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Full Length Article

Isolation and Expression Analysis of *OfLIS* Gene in *Osmanthus fragrans*

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Abstract

Osmanthus fragrans Lour., originating from the Yangtze River valley of China, has high ornamental value and is an important source of floral scent materials. Linalool is an important marker of the floral scentin *O. fragrans*. In this study, a linalool synthase-encoding gene (*OfLIS*, GenBank No. MK563985) was cloned from *O. fragrans* var. Jinyu Taige by rapid amplification of cDNA (complementary DNA) ends (RACE). The full-length of OfLIS was 2911 bp in length and consisted of an open reading frame (ORF) of 2523 bp, encoding a protein consisting of 840 amino acid residues. Sequence alignment indicated that *OfLIS* contains the conserved DDxxD motif and RLxxDxxxxxxExxxG motif. The phylogenetic analysis revealed OfLIS belongs to subfamily II of the dicot group and has the closest relationship with *PaLIS*. Tissue-specific expression analysis showed that the *OfLIS* gene is specifically expressed in flowers and its expression is highest at peak flowering stage (PFS). In addition, 17 potential phosphorylation sites were found in OfLIS, and 531Y is located in the DDxxD motif. The cloning and expression analysis of *OfLIS* gene helps further our understanding of the mechanism of floral scent formation in *O. fragrans*.

Keywords: *Osmanthus fragrans* var. JinyuTaige; Floral scent; Linalool synthase; Expression level; Phosphorylation

Introduction

Floral scent, which is a mixture of low molecular weight volatile compounds, is a key factor considered in the use of garden plants due to the aesthetic and economic value (Giusto *et al*. 2010). In addition to attracting insects for pollination, the compounds of floral scent are important for fragrance and medicinal products (Zhu *et al*. 2016). A mass of volatile compounds were recently identified, and a number of biosynthesis-related genes were cloned in plants (Lavid *et al*. 2002; Shalit *et al*. 2003; Verdonk *et al*. 2003; Wu *et al*. 2004; Boatright *et al*. 2004; Dexter *et al*. 2007).

Linalool is an acyclic monoterpene found in the floral scents of many plants. As an important fragrance material, linalool is widely used in cosmetic products such as perfumes and shampoos, and in non-cosmetic products like household cleaners and detergents (Karuppiah *et al*. 2017). The annual worldwide use of linalool exceeds 1000 tons, which is mostly extracted from plants (Bickers *et al*. 2003). Linalool synthase (LIS) is the enzyme that catalyzes the formation of linalool using geranyl pyrophosphate as a substrate. LIS has two conserved regions, the aspartate-rich (DDxxD) motif and the NSE (RLxxDxxxxxxExxxG) motif, which are essential for binding Mg^{2+} ions (Karuppiah *et al*. 2017). In plants, the *LIS* gene is firstly cloned from the flower of *Clarkia breweri* (Pichersky *et al*. 1995). Until now, *LISs* have been widely isolated from the species such as Arabidopsis, cotton, papaya, grape, and citrus (Shimada *et al*. 2014; Zhu *et al*. 2014; Boachon *et al*. 2015; Gomes *et al*. 2016; Huang *et al*. 2018).

Sweet osmanthus (*Osmanthus fragrans* Lour.) is an evergreen shrub species belonging to the Oleaceae family. As its unique fragrance and cultural values, sweet osmanthus is a well-known flower in East Asia, especially in China. Based on their flowering season and corolla coloration, sweet osmanthus can be classified into four groups: Yingui, Jingui, Dangui, and Sijigui (Han *et al*. 2019). The fragrance of the osmanthus flower contains more than 30 chemical substances, and several related genes have been isolated in the past decade (Wang *et al*. 2009). Baldermann *et al*. (2010) cloned a carotenoid cleavage dioxygenase 1 encoding gene, and showed its relationship to carotenoid accumulation and volatile emission during floral development. Han *et al*. (2016) characterized an OfWRKY3 positively regulating the carotenoid cleavage dioxygenase gene *OfCCD4*. Xu *et al*. (2016) identified 10 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway-related genes in *O. fragrans*, and *OfDXS1*, *OfDXS2*, and *OfHDR1* expression patterns have a clear diurnal oscillation. *O. fragrans* var. Jinyu Taige is a cultivar of the Sijigui group that has a large

number of flowers and a long flowering period (Qin *et al.* 2012). Based on the results of previous transcriptome sequencing of *O. fragrans* var. Jinyu Taige at different stages (Xian *et al.* 2019), we isolated the *OfLIS* gene using rapid amplification of cDNA ends (RACE) technology in this study. These results provide a theoretical basis for research on the mechanism of aroma formation, the selection of new species, and improvement of the processing and use of *O. fragrans* products, which will support the industrial development of *O. fragrans* and accelerate the structural reform of the *O. fragrans* supply industry.

