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# 荔枝GA信号途径基因*LcPIF4*的克隆及其功能分析

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**摘 要：【目的】**克隆荔枝GA信号途径的*LcPIF4*基因，并对其序列特征、表达特点、基因功能、亚细胞定位及互作蛋白进行研究。**【方法】**利用花穗RNA-seq数据对*LcPIF4*基因进行生物信息预测及分析，通过qRT-PCR对*LcPIF4*基因在不同组织的表达进行研究，并在表达水平上分析其对烯效唑的响应。通过拟南芥转基因株系的构建对其基因功能及亚细胞定位进行分析，同时利用酵母双杂分析其与LcDELLA-1蛋白的互作。**【结果】**克隆的荔枝*LcPIF4*基因ORF长1 617 bp，编码539个氨基酸，且具有经典的APB结构域和HLH结构域。qRT-PCR结果表明，*LcPIF4*基因在花穗、叶片、果皮等器官表达水平较高，且烯效唑处理后其表达水平显著下降。转基因试验表明，在拟南芥中过表达*LcPIF4*基因可促进下胚轴生长，且过表达*LcPIF4-YFP*的转基因材料可在细胞核位置检测到荧光信号。酵母双杂交结果表明，LcPIF4与赤霉素途径阻遏蛋白LcDELLA-1存在直接的蛋白互作。**【结论】**荔枝LcPIF4蛋白具有拟南芥PIF4蛋白相同的结构域；*LcPIF4*在花穗中高表达，且其表达被烯效唑所抑制。*LcPIF4*可促进拟南芥下胚轴生长，其编码蛋白定位于细胞核。LcPIF4蛋白与LcDELLA-1在酵母中存在互作。

**关键词：**荔枝；*LcPIF4*；烯效唑；花穗；表达模式

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## Cloning and function analysis of GA signal pathway gene *LcPIF4* in litchi

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**Abstract**: 【Objective】Phytochrome-interacting factors (PIFs) family proteins are a small subset of basic helix-loop-helix transcription factors that interact specifically with the active form of phytochrome (phy) photoreceptors and play an important role in GA signaling. *PIFs* control diverse aspects of plant growth and development, such as hypocotyl elongation, cotyledon growth, circadian rhythms, seed germination, high temperature tolerance and anthocyanin synthesis. *LcPIF4*, a homolog of *Arabidopsis PIFs* was identified in Litchi from RNA-seq data of panicle. Our study showed that *LcPIF4* was highly expressed in panicle. In order to explore the function of *LcPIF4* in the uniconazole induced panicle growth inhibition, its sequence characteristics, gene function and expression pattern were analyzed by bioinformatics analysis, agrobacterium-mediated transformation and RT-qPCR.【Methods】The sequence of *LcPIF4* was obtained from RNA-seq data and the full length ORF was predicted through Open Reading Frame Finder software (https://www.ncbi.nlm.nih.gov/orffinder/). For gene cloning, total RNA was extracted from root, stem, leaf, female flower, male flower, pulp, pericarp and panicle. About 5 μg of total RNA was used to synthesize first-strand cDNA using the M-MLV reverse transcriptase (Life technologies). The cDNA were used as a template for amplification of the open reading frame (ORF) of *LcPIF4* with pair of gene-specific primer. To analyze the expression pattern of *LcPIF4*, semi-quantita-

