

# Identification of differentially expressed sequences in bud differentiation of Oriental hybrid lily Sorbonne by suppression subtractive hybridisation (SSH)

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## Abstract

The developmental process of lily flower bud differentiation has been studied in morphology thoroughly, but the mechanism in molecular biology is still ambiguous and few studies on genetic expression have been carried out. Little is known about the physiological responses of flower bud differentiation in Oriental hybrid lily Sorbonne(*Lilium* spp.) during the stages of flower bud differentiation and the genes that are involved in these responses. In this study, the differences in gene expression between two stages of lily bud differentiation—the stage before bud differentiation(SB) and the stage during bud differentiation(SD) were studied. The suppression subtractive hybridization (SSH) method conducted to generate large-scale expressed sequence tags (EST) is designed to identify gene candidates related to the morphological and physiological differences between the stage before bud differentiation and the stage during bud differentiation of Oriental hybrid lily Sorbonne. The results showed that the SD could induce differential expression of the genes related to lily flower bud differentiation. EST were isolated, cloned, sequenced and identified using BlastN and BlastX, and indicated that at the stage of the flower bud differentiation there is a activation of a floral development response at a molecular level, mainly related to low temperature and posttranscriptional regulation of nucleic acids. 24.1% of the isolated sequences are not yet described, which shows the lack of genomic information currently available for lily. Sequence analysis revealed that most of the differentially expressed genes are related to metabolism and regulation such as protein synthesis and catabolism of carbohydrate. Some genes also encoded transcription factors. These genes showed high mRNA transcript levels in the stage during flower bud differentiation.

This study revealed hitherto unknown genes putatively involved in the stage of lily flower bud differentiation, which serve as a starting point for understanding the differentiation of lily flower bud.

**Keywords** lily flower bud differentiation, Gene expression, SSH

## Abbreviations

EST Expressed sequence tag

SSH Suppression subtractive hybridization

## Introduction

The progress of floral development included the formation of flower meristem, floral organ differentiation, flower primordium differentiation and organofaction followed(Weigel 1998; Zhao et al.2001). Over the last few years, through the molecular genetics study of model plant *Arabidopsis thaliana*, *Petunia hybrid*, *Antirrhinum majus*, many genes of important role in floral development and chromogenesis were isolated (Sessions et al.1998; Siegfried et al.1999; Kieffer and Davies 2001; Zhao et al.2001; Lohmann and Weigel 2002; Sung et al.2003)

The genus *Lilium* comprises more than 90 species (Asano 1989) and is classified into sections (Comber 1949; Smyth et al. 1989). All species of section Archelirion (*L. auratum*, *L.*

*speciosum*, *L. rubellum*, *L. japonicum*, *L. alexandrae* and *L. nobilissimum*) are distributed mainly in Japan (Shimizu 1987). The Oriental hybrid lily (*Lilium spp.*) derived from inter-specific crosses of species of section Archelirion (Leslie 1982) is one of the most popular ornamental plants world-wide.

SSH has emerged as a widely used technology to identify genes that are differentially regulated between two biological situations. Because it includes a normalisation step, it is used for preference to clone low abundance differentially expressed transcripts. It does not require previous sequence knowledge and may start from PCR amplified cDNAs. It is thus particularly well suited to biological situations where specific genes are expressed and tiny amounts of RNA are available. The SSH technique is believed to generate an equalized representation of differentially expressed genes and provides a high enrichment of differentially expressed mRNA (Diatchenko et al. 1996; Marenda et al. 2004). The efficiency and reproducibility of SSH are very useful in studies of tissue-specific, developmental, or induced differentially expressed genes (Von Stein et al. 1997; Basyuni et al. 2011; Prabu et al. 2011; Yang et al. 2011b). In addition, in some plant species, SSH has proved useful for identifying genes differentially expressed during zygotic and somatic embryogenesis (Bishop-Hurley et al. 2003; Namasivayam and Hanke 2006; Legrand et al. 2007; Tsuwamoto et al. 2007; Wang et al. 2007; Geng et al. 2009). Despite its utility and efficiency in isolating differentially expressed genes, SSH has not yet been widely applied in lily. In this study, differentially expressed genes during the stage of flower bud development were identified using SSH. The paper discusses the putative roles of the identified genes.