Materials and Methods

Plant materials

Osmanthus fragrans Lour. var. Jinyu Taige was planted in 2010 in the garden of the germplasm resource for woody flowers at the Horticulture Research Institute, Modern Agricultural Science and Technology Innovation Demonstration Park, Sichuan Academy of Agricultural Sciences, China. Flowers (3 g) from 3 different plants with the same growth status and health at early flowering stage (EFS), peak-begin flowering stage (PBFS), peak flowering stage (PFS), and peak-end flowering stage (PEFS) were collected status from September to October 2017 and quickly frozen with liquid nitrogen for storage at –80°C. The roots and leaves were rinsed with ddH_2O (double distilled H_2O), cleaned with aseptic paper and then stored at –80°C.

Extraction of total RNA and synthesis of cDNA

According to the instructions of the RNA prep Pure Plant Kit (DP437, Tiangen Biotech Co., Ltd., Beijing, China), total RNA was extracted from *O. fragrans* var. Jinyu Taige. Then, the purity and integrity of the extracted total RNA were examined by spectrophotometry (UV757CRT, INESA Analytical Instrument Co., Ltd., Shanghai, China) and 1% agarose gel electrophoresis (DYCP-32A, Liuyi Biotechnology Co., Ltd., Beijing, China), respectively. Finally, according to the instructions of the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), 1.5 μg of total RNA was used for the synthesis of first-strand cDNA.

Cloning of full-length cDNA of the *OfLIS* gene

Based on an analysis of transcriptome data (Xian *et al.* 2019), c162522_g1 in *O. fragrans* with a length of 1463 bp was found to have certain homology with the LIS proteins in other plants. To obtain the full-length sequence of the gene, the following primers were used for amplification: 5'-RACE-F: AAG CAG TGG TAT CAA CGC AGA GT, 5'-RACE-R: CAA GTG TTT TCT CGA TCT CTT, 3'-RACE-F: ATC CGA GCT TAA CAA ATG AAA GGC, and 3'-RACE-R: TAC GTT TTT TTTTTT TT. Then, the amplified fragments were recovered with an agarose gel DNA recovery kit (DP 209,Tiangen Biotech Co., Ltd., Beijing, China) and ligated into the pEASY-Blunt Simple Cloning Kit (CB111-01, Trans Gen Biotech Co., Ltd., Beijing, China) cloning vector and used to transform *Escherichia coli* strain DH5α. Three clones cultured overnight at 37°C were selected for sequence determination by Gen Script Co., Ltd. (Nanjing, China). The sequencing results were spliced with the c152118_g1 sequence with DNAMAN to obtain the complete full-length *OfLIS* cDNA.

Bioinformatics analysis of the *OfLIS* gene

The open reading frame (ORF) of the *OfLIS* gene was predicted using ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html, NCBI, Bethesda, Maryland, USA). The derived protein molecular weight and isoelectric point of *OfLIS* were predicted using Prot Param software (http://web.expasy.org/protparam, SIB Swiss Institute of Bioinformatics, Switzerland). The three-dimensional homologous modelling of *OfLIS* was performed by SWISS-MODEL (http://swissmodel.expasy.org/, SIB Swiss Institute of Bioinformatics, Switzerland). The phosphorylation sites in the protein sequence derived from *OfLIS* were predicted using Kinase Phos software (http://kinasephos.mbc.nctu.edu.tw/index.php, Bid Lab, Institute of Bioinformatics, National Chiao Tung University, Taiwan). *OfLIS* was compared with *FrLIS* (KX452731), *OsLIS* (AK110925), *Lllis* (ABD77417), *AtLS1*(AAO85533), *AaLS1* (GQ338153), *CuSTS3-1* (AB857230), *PhLS* (FJ644546), *AmNES/LIS* (EF433761), *CbLIS* (U58314), *VvRiLinNer* (JQ062931), *GhTPS12* (KJ957818), *PaLIS* (AAL24105), *CsLIS/NES* (KF006849), and *OfLis* (FJ645727) sequences using DNAMAN 5.22 software (Lynnon Biosoft, San Ramon, CA, USA). The Multiple Sequence Alignment website (https://www.ebi.ac.uk/Tools/msa/, Agilent, Santa Clara, CA, USA) was used for LIS protein phylogenetic tree construction with 1000 bootstrap replicates. LIS sequence information from different species was obtained from the National Center for Biotechnology Information (NCBI).

