Multiple sequence alignment and phylogenetic tree analyses of ADH

Multiple sequence alignment analysis reveals that *OfADH* protein shares 96.2% identity with the ADH from *Oleaeuropaea* (JN200815.1) (Fig. 7). Phylogenetic analysis (Fig. 8) further reveals that the *OfADH* protein is most closely related to ADH from *Oleaeuropaea*, and as a major branch of ADH1 cluster with *Arabidopsis thaliana*, *Oryza sativa*, and *Zea mays*. However, *OfADH* shares low homology of the amino acid sequence with *Musa acuminata*, and *Populus tremuloides*. According to sequence similarity and phylogenetic analyses, we presume that ADH family

Table 1: Primers used for gene cloning and expression of *O. fragrans* ADH

Names	The primers	Nucleotide
Cloning of the main fragment	ADH-F	TCGCAATGGACTCCCTGT
	ADH-R	GGGAGGTCTGTTTGAGGTCTT
3'RACE	ADH-3'gsp2	CTGGAGGGAAGTCACTATTGAAAAGG
	ADH-3'gsp3	GACTGGGGGTGGATTACTGTTATGA
5' RACE	ADH-5'gsp1	CCACCTGTAGTATCTTTG
	ADH-5'gsp2	TCCAACAGCACCAACACCAATA
	ADH-5'gsp3	ACATTCCCCACATTCTCCCA
Cloning of full-length of *OfADH*	FI-ADH-F	ACGGGACAATATTCTCCCA
	FI-ADH-R	CTCTCATTAATTTTCTCCATTC

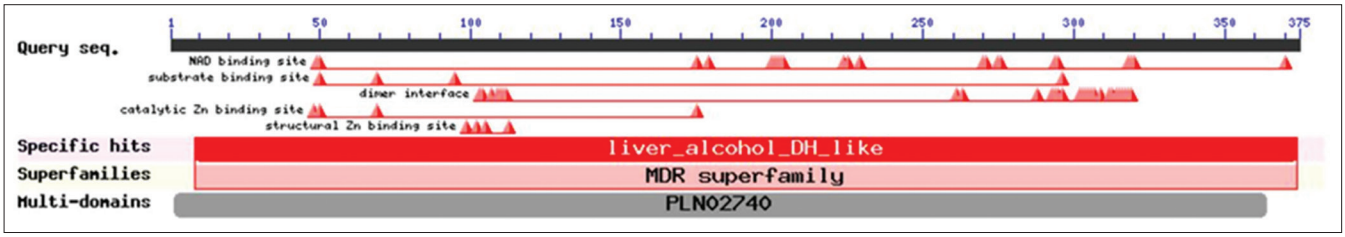

Fig 5. Profiling of conserved domains of *OfADH* protein.

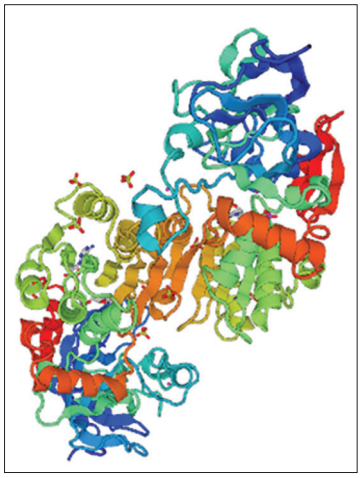

Fig 6. Prediction of three-dimensional structure of *OfADH* protein.

from plants represent significant differences related to their functional diversity in different plants.

OfADH gene expression patterns

The *OfADH* gene expression patterns of flowers and leaves in Mar, Jun, Sep and Dec were studied by semi-quantitative RT-PCR (Fig. 9). *OfADH* expression level of flowers was the highest in March, and then decreased significantly in June. In September, *OfADH* expression had rose again a bit, while the expression was similar to March in December. The *OfADH* expression of flowers performed a high-low-high pattern in different season. However, the *OfADH* gene expression pattern of leaves was different from flowers. *OfADH* expression level of leaves was the lowest in March, and then increased in June. In September, the highest expression level was reached. So, the gene expression pattern of *OfADH* in leaves was low-high-low trend.

During flowering, the expression of *OfADH* gene increased continuously from the budding stage and reached the highest expression level at the blooming stage. The expression of *OfADH* gene decreased rapidly from full-flowering to drop-off stage (Fig. 10).

In one of the full flowering days, the *OfADH* gene expression level was the highest at 14:00, which was 3 times that at 2:00 (the lowest expression level). And the expression level of the other time points was not significant (Fig. 11).

DISCUSSIONS

The studies of ADH gene about humans, animals and microorganisms were very more (Pan et al., 2014) than this about plants (Chao et al., 2014). At present, ADH gene have been identified in a lot of herbaceous and woody plants, such as *Arabidopsis* (Chung et al., 1999), *Legumes* (Fukudaet et al., 2005), *Poplar* (Bomatia et al., 2005), and *Pyrus* (Zheng et al., 2011). ADH gene are widely expressed in leaves, flowers, stems, roots and seeds, and demonstrate diversity of biological function in plants.