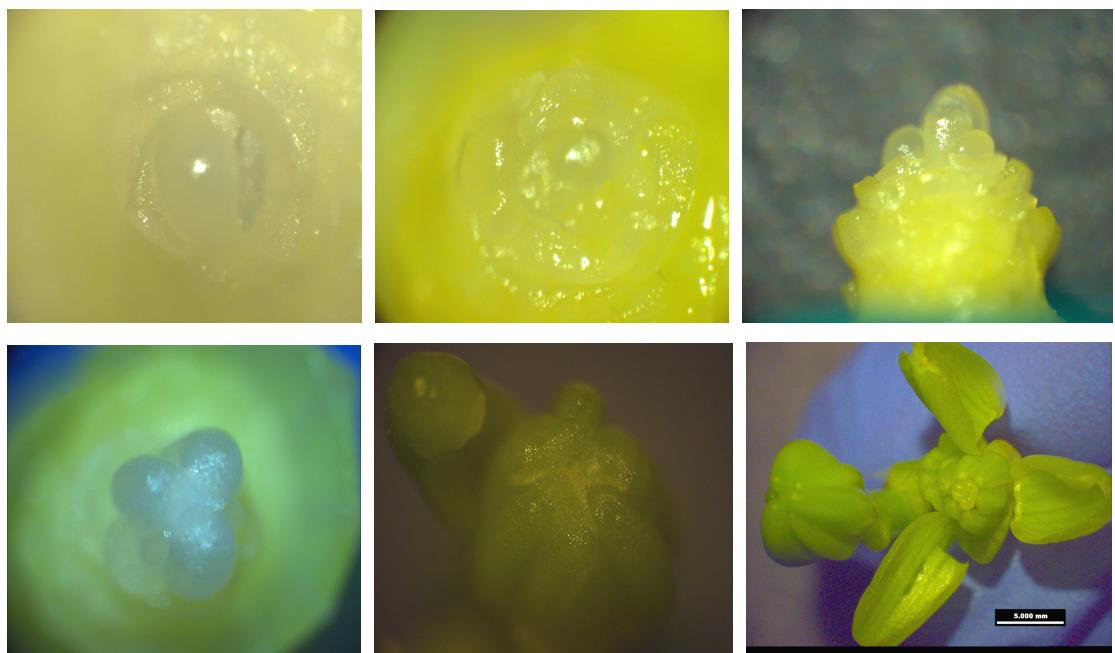
The development of EST libraries associated with differential gene expression technologies provides a panoramic view of many biological processes (Green et al. 2001). On the basis of cDNA, to probe for DNA fragments or through specific antibody combined recombinant cDNA to clone protein, the target gene could be isolated from the library(shchennikova et al. 2003).In addition, the library also was used to establish EST database, make gene chip to understand the gene expression. (Hu et al.2003; Laitinen et al. 2005; Shimamura et al. 2007)

Our aim in this work was to contribute to the wider picture of lily flower bud differentiation, by characterizing genes preferentially expressed between SB and SD. SSH is a PCR-based method (Diatchenko et al. 1996) that was developed to enrich rare transcripts and low abundance genes in plant development process. This study provides an insight into the transcriptomes that drive the flower bud differentiation process in Oriental hybrid lily. The main objective of this study was to isolate and characterize cDNAs differentially expressed during lily flower bud differentiation. The identification of specific genes required to contribute to study the molecular mechanism of lily flower bud differentiation.