Expression pattern analysis of the *OfLIS* gene

The following qPCR (quantitative real-time PCR) primers for the *OfLIS* gene were designed using Primer Premier 5.0 software (Primer Biosoft International, San Francisco, CA, USA): *OfLIS*-qF (TTC TGA TGG ATG GAT T) and *OfLIS*-qR (AAG GTC TGG ACG AGT G). The primer sequences used to amplify *OfActin*, an internal reference gene, were *OfActin*-qF (CAA GAA GAC CAC CAT GCC AAA) and *OfActin*-qR (AAA GCT CAC TGC TCA AAC AAC). The qPCR was performed on a CFX96 real-time

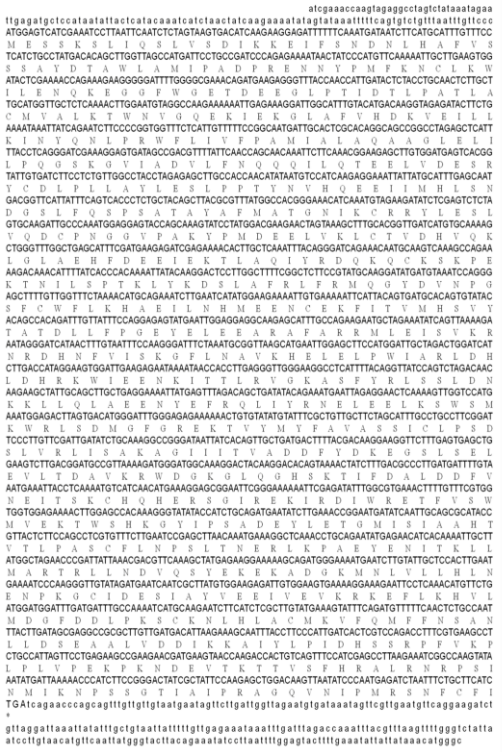

Fig. 1: Full length cDNA and deduced amino acid sequences of *OfLIS*. The predicted amino acid sequence is listed below the nucleotide sequence. Note: *, stop codon

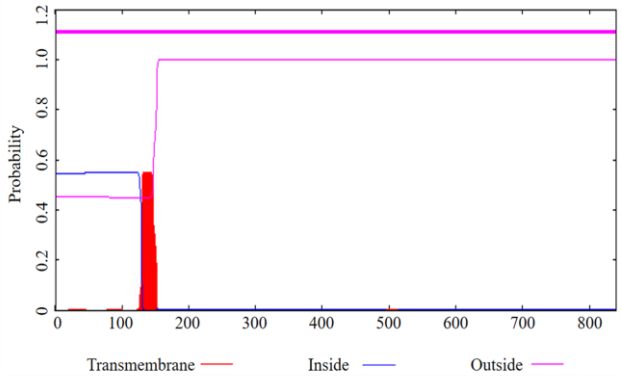

Fig. 2: Transmembrane domain analysis of *OfLIS*

quantitative PCR system (Bio-Rad, Hercules, CA, USA) following the SYBR® Premix Ex Taq instructions (TAKARA Bio, Otsu, Shiga-ken, Japan) with an annealing temperature of 58°C. The relative expression level of the *OfLIS* gene was calculated using the$2^{-\triangle\triangle Ct}$ method.

Data analysis

Data were analyzed using S.P.S.S. 19.0 software (IBM, Armonk, NY, USA) by Duncan's multiple range test. The capital or lower-case letters represented $P < 0.01$ or $P < 0.05$, respectively. Excel 2013 (Microsoft, Redmond, Washington, USA) was used for mapping.

