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Molecular Analysis of Anthocyanidin Synthase Gene from *Lilium oriental* 'Sorbonne'

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Abstract: As one of the key rate-limiting enzymes in anthocyanin biosynthetic pathway, anthocyanidin synthase (ANS) catalyse leucocyanidin into colour anthocyanin. Using Lilium oriental 'Sorbonne' as the material, an ANS gene conservative district sequence was cloned from fresh petals of L. oriental 'Sorbonne' by RT-PCR and rapid amplification of cDNA ends. Sequence analysis and homology comparison of the cDNA was performed using DNAman software and the BLASTN programs. Results showed that the length of the ANS gene core fragment was 804 bp (GenBank Accession No. JX427406) and encode 264 amino acids. Phylogenetic analysis showed that the cloned LiANS core fragment was genetically close to monocots of Tulipa and Iris.

Key words: Anthocyanidin synthase · *Lilium oriental* 'Sorbonne' · Phylogenetic tree

INTRODUCTION

The lily, one of the most popular ornamental plants in the world, is a perennial herb ball root flower. It belongs to the genus *Lilium* of the family Liliaceae. The demand for lily has been increased recently as a growing number of customers are attracted to its colourful flowers. Thus, breeding lily cultivars with new colours is important.

The flowers and leaves of higher plants turn red in relation to anthocyanin accumulation [1]. Anthocyanin is a water-soluble natural pigment in plant that can turn red, purple, blue, pink and others [2]. It is catalysed by anthocyanidin synthase (ANS, also named leucoanthocyanidin dioxygenase, LDOX) from leucocyanidin into colour anthocyanin [3]. In addition, ANS, one of the key rate-limiting enzymes in Anthocyanin Biosynthetic Pathway, was located in the end of the anthocyanin synthesis pathway. It was specifically expressed in the flower and the flower colour variation was changed with the expression level of ANS gene. Therefore, ANS gene was cloned from *L. oriental* 'Sorbonne' in this research. The sequence comparison and bioinformatics analysis were conducted in order to lay a foundation for further exploration the reason and molecular mechanism of lily flower colour formation.

MATERIALS AND METHODS

Materials: The fresh petals were collected from *L. oriental* 'Sorbonne', which were immediately frozen in liquid N_2 and stored in -80°C until needed. The RNA extraction kit, vector PGM-T, mini plasmid-DNA extraction kit, DNA polymerase, dNTPs, DNA Marker D2000 and *Escherichia coli Top10* used in the experiments were purchased from Tiangen Biotech Co. Ltd. (Beijing, China). The agarose gel and DNA gel extraction kit were bought from AxyGen Co., Ltd. M-MLV as the reverse transcriptase was procured from Promega. All other chemicals were of analytical grade. All the primers were synthesised by SBS Genetech Co. Ltd. (Beijing, China).

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RNA Extraction and cDNA Synthesis: Petals of *L. oriental* 'Sorbonne' weighing 100 mg were quickly ground to powder in liquid N_2 and dissolved in lysis buffer. The total RNA was extracted by an RNA extraction kit. First-strand cDNA was synthesised with using total RNA as the template, Oligo dT as the primer and M-MLV as the reverse transcriptase. Where is the reference [4].

Amplification of LiANS Core Fragment: After querying and analysing the information of ANS genes from Genbank and plant EST databases, a pair of primers by Primer 5.0 and BLASTN sequencing analysis were designed:

AF:5'-A(T/C/G)TGGGG(C/G/T)GT (G/A) ATGCA (C/T) (G/A/C) T(C/T/G)(G/A)T(C/G/T)AA- 3'

AR:5'-(A/C) TGAACAGCTT (A/G) TGCT (G/T) (G/A) A(T/C) (G/A)TGCT-3'

This system of PCR was performed by reverse-transcript cDNA as template, AF and AR as primers. The reaction mixture having volume of 25 µl containing DNA template 1µl, 10 × Taq Buffer 2.5 µl, Primer 1 µl (10 mM/l), Taq 0.5 (2.5U/µl), dNTPs 0.5µl (10 mM/l) and sterilized deionized water 18.5 µl. The amplification procedure conditions were initial denaturation at 94℃ for 5 min, 35 cycles of denaturation at 94℃ for 30 sec, annealing at 58℃ for 30 s, extension at 72℃ for 1 min and final extension at 72 ℃ for 10 min. 1.0% Agarose gel electrophoresis was performed to detect the PCR products. The cloned core fragment was later sequenced by a TA cloning kit.