## Materials and Methods

### Plant Material and Growth Conditions

The Oriental hybrid lily Sorbonne was planted with routine maintenance and management in greenhouse of Beijing Forestry University, through the microscope, some growing points of six stages (including the stage before flower bud differentiation(SB); the stage of flower bud differentiation start-up, the flower primordium differentiation stage, the perianth primordium differentiation stage, the stamen primordium differentiation stage and the pistil primordium differentiation stage (SD) ) were flash frozen in liquid nitrogen as they were harvested and stored at -80°C.



**Fig.1 Six stages of lily flower bud differentiation**

### **RNA Isolation and poly(A)<sup>+</sup> RNA purification**

Tester (SD) and driver (SB) total RNA were extracted separately and purified using the RNeasy Plant Mini Kit combined with RNase-free DNase (Qiagen, Tokyo, Japan). RNA purity and quality were checked by both agarose gel electrophoresis and spectrophotometry. Blossom bud materials were ground in liquid nitrogen, and poly(A)<sup>+</sup> RNA was subsequently purified using an Oligotex<sup>TM</sup> mRNA Mini kit (Qiagen, Valencia, Calif.) following the guidelines of the manufacturer.

Double-strand cDNAs were synthesized using the cDNA synthesis system Kit (Roche) with 2 µg of mRNA. The resulting cDNAs were dissolved in 20 µl of H<sub>2</sub>O.

### **Driver and Tester Preparation**

Driver and tester double-strand cDNAs were digested by RsaI in a 50-µl reaction mixture containing 15 U of enzymes (Fermentas) for 3 h. The cDNAs were then phenol-extracted, ethanol precipitated, and re-suspended in 6 µl of H<sub>2</sub>O. The digested tester cDNA (1 µl) was diluted in 5 µl of H<sub>2</sub>O.

### **Suppressive Subtractive Hybridization**

A PCR-Select<sup>TM</sup> cDNA Subtraction Kit (Clontech, USA) was used for adaptor ligation: the diluted tester cDNA (2 µl) was ligated to 2 µl of adapter 1 and adapter 2 (10 µM) in separate ligation reactions in a total volume of 10 µl at 16°C overnight, using 15 U of T4 ligase (Roche) in the buffer supplied by the manufacturer. After ligation, the samples were heated at 70°C for 5 min to inactivate the ligase and then stored at -20°C. Two microliters of 5× hybridization buffer [50 mM Hepes pH 8, 0.5 M NaCl, 0.02 mM EDTA pH 8 and 10% (w/v) PEG 8000] were added to each of the two tubes containing adapter 1 and adapter 2-ligated tester cDNA. The solution was overlaid with mineral oil, and the samples were denatured for 1.5 min at 98°C and then allowed to anneal for 8 h at 68°C. After this first hybridization, the two samples were combined and a fresh portion of heatdenatured driver in 1.5 µl of hybridization buffer was added. The sample was allowed to hybridize for an additional 10 h at 68°C. The final hybridization was then diluted in 200 µl of dilution buffer (20 mM Hepes pH 8, 50 mM NaCl, 0.2 mM EDTA), heated at 72°C for 7

min and stored at -20°C.

After subtraction, two PCR amplifications were performed. The first was conducted in 25 µl and contained 1 µl of diluted subtracted cDNA, 0.3 µM of P1 primer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 1 U of Taq polymerase (Invitrogen) and 1× reaction buffer. PCR was conducted using the following parameters: 72°C for 2 min, 30 cycles at 94°C for 30s, 68°C for 30s, 72°C for 2 min, followed by a final extension at 72°C for 7 min. The amplified products were diluted tenfold in H<sub>2</sub>O. Some of the product (1 µl) was then used as a template in secondary PCR with nested PCR primers 1(5'-TCGAGCGGCCGCCGGCAGGT-3') and nested PCR primers 2(5'-AGCGTGGTCGC GGCGAGGT-3'). PCR was performed for 20 cycles (94°C for 30 s, 66°C for 30 s, 72°C for 2 min). The product of the second PCR was analyzed on 2% agarose gel stained with ethidium bromide.