Results

Cloning of the *OfLIS* gene from *O. fragrans*

The 5'-RACE and 3'-RACE amplification products were 987 and 862 bp in length, respectively. After spliced with c162522_g1, the 2911 bp full-length *OfLIS* gene sequence (Gen Bank No.MK563985) was obtained. The sequence encodes an acidic protein composed of 840 amino acid (aa) residues with a molecular weight of 96.2 kDa and a theoretical isoelectric point of 5.93. The 5' and 3' untranslated regions of the gene are 126 and 262 bp in length, respectively, whereas the ORF is 2523 bp (Fig. 1). Transmembrane domain analysis indicated that 120–150 aa of the N-terminus comprised transmembrane domain (Fig. 2).

Sequence alignment of *OfLIS* with other homologous plant protein sequences

The *OfLIS* from *O. fragrans* var. Jinyu Taige and the related protein sequences from *Oryza sativa*, *Arabidopsis thaliana*, *Carica papaya*, *Clarkia breweri*, and *O. fragrans* var. thunbergii were aligned using DNAMAN software ((Lynnon Biosoft, San Ramon, CA, USA). The sequence homologies between *OfLIS* and *PaLIS*, *CbLIS*, *AtLS*1, *OsLIS* and *OfLis* are 93, 92, 52, 63 and 53%, respectively. Conserved domain analysis clearly showed that all proteins contained the conserved DDxxD and RLxxDxxxxxExxxG motifs (Fig. 3). To analyze the phylogenetic relationship between OfLIS and other homologous proteins, a phylogenetic tree was constructed among 17 LIS members from XXX plant species using the neighbor joining method. The phylogenetic analysis showed that all members are divided into monocot group and dicot group (Fig. 4). The members of the dicot group were further grouped into subfamily I and subfamily II (Fig. 4). OfLIS belongs to subfamily II and has the closest relationship with *PaLIS*.

Expression pattern analysis of *OfLIS*

Tissue expression analysis showed the *OfLIS* expression level is very low in roots and leaves, but is extremely high in flowers (Fig. 5A). Different stages of flowering expression pattern indicated that the expression level of *OfLIS* gradually increases over the four flower stages and peaks at PFS but then declines at PEFS (Fig. 5B).

OfLIS tertiary structure model construction and potential phosphorylation sites prediction

The tertiary structure of the OfLIS was predicted using the

Table 1: Putative phosphorylation sites determined by OfLIS analysis

Amino acid position	Amino acid types
6	Serine
34	Tyrosine
181	Tyrosine
189	Tyrosine
217	Serine
223	Tyrosine
306	Serine
371	Tyrosine
461	Tyrosine
504	Serine
531	Tyrosine
536	Serine
611	Tyrosine
618	Tyrosine
653	Tyrosine
703	Tyrosine
767	Tyrosine

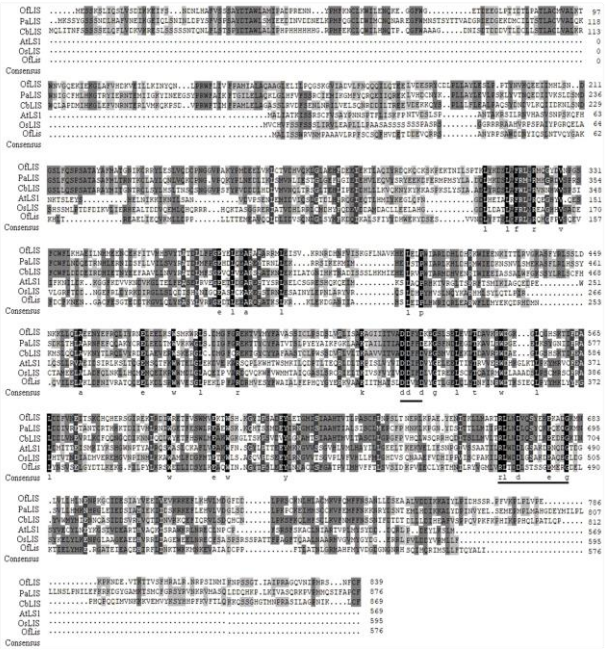

Fig. 3: Alignment of the OfLIS amino acid sequence with LIS from other five plants. Gene accession numbers: OfLIS, MK563985 for *Osmanthus fragrans* var. Jinyu Taige; PaLIS, AAL24105 for *Carica papaya*; CbLIS, U58314 for *Clarkia breweri*; AtLS, AAO85533 for *Arabidopsis thaliana*; OsLIS, AK110925 for *Oryza sativa*; OfLis, FJ645727 for *Osmanthus. fragrans* var. thunbergiiin. The shading color from black to white represents homology level: 100%, 75%, 50%, and 0%. The conserved motifs are underlined in black.

online software SWISS-MODEL (Fig. 6). We found that the protein mainly consisted of α helices and random coils. Potential phosphorylation sites prediction identified 17 potential phosphorylation sites (Table 1), and 531Y is located in the DDxxD motif (Fig. 6).