Sequencing of the LiANS Core Fragment: The cloned fragment of ANS gene was purified by DNA gel extraction kit and later ligated to PGM-T. The ligation product was transformed into *E. coli* competent cells. The correct colony screened by blue-white selection and colony PCR was subsequently sequenced by Tiangen Biotech Co. Ltd. (Beijing, China).

Bioinformatic Analysis : Primer Premier 5 software (http://www.Premierbiosoft.com) was used to design all the primers. Sequences were aligned using the software DNAman 5.2.2 (http://www.lynnon.com) and CLUSTAL W 1.81 [5]. The phylogenetic tree was constructed by Neighbour-Joining method using MEGA 4.0 (Molecular Evolutionary Genetics Analysis, version 4.0) software [6].

RESULTS

Cloning of the LiANS Core Fragment: The ANS gene was reversebly transcribed in the total RNA of the lily plantlet petal and its cDNA was used as the template for PCR reactions. An expected fragment of about 800 bp was observed in the agarose gel electrophoresis (Fig. 1). Cloning and sequencing of this fragment was conducted.

Analysis of the LiANS Nucleotide and Peptide: Sequencing and BLAST analysis showed that the length of ANS obtained was 804 bp and encode 264 amino acids (Fig. 2). This fragment shares high homology with the other known ANS gene. Homology search results in GenBank (NCBI) showed that LiANS nucleotide had high identity to other plants such as *Tulipa fosteriana* (86% identities, accession number KC261507), *Strelitzia reginae* (74% identities, accession number KC484623), *Ipomoea eriocarpa* (73% identities, accession number HQ141977), *Paeonia suffruticosa* (73% identities, accession number KJ466969), *Solenostemon* (73% identities, accession number EF522157).

In addition, a domain of the 20G-Fe II_oxy superfamily was observed in this fragment by analysing with BLASTp (Fig. 3). This gene obtained was tentatively named *LiANS* and submitted to GenBank (JX427406).

Sequence analysis revealed that *LiANS* was highly homologous to other species. *LiANS* shared 86%, 74% and 73% nucleotide homology, respectively with ANS genes of *Tulipa gesneriana* (AB456683.1), *Iris×hollandica* (AB284174.1) and *Solenostemon svutellarioides* (EF522157.1). It shared 72% nucleotide homology with *Paeonia lactiflora* (JQ070805.1), *Prunus salicina* var. *cordata* (JN560957.1), *Fragaria×ananassa* (AY695817.1), *Malus domestica* (AF117269.1) and 71% homology with *Arabidopsis lyrata* subsp. *Lyrata* (XM002867699.1) (Fig. 4).

Phylogenetic Analysis of the Amino Acid Sequence of *Lians*: Phylogenetic analysis was done by aligning amino acid sequence using software Clustalx 1.83 and Mega 4.0 software to construct a tree (Fig. 5).This study discovered that the ANS gene of *M. domestica* and *P. salicina* var. *cordata* were grouped togather. Both belongs to Rosaceae family. The ANS genes of *Matthiola incana* and *A. lyrata* subsp. *Lyrata* were also grouped and both were in Cruciferae family. *T. gesneriana*, *L. oriental* 'Sorbonne' and *Iris×hollandica*, which were all monocotyledonous, were clustered into one group. *T. gesneriana* and *L. oriental* 'Sorbonne', belong to Liliaceae, have a close genetic relationship.

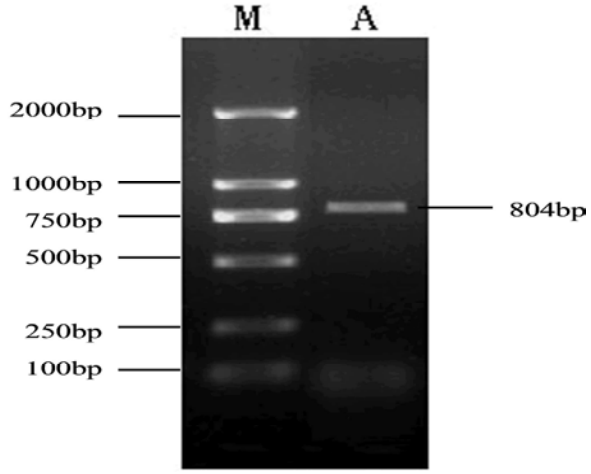

Fig. 1: Agarose electrophoresis of amplification of ANS conserved sequence from *Lilium oriental* 'Sorbonne' M: DL 2, 000TM DNA Marker; A: The fragment of *LiANS*