Amplified fragments from the second PCR were purified using the Gene Elute Gel Extraction Kit (Sigma), cloned into a T-easy vector(Promega) and subsequently transformed into competent Escherichia coli (DH5 $\alpha$ ), plated on solid Luria– Bertani (LB)/1.5 Difco agar plates supplemented with 50 mg/l ampicillin, and then grown overnight at 37°C.

Some 37 colonies were randomly selected and grown in 200 µl LB medium in standard 96-well plates and was performed PCR amplification. Then the positive clones were sequenced by Beijing SAN Po vision biological technology Co., LTD

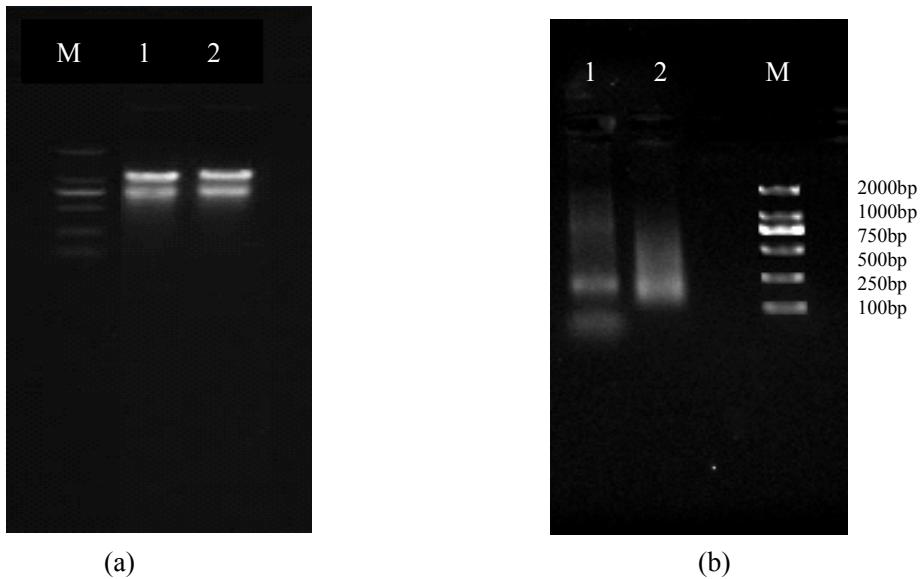
### **Bioinformatic analysis for gene identification**

The Expressed Sequence Tags (EST) were wiped off vector, host cell and repeated sequence by Cross-match program. The sequences of high quality were clustering to join together by Phrap program. The available Expressed Sequence Tags (EST) were functionally annotated using BlastN and BlastX at NCBI.

## **Results**

### **Extracted RNA and subtraction**

The extracted total RNA obtained was quantified by spectrophotometry and purity evaluated by A<sub>260</sub>/A<sub>280</sub> ratio (1.8>2.0). The results suggested that the extracted RNA was of suitable quality to proceed with the experiments (Fig.2(a)). The purified poly(A)<sup>+</sup> RNA was dispersion in 100bp~1500bp. Double strand cDNA was digested by Rsa I and 100 ~ 1500bp cDNA fragments were obtained. Through two subtractive hybridization and two suppression PCR , the fragments with specific genes were further enrichment. From Fig.2(b), it was indicated that amplified cDNA fragments were mainly in 100~1500bp , in accord with anticipation.