The interaction between ADH and ethylene could promote the persimmon deastringency during maturation process (Min et al., 2012). ADH gene also regulated the synthesis of fruit aroma components through the fatty acid derivatives metabolic pathway (Dudareva et al., 2008). Especially, it would affect fruit aromatic components and flavor of fruit a lot that the content of ADH had a significant change during fruit maturation and ripening period (Imahon et al., 2002;

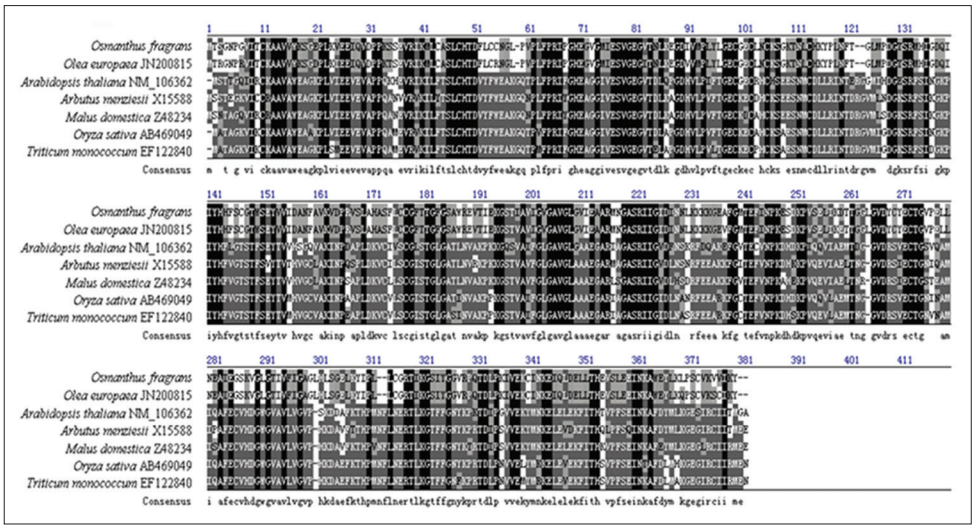

Fig 7. Amino acid multiple alignment of ADHs between *O. fragrans* var. *semperflorens* and other plants.

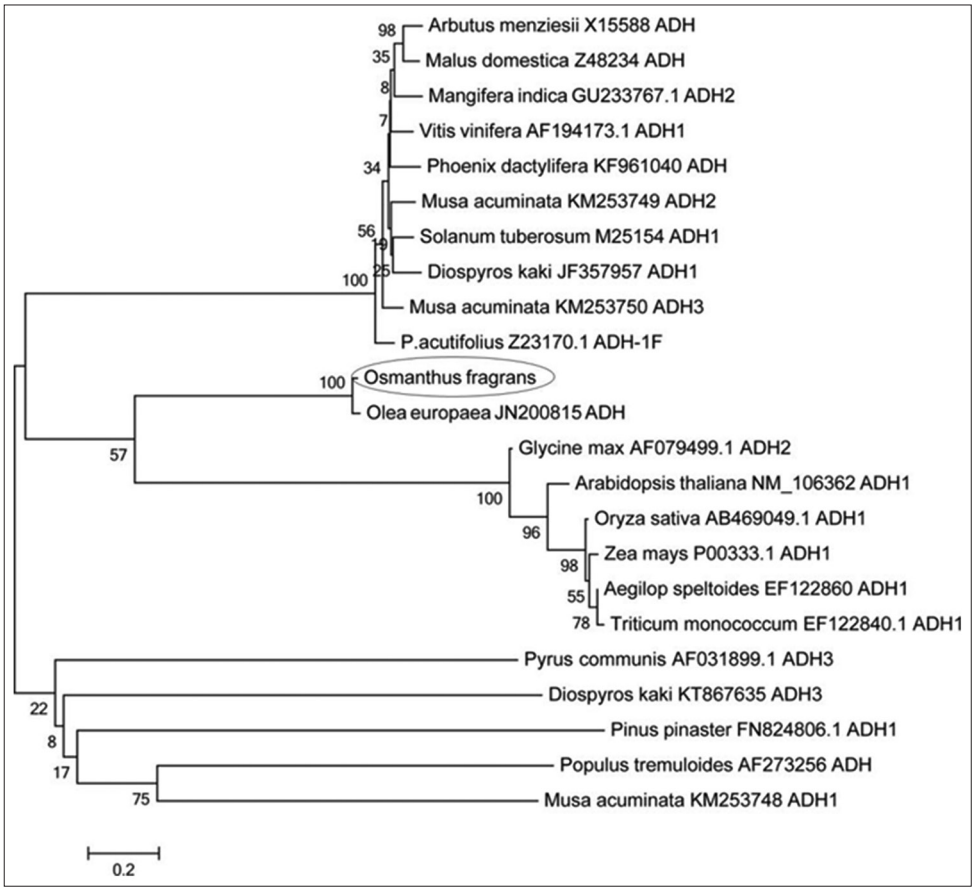

Fig 8. Phylogeny tree of ADHs from different plants.