Discussion

Linalool is one of the main components of plant floral scents and is widely used in the cosmetic industry (Jiang *et al*. 2015; Huang and Hou 2017). Recently, linalool was found to have unique effects on human health, such as hypnotic (Linck *et al*. 2010), anti-inflammatory (Huo *et al*. 2013), analgesic (Kuwahata *et al*. 2013), anti-tumor (Chang and Shen 2014), and anti-anxiety (Cheng *et al*. 2015) effects. LIS is a key enzyme in the linalool synthesis pathway that directly catalyses the formation of linalool from GPP (L-galactose-1-phosphate phosphatase) (Nagegowda *et al*. 2008). Deng *et al*. (2016) co-expressed AaLS1 with FPPS (farnesyl pyrophosphate synthase) in *Saccharomyces cerevisiae*, and produced 240 μg/L (*S*)-linalool. Over-expression of a linalool synthase GhTPS12 in tobacco can significantly increase the content of linaloolin comparison to mock (Huang *et al*. 2018). In *Lavandula officinalis*, the result of Southernblotting suggests *LIS* has two copies (Zhang 2006). As one of the top 10 traditional flowers in China, *O. fragrans* has a pleasant fragrance (Zheng *et al*. 2017). Tang *et al*. (2009) isolated an OfLis with 576 aa in *O. fragrans* var. thunbergiiin. In this study, a *OfLIS* gene encoding 840 aa was obtained in *O. fragrans* var. Jinyu Taige. Despite the differences in length, the two proteins both have a core conserved aspartate-rich motif and an NSE motif, suggesting the diversification of LIS in *O. fragrans*.

Phosphorylation is a kind of protein post-translational modification that plays an important role in the regulation of protein activity (Eberhardt *et al*. 2012; Hess and Stamler 2012). The protein phosphorylation is manipulated by adding phosphate groups through protein kinases (MAPK, CDPK, *etc*.) and removing phosphate groups through phosphatases (PP1, PP2A, *etc*.) (Xu and Zhang 2015; Hou *et al*. 2016; Tiffany and Boudsocq 2019; Chao *et al*. 2020). In winter wheat, Xu *et al.* (2019) perform phosphoproteomic analyses on the different flowering stages and identify 124 differentially expressed phosphorylated proteins that participate in translation, transcription, and metabolic processing. Additionally, there are studies show that phosphorylation is involved in the regulation of plant aroma biosynthesis (Fallon and Trewavas 1993). 3-hydroxy-3-methylglutary CoA reductase (HMGR) is a key regulatory enzyme that controls the synthesis of isopentenyl diphosphate (IPP), an aromatic precursor. In Arabidopsis, the 577 site of AtHMGR1 was found to be phosphorylated by BoHRK from *Brassica oleracea* (Dale *et al*. 1995). In the present study, 17 potential phosphorylation sites were detected in OfLIS, of which we found that 531Y is located in the conserved DDxxD domain. Considering the DDxxD domain is the crucial catalytic site for binding Mg^{2+} ions, we deduced the phosphorylation of 531Y may regulate the catalytic activity of OfLIS.