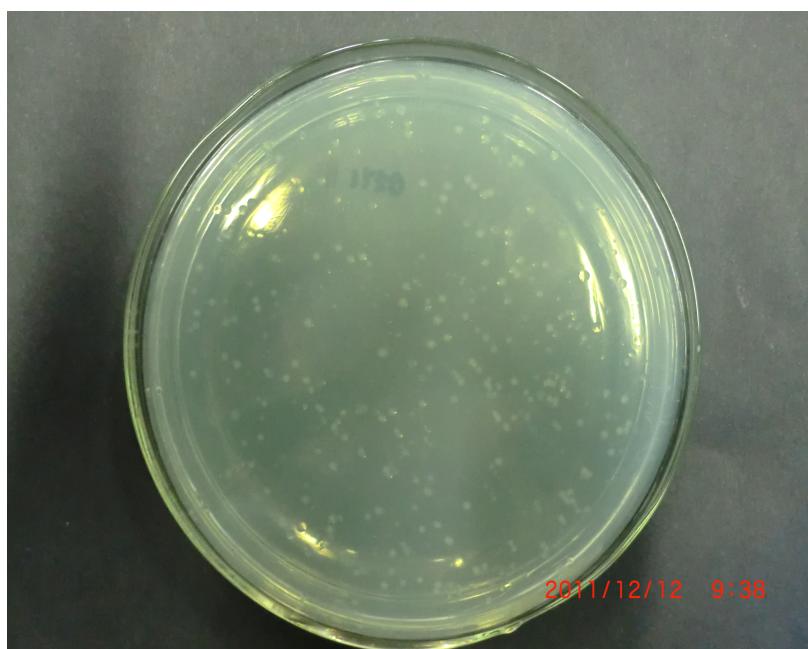


**Fig.2**Agarose gel electrophoresis of total RNA(a), M:RNA Marker, 1,2: Total RNA;

Agarose gel electrophoresis of the second SSH-PCR products(b), M:RNA Marker, 1: unsubtracted sample; 2: subtracted sample

#### Quality analysis of the subtracted library

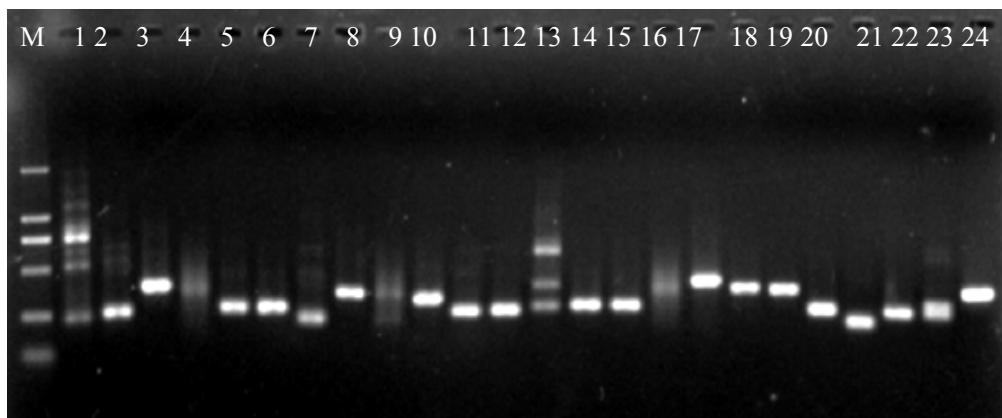
The result of the library clone was shown in Fig.3, it was indicated that the total number of cloning CFU was up to  $9.10 \times 10^3$ , (namely  $3.64 \times 10^5$  CFU/ml), it demonstrated the library titer was high , the transformation efficiency was good enough to clone low abundance genes.



**Fig.3** The plate of the blue/white screening

#### Appraisal confirmation of PCR insert-size

A SSH library with SB cDNA as the driver and SD as the tester was constructed. Selecting randomly positive clones to conduct PCR amplification, the agarose gel electrophoresis result was shown below, the subtracted library fragments were between 100bp~1500bp; the recombination frequency was more than 85% which indicated that the obtained library had high recombination frequency and integrity.