Li et al., 2011). To explore the expression pattern of gene in the aroma synthesis pathway will be an effective method, because the difference of floral composition is significant among plants and the fragrance biosynthetic pathway is unclear. In this study, the full-length cDNA of *OfADH* have been cloned and sequence analyzed. The *OfADH* gene expression pattern, a trend of increasing from budding stage to blooming stage and then decreasing, was positive correlation with the aroma formation during flowering development (Li et al., 2008). This conclusion was similar with observations that ADH gene had a certain promotion effect on aroma formation (Bellincontro et al., 2005).

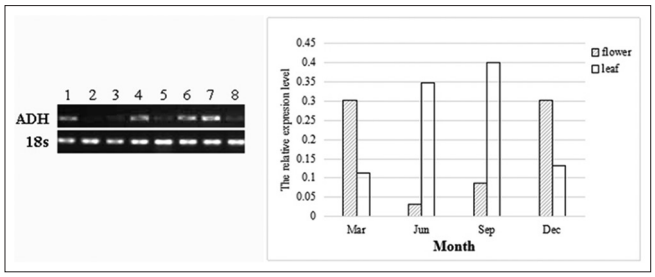

Fig 9. Expression level of *O. fragrans* var. *semperflorens* ADH gene in different months and tissues. 1-4: The *OfADH* expression of flowers in Mar, Jun, Sep, and Dec; 5-6=The *OfADH* expression of leaves in Mar, Jun, Sep, and Dec.

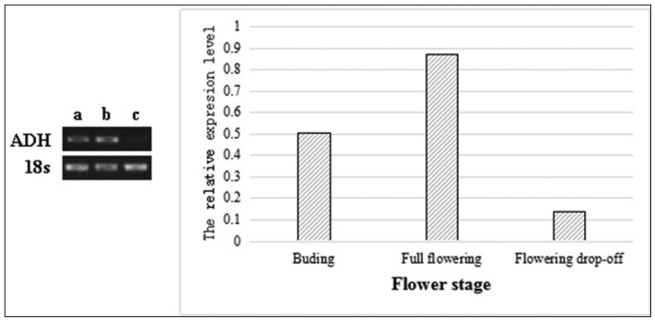

Fig 10. Expression level of ADH gene in different *O. Fragrans* flowering stages a: Budding stage; b: Full flowering; c: Flowering drop-off.

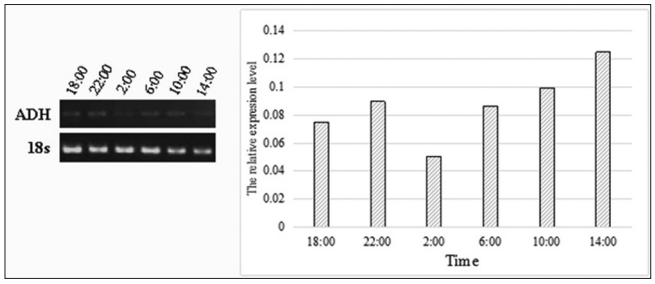

Fig 11. Expression level of *OfADH* gene in different times.

At the same time, a large of researches indicated that stress can promote expression of ADH in order to increase plant resistance and adaptability to the environment. ABA could induce ADH expression in *Arabidopsis* (De Bruxelles et al., 1996). The expression of ADH would increase in the water logging peanut roots (Liu et al., 2007). The certain concentration of salt stress could make ADH increasing significantly in rice roots (Liang, et al., 2012). ADH was up-regulated in the leaves of Banana Seedlings under cold stress (Jia et al., 2014). In this study, the expression level of *OfADH* was higher in June and September with relatively high temperatures than it was in March and December. When leaves of *O. Fragrance* were under high temperature stress, ADH gene expression would be increased because of the resistance response mechanism activation in order to avoid from heat damage. Many researches about functional components in the leaves, flowers (Ge et al., 2018a and 2018b), barks (Ge et al., 2017a and 2017b), stems (Ge et al., 2017c and 2017d), roots (Ge et al., 2017e; Chen et al 2017), fruits (Jiang et al., 2018a and 2018b), timbers (Peng et al., 2017a and 2017b) and byproducts (Liu et al., 2017; Xie et al., 2018) of plants showed that they contain many bioactive components (Peng et al., 2016; He et al., 2017; Cheng et al., 2017; Yang et al., 2017; Qin et al., 2017; Xu et al., 2018; Wang et al., 2018). This result was consistent with the resistance response function of ADH (Strommer et al., 2011).

This finding indicated that the *OfADH* has a certain function in the aroma synthesis pathway in flowers and heat shock response in leaves.

There is obvious functional diversity of *OfADH* in *O. fragrance* var. *Semperflorens*. The molecular mechanism of OfADH remains to be further research in order to find out how OfADH perform different function in flowers and leaves.

ACKNOWLEDGEMENTS

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