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1       AGTGGGGGGTGATGCATGTGATGAACCATAGAATTCCGTTGGAGTTGATAGATAGGGTGA
1                  M  H  V  M  N  H  R  I  P  L  E  L  I  D  R  V
61      GGGAGGTGGGGAAGGGCTTTTTCGACCTTCCGGTAGAGCAGAAAGAGAAGTATGCCAATG
17      R  E  V  G  K  G  F  F  D  L  P  V  E  Q  K  E  K  Y  A  N
121     ATCAGGCGTCTGGAGAGATACAGGGGTACGGGAGTAAGCTGGCGAATAATGAAAGTGGGC
37      D  Q  A  S  G  E  I  Q  G  Y  G  S  K  L  A  N  N  E  S  G
181     AGCTTGAGTGGGAGGATTACTATTTTCACCTTATATTTCCAGAGGAAAAGACCGATTTGT
57      Q  L  E  W  E  D  Y  Y  F  H  L  I  F  P  E  E  K  T  D  L
241     CCCGCTGGCCCAAAGAACCAGAAGACTATACAGAAGCAACTAAGGAGTTTGCTAAGGAGC
77      S  R  W  P  K  E  P  E  D  Y  T  E  A  T  K  E  F  A  K  E
301     TTAGAGTGGTGGTGACCAAGATGCTATCCATGCTCTCTCAAGGTCTCGGCCTCGAATCCG
97      L  R  V  V  V  T  K  M  L  S  M  L  S  Q  G  L  G  L  E  S
361     GCAAGCTCGAGAAAGAGCTCGGGGGAATGGATGACCTACTCATGCAGATGGAGATCAACT
117     G  K  L  E  K  E  L  G  G  M  D  D  L  L  M  Q  M  E  I  N
421     ACTATCCCAAATGTCCGCAACCTGAGCTCGCCCTCGGTGTCGAAGCCCACACCGATGTCA
137     Y  Y  P  K  C  P  Q  P  E  L  A  L  G  V  E  A  H  T  D  V
481     GCTCCCTCACCTTCCTCCTCACCAACATGGTACCGGGCCTTCAGCTCTACTATGGTGGCA
157     S  S  L  T  F  L  L  T  N  M  V  P  G  L  Q  L  Y  Y  G  G
541     AATGGGTCATCGCCCAGTGCGTCCCCGATTCGCTTCTCGTCCACATTGGCGACACACTAG
177     K  W  V  I  A  Q  C  V  P  D  S  L  L  V  H  I  G  D  T  L
601     AGATTCTCAGCAATGGTAGATATAGGAGCATTTTGCATAGGAGTTTGGTGAACAAGGAGA
197     E  I  L  S  N  G  R  Y  R  S  I  L  H  R  S  L  V  N  K  E
661     GGGTCAGGATCTCGTGGGCAGTGTTCTGCGAGCCGCCGAAGGAGACGATCGTGCTGAAAC
217     R  V  R  I  S  W  A  V  F  C  E  P  P  K  E  T  I  V  L  K
721     CATTGCCGGAGCTGGTGACGGAGGGGGCGCCGGCGAAGTTCCCGCCGAGGACTTTCAAGC
237     P  L  P  E  L  V  T  E  G  A  P  A  K  F  P  P  R  T  F  K
781     AGCACGTTCAGCACAGCTGTTCAT
257     Q  H  V  Q  H  S  C  S
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Fig. 2: Conserved nucleotides and deduced amino acid sequences of ANS from *Lilium oriental* 'Sorbonne'