**Fig.4** PCR analysis of clones from the subtracted library. Lanes 1–24: PCR products from different clones. Lane M: DNA size markers

### DNA Sequencing and analysis of the subtracted library

A SSH library with SB cDNA as the driver and SD as the tester was constructed. 37 cDNA sequencing was sequenced. Homology searches in the GenBank database were conducted using the BLASTX and BLASTN search programs(<http://www.ncbi.nlm.nih.gov/BLAST/>). In total, 29 independent sequences (unigenes) were obtained and analysed by comparing them to non-redundant protein (nr) and EST databases, using BLASTX, BLASTN programmes. Namely about 78% clones in the library represented different genes. In the 37 cDNA, the longest was 807bp, the shortest was 121bp and the average was 389bp, which was according to the result of PCR amplification detection, including 6 sequences matching with known genes of lily, and 7 sequences with no similarity to known sequences in GenBank, which maybe were new genes or they couldn't be found with similarity to other species genes as they were located at 3'end of abundant genes. 22 sequences were annotated, These genes were related to 26S proteasome non-ATPase regulatory subunit, ribonuclease E, Gibberellins-regulated protein, Photosystem II protein K, Malate dehydrogenase, Polyubiquitin 2, GAD5, calmodulin binging, Cryptochrome I, ATPase subunit 4, 60S ribosomal protein L38, phosphoglycerate mutase, lipid transfer protein, pathogenesis-related protein PR-106 and nucleoside diphosphate kinase, signal transduction mechanisms and so on. A list of ESTs expressed with their annotation based on similarity to databases is shown in Table 1.

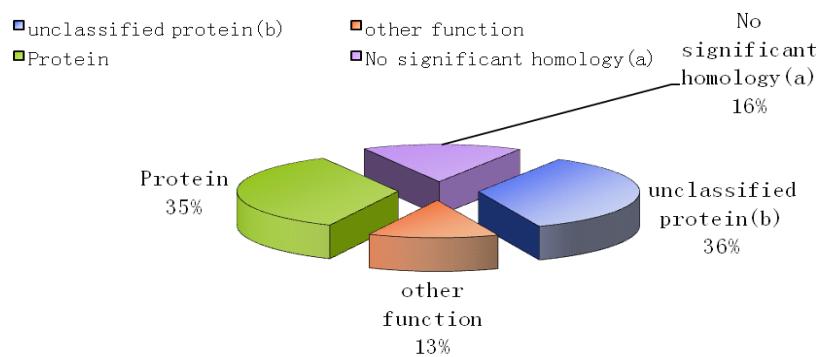
**Table 1 Specification of the identified ESTs and their putative function**

clone	Length(bp)	Number of identical clones	Homology analysis	Putative function	Accession no of Match EST	Identities(aa/aa or bp/bp)	E-value
S1	123	1	BLAST x	Predicted protein( <i>Populus trichocarpa</i> )	EEE75450.1	60/129(54%)	8e-28
S2	398	3	BLAST x	Putative plastid protein( <i>Oryza sativa Japonica Group</i> )	BAZ98694.1	36/106(34%)	0.0
S3	290	1	BLAST x	26S proteasome non-ATPase regulatory subunit	P93768	289/290(99%)	2e-94
S4	385	2	BLAST x	ribonuclease E ( <i>Lilium longiflorum</i> )	ABO0849.1	232/286(81%)	6e-55