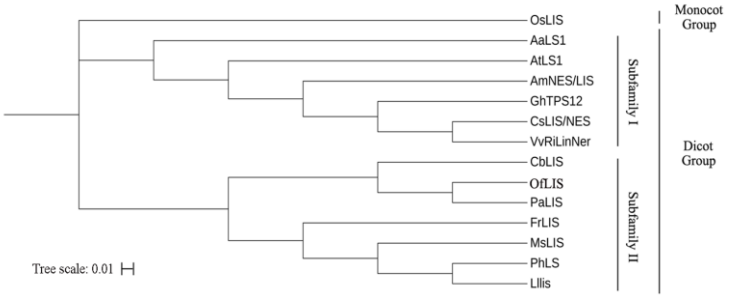

Fig. 4: Phylogenetic analysis of LIS proteins. The bar represents the evolutionary distance. Gene accession numbers: OfLIS, MK563985 for *Osmanthus fragrans* var. Jinyu Taige; PaLIS, AAL24105 for *Carica papaya*; CbLIS, U58314 for *Clarkia breweri*; AtLS, AAO85533 for *Arabidopsis thaliana*; OsLIS, AK110925 for *Oryza sativa*; AaLS1, GQ338153 for *Actinidia arguta*; AmNES/LIS, EF433761 for *Antirrhinum majus*; GhTPS12, KJ957818 for *Gossypium hirsutum*; CsLIS/NES, KF006849 for *Camellia sinensis*; VvRiLinNer, JQ062931 for *Vitis vinifera*; FrLIS, KX452731 for *Freesia hybrida*; MsLIS, AAC37366 for *Mentha spicata*; PhLS, FJ644546 for *Perilla frutescens*; and Llis, ABD77417 for *Lavandula latifolia*

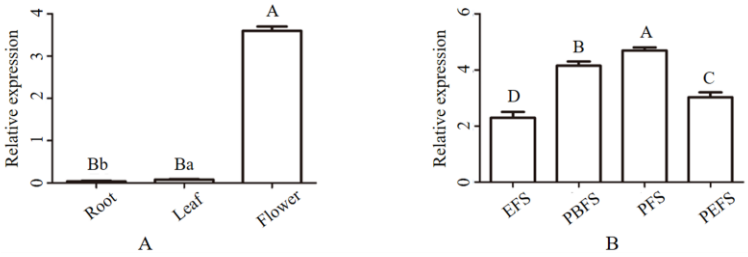

Fig. 5: Expression pattern of *OfLIS* in different tissues (**A**) and different flowing stages (**B**) in *O. fragrans*. Error bars for qRT-PCR showed the standard deviation of three replicates. The capital letters represent $P < 0.01$ lower case letters indicate $P < 0.05$

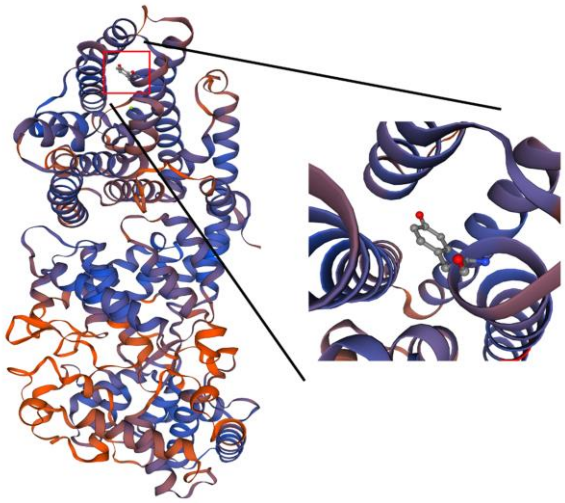

Fig. 6: Tertiary structure model construction of OfLIS. The upper box (labeled with red) is enlarged, with the right panel representing the 531Y located in the DDxxD motif

Conclusion

In this study, we cloned an *OfLIS* gene from *O. fragrans* var. Jinyu Taige. Sequence alignment indicated that *OfLIS* contains the conserved DDxxD motif and RLxxDxxxxxxExxxG motif. The phylogenetic analysis revealed OfLIS belongs to subfamily II of the dicot group and has the closest relationship with *PaLIS*. Tissue-specific expression analysis showed that *OfLIS* is specifically expressed in flowers and its expression is highest at peak flowering stage (PFS). We found 17 potential phosphorylation sites in OfLIS, and 531Y is located in the

DDxxD motif. The cloning and expression analysis of *OfLIS* gene helps further our understanding of the mechanism of floral scent formation in *O. fragrans*.

Acknowledgements

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Author Contributions

Rui Chen, Yuanzhi Pan and Xiaolin Xian conceived and designed the experiments; Rui Chen and Yuanzhi Pan performed the experiments; Haiyan Song analyzed the data; Xiaolin Xian contributed materials; Ju Hu and Rui Chen wrote the paper.

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