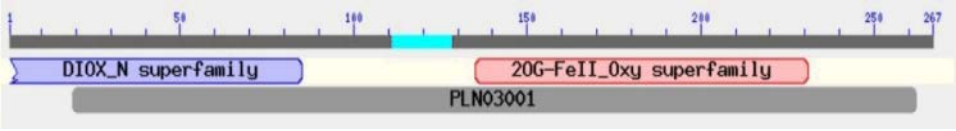

Fig. 3: Domain of 20G-FeII_ oxy superfamily of *LiANS*

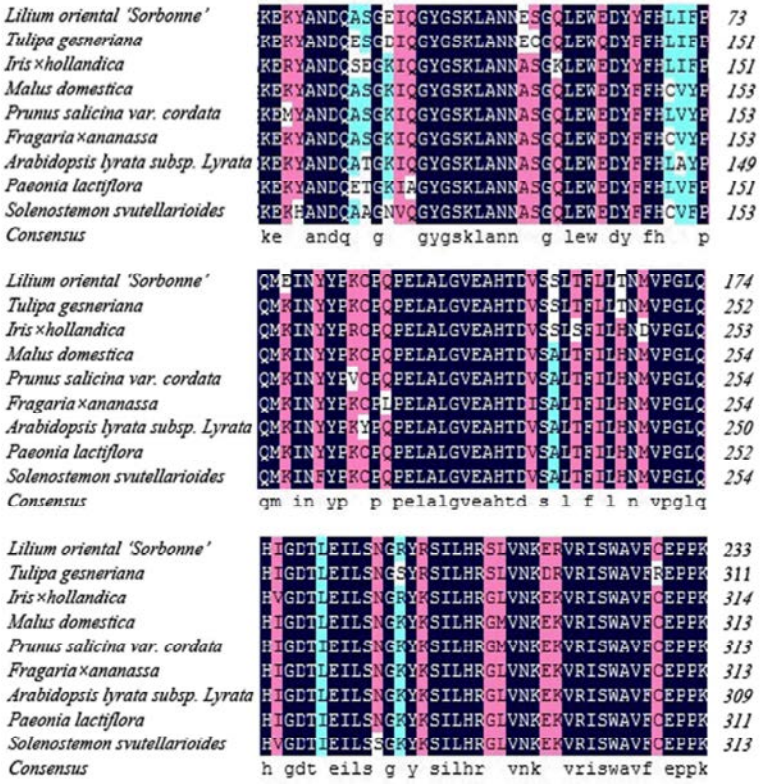

Fig. 4: Alignment of deduced amino acid sequences of *LiANS* and its homologous genes

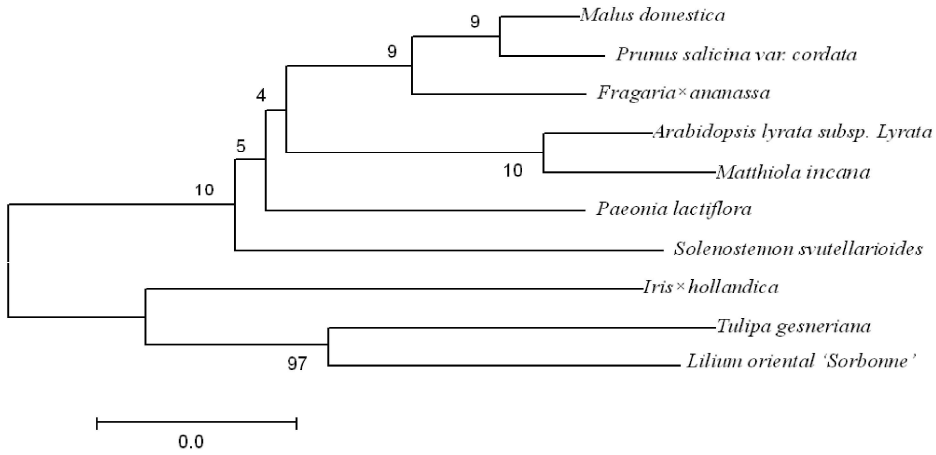

Fig. 5: Phylogenetic tree based on LiANS homologous sequences using Neighbour-Joining methods

DISCUSSION

ANS is an enzyme which is involved in biosynthetic pathway of anthocyanin. As a part of flavonoids metabolism, ANS protein belonged to the family of oxoglutarate dependent dioxygenases. It had high homology with the other flavonol synthetase of this family in the biosynthesis pathway of flavonoids[7]. Anthocyanin biosynthetic pathway and ANS gene had been conducted extensive research in the field of genetics, enzymology and molecule level [8-10] since ANS gene was first separated from maize [11]. Seitz *et al.* [11] believed that the white spate colour of Zantedeschia aethiopica was due to lack of the activity of ANS. ANS gene and promoter were cloned from Forsythia×intermedia by Rosati *et al.* [7] and there was no anthocyanin in the petal of the Forsythia× intermedia in his study, which was also caused by the absence of expression of ANS gene.

Nakatsuka *et al*. [13] reported that the flower of Gentiana turning white was related to the mutation of the ANS gene.