S5	484	1	BLAST x	Gibberellins-regulated protein( <i>Arabidopsis thaliana</i> )	BAF00966.1	474/484(98%)	0.0
S6	125	2	BLAST x	Photosystem II protein K( <i>Oryza sativa</i> )	AAS46106.1	75/125(60%)	2e-28
S7	123	1	BLAST x	Malate dehydrogenase( <i>Citrullus lanatus</i> )	P177883	121/123(98%)	7e-11
S8	125	1	BLAST x	Polyubiquitin ( <i>Deschampsia antarctica</i> )	AAM22748.1	120/123(97%)	4e-23
S9	121	1	BLAST x	GAD5,calmodulin binding( <i>Arabidopsis thaliana</i> )	EEF33884.1	80/91(87%)	1e-52
S10	124	1	BLAST x	Cryptochrome I ( <i>Vitis riparia</i> )	ABX80391.1	118/124(95%)	2e-15
S11	308	2	BLAST x	ATPase subunit 4( <i>Eichhornia crassipes</i> )	AAW30200.1	257/289(88%)	8e-93
S12	395	1	BLAST x	60s ribosomal protein L38( <i>Arabidopsis thaliana</i> )	NP181874.1	357/395(90%)	3e-84
S13	285	1	BLAST x	lipid transfer protein ( <i>Lilium longiflorum</i> )	ABK41612.1	236/285(82%)	4e-71
S14	439	3	BLAST x	pathogenesis-related protein PR-106( <i>Lilium longiflorum</i> )	AAF21625.1	300/382(78%)	8e-15
S15	397	1	BLAST x	nucleoside diphosphate kinase( <i>Hyacinthus orientalis</i> )	AAT08712.1	299/369(81%)	3e-37

### Overview of expressed sequences

In order to provide an overview on the type of expressed sequences, these 29 sequences were grouped by origin and function. As presented in Fig.5, 17.2% of the sequences were clustered into “no significant homology” and other 6.90% into “unclassified protein”, as their hit in Blast N was ‘unknown’.



**Fig. 5 Functional categorization of genes.** All 29 sequences were functionally annotated using Blast N and Blast X at NCBI, and categorized based on GO annotation. Percentages of genes in each category are also presented. These genes had no significant hits in Blast N and Blast X searches of NCBI .b These genes matched proteins annotated as ‘unknown protein’.

EST analysis reported in this study provides an efficient means of gene discovery in the oriental hybrid lily for which molecular and genetic information is not available. It is therefore likely that some of the sequences represent newly discovered genes. However, it cannot be completely discounted that part of these EST sequences belongs to 5' and 3' untranslated regions.

Overall the number of novel sequences found here suggests that flower bud differentiation in oriental hybrid lily is likely to involve many more genes than so far described in this study.

## Discussion

Lily is one of the important ornamental plants in the world. Dissection of the mechanism of lily floral development may provide tools for potential applications in floral morphology manipulations. Lily, like many other monocots, has two whorls of almost identical petaloid tepals. The morphological and physiological differences between the stage of lily flower bud differentiation and the stage during lily flower bud differentiation in the life cycle of *Lilium* species are so great that many studies have been performed. Although many genes have been cloned from lily, the mechanism of flower development still remains unclear.

Flowers are unique structures housing reproductive parts of higher plants, and also have a huge variety of shapes, colors, sizes, and fragrances. Many breed varieties were cultivated by selective breeding, which cost people much time for some limit factors, such as cross compatibility between parents. Fortunately, genetic engineering applied into plants would enhance the specificity and efficiency of genetic improvement. However, rare species were produced in the commercial plants by this technique, except for some model plants, such as *Arabidopsis*, *tobacco*, and *petunia*. One of the major obstacles is the lack of effective candidate genes. In this article, the subtracted library obtain some candidate genes in lily because it provides insight into genetic basis of lily floral development and potential genetic improvement for cultivating novel variety by manipulating flower morphology.

This research conductde lily flower bud differentiation subtracted cDNA library with high quality and reflected the condition that many genes were involved in the lily flower bud differentiation, laying the foundation for researching the gene expression technology, such as gene chip technology and EST technology. The result provide reference value for researching lily blossoming, flower color forming and related genes cloning. Further research is required to characterise the functions of those genes obtained from the library so as to understand their role at theflower bud differentiation stage, during floral development, and normal plant development.

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The Ph. D. Programs Foundation of Ministry of Education of China: "The subzero treatment of Lily bulb and the research of growth pattern after planting(20110014110006)

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