ANS gene isolated from *Lilium* in this study encodes polypeptide of 264 amino acids. Previous studies on ANS gene reported high number of amino acids encoding genes from various plants species. ANS gene from Saussurea medusa [14,8] and Torenia fiournieri [14] are encodes polypeptide of 354,356 and 376 amino acids, respectively. Therefore, the expressing of ANS gene was important for the formation of flower's color.

CONCLUSION

In this study, sequence comparison and bioinformatics analysis of ANS genes were conducted to explore the reason and molecular mechanism of lily flower colour formation. *LiANS* was cloned from *Lilium oriental* 'Sorbonne'. Results showed that the length of the LiANS core fragment is 804 bp (GenBank Accession No. JX427406) and can encode 264 amino acids. Phylogenetic tree analysis revealed that the cloned LiANS core fragment is genetically close to the monocots of *Tulipa* and *Iris*. The complete gene needed further studies.

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REFERENCES

1. Yoshida, K., Y.T. Kato, K. Kameda and T. Kondo, 2003. Sepal Color Variation of *Hydrangea macrophylla* and vacuolar pH Measured with a Proton-Selective Microelectrode. Plant Cell Physiol., 44: 262-268.
2. Schaefer, H.M. and D.M. Wilkinson, 2004. Red leaves, insects and coevolution: a red herring. Trends Ecol. Evol., 19: 616-618.
3. Wilmouth, R.C., J.J. Turnbull, R. Welford, I.J. Clifton, A.G. Prescott and C.J. Schofield, 2002. Structure and mechanism of anthocyanidin synthase from *Arabidopsis thaliana*. Structure, 10: 93-103.
4. Li, X.Y., C.X. Wang, H.M. Sun and T.L. Li, 2011. Establishment of the total RNA extraction system for lily bulbs with abundant polysaccharides [J]. African Journal of Biotechnology, 10(78): 17907-17915.
5. Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. Clustal-W improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic. Acids Res., 22: 4673-4680.
6. Tamura, K., J. Dudley, M. Nei and S. Kumar 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol., 24: 1596-1599.
7. Rosati, C., A. Cadic, M. Duron, M. Ingouff and P. Simoneau, 1999. Molecular characterization of the anthocyanidin synthase gene in *Forsythia* × *intermedia* reveals organ-specific expression during flower development. Plant Sci., 149: 73-79.
8. Cheng, L.Q., Y.J. Xu, E. Grotewold, cZ. Jin, F. Wu, C. Fu and D. Zhao, 2007. Characterization of Anthocyanidin Synthase (ANS) Gene and anthocyanidin in rare medicinal plant- *Saussurea medusa*. Plant Cell Tissue Organ Culture, 89: 63-73.
9. Pang, Y.Z., G.J. Peel, E. Wright, Z. Wang and R.A. Dixon, 2007. Early Steps in Proanthocyanidin Biosynthesis in the Model Legume *Medicago truncatula*. Plant Physiol, 145: 601-615.
10. Reddy, A.M., V.S. Reddy, B.E. Scheffler, U. Wienand and A.R. Reddy, 2007. Novel transgenic rice overexpressing anthocyanidin synthase accumulates a mixture of flavonoids leading to an increased antioxidant potential. Metab Eng., 9: 95-111.
11. Ramirez-Tortosa, C., Ø.M. Andersen, P.T. Gardner, P. C. Morrice, S.G. Wood, S.J. Duthie, A.R. Collins and G.G. Duthie, 2001. Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. Free Radic. Biol. Med., 31: 1033-1037.
12. Seitz, C., N. Oswald and D. Borstling, 2003. Being acyanic:an unavoidable fate for many white flowers. Acta Horticulturae, 612: 83-88.
13. Nakatsuka, T., M. Nishihara, K. Mishiba and S. Yamamura, 2005. Two different mutations are involved in the formation of white-flowered gentian plants. Plant Sci., 169: 949-958.

14. Xu, F., H. Cheng, R. Cai, L.L. Li, J. Chang, J. Zhu, F.X. Zhang, L.J. Chen, Y. Wang, S.H. Cheng and S.Y. Cheng, 2008. Molecular Cloning and Function Analysis of an Anthocyanidin Synthase Gene from *Ginkgo biloba* and Its Expression in Abiotic Stress Responses. Mol. Cells, 26: 536-547.

15. Nakajima, J., Y. Tanaka, M. Yamazakl and K. Saito, 2000. cDNA Clonlng and Gene Expression ofAnthocyanidin Synthase from *Torenia fiournieri*. Pant Biotechnology, 17: 331-335.