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tive reverse transcriptase PCR (qRT-PCR) was conducted. The primer used for qRT-PCR was designed by Primer Premier 5.0, and qRT-PCR were performed on ABI 7500Real-Time PCR System. *LcACTIN* was used as internal control. Relative expressions of all replicas of each sample were calculated by the $2^{-\Delta\Delta Ct}$ method. pCAMBIA2300 and pCAMBIA2300-YFP were used to construct overexpression and YFP-fused vectors, *LcPIF4* was cloned into the pCAMBIA2300 and pCAMBIA2300-YFP vector by recombination using the pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech) respectively. The sequencing-confirmed pCAMBIA2300-YFP-LcPIF4 vectors were transformed into *agrobacterium tumefaciens* GV3101, and then selected *A. tumefaciens* GV3101 clones were grown overnight for *Arabidopsis* transformation using flower dipping method. To analyze hypocotyl length, plates containing *Arabidopsis* seeds were germinated and incubated vertically under 14/10 h light/dark cycle. observation and measurement were conducted 7 days after sowing. To monitor the subcellular localization of LcPIF4, transgenic *Arabidopsis* roots were checked for YFP signal of fusion protein after 7 days of sowing. To analyze the interaction between LcPIF4 and LcDELLA-1, *LcDELLA-1* was inserted into the pGADT7 prey vector (GAL4 activation-domain; AD), *LcPIF4* was fused into the pGBKT7 bait vector (GAL4 binding-domain; DBD), Prey and bait vectors were then introduced into AH109 yeast strain. Transformed colonies were selected on synthetic complete medium lacking Leu and Trp. Transformants were maintained on the same medium and transferred to synthetic medium without Ade, His, Leu and Trp.【Results】The ORF of *LcPIF4* was 1 617 bp length and encoded a protein containing 539 amino acids. The protein sequence of LcPIF4 shared similarity of 42.7% with the PIF4 from *Arabidopsis*. sequence analysis showed that LcPIF4 protein contained a typical conserved APB motif at the N-terminal regions and a conserved HLH domain at the C-terminal regions, suggesting that LcPIF4 could have a typical function of PIF4 protein. Expression pattern of *LcPIF4* was analyzed by qRT-PCR, expression level of *LcPIF4* was higher in leaf, panicle, male flower, female flower than that in stem, pericarp, pulp and root. In addition, Uniconazole was able to inhibit the expression *LcPIF4*, five days and ten days after Uniconazole treatment the expression levels of *LcPIF4* were only 1/3 and 1/5 of the control, respectively. To determine the roles of *LcPIF4* in plant growth and development, More than 10 independent T1 transgenic lines were generated with the *35S:LcPIF4* construct. Three homozygous lines were selected in the T3 generation to examine the phenotypes. Compared with the WT plants, the hypocotyl of *Arabidopsis* overexpressing *LcPIF4* were much longer. The hypocotyl length of transgenic *Arabidopsis* were 8.3 mm on average, while the hypocotyl length of WT plants was only 2.1 mm on average. To determine the subcellular location of LcPIF4 protein, its ORF was fused to YFP and then, the fusion gene under the control of the 35S promoter was transformed into *Arabidopsis* using an *agrobacterium*-mediated transformation. YFP fluorescence was observed in the roots of *35S:LcPIF4-YFP* seedlings using spinning disk confocal microscopy and it was found that LcPIF4-YFP was localized at nucleus, suggesting that LcPIF4 could be targeted to the nucleus of cells. *PIFs* could play an important role in the GA signal pathway, GA promotion of hypocotyl elongation would require *PIFs*. previous studies showed that PIFs participated in GA signaling through direct interaction with DELLA proteins. To confirm the interaction between LcPIF4 and LcDELLA-1 in litchi, *LcPIF4* was fused with the AD domain of the pGADT7 vector and *LcDELLA-1* was fused with the BD domain of the pGBKT7 vector. The bait and prey vectors were co-transformed into yeast through LiAc-mediated yeast transformation method, and the interaction between LcPIF4 and LcDELLA-1 was reconstructed.【Conclusion】In this study, a homolog of the phytochrome-interacting factor *LcPIF4* was identified. *LcPIF4* could promote the elongation of *Arabidopsis* hypocotyl. LcPIF4 protein was localized at nuclear. *LcPIF4* was highly expressed in pan-

icle and responsed to uniconazole at expression level. Yeast two hybrid assay showed LcPIF4 protein could interact with LcDELLA-1.

**Key words**: Litchi; *LcPIF4*; Uniconazole; Panicle; Expression pattern

荔枝(*Litchi chinensis* Sonn.)是我国华南地区种植面积最大的木本果树,是华南农村经济结构中的支柱产业之一。但是'妃子笑'等主栽品种花穗长花量大,存在花穗过度生长消耗过多能量导致荔枝坐果率偏低的问题。因此,对荔枝花穗生长的分子机制进行研究对于提高坐果率有重要的理论和生产价值。笔者所在实验室开发了以GA合成抑制剂烯效唑为核心的花穗调控技术,可有效抑制花穗过度生长并提高荔枝坐果率[1],但目前对烯效唑通过哪些基因抑制荔枝花穗生长还不清楚。qRT-PCR试验结果显示,荔枝*LcPIF4*基因的表达水平在烯效唑处理后被显著抑制,暗示其表达水平与荔枝花穗生长密切相关。

*PIFs*属bHLH转录因子家族第15亚家族,在拟南芥中的研究表明,该基因家族在GA信号途径中具有重要的作用[2]。该基因编码蛋白有2个重要功能结构域,一个是位于N端的APB(active phyB binding)结构域,该结构域具有核定位信号,且参与phyB的互作[3];另一个是位于C端的bHLH结构域,主要负责PIFs蛋白的二聚化及与DNA的结合[4-7]。拟南芥*PIFs*基因参与多个生理活动,如下胚轴伸长、子叶生长、生物钟、种子萌发、高温耐受、叶绿素合成及花青苷合成等[8-9]。PIFs蛋白在暗下大量积累以促进暗形态建成,在光下其被磷酸化并被具有活性的光敏色素降解[10-12]。过表达*PIFs*的拟南芥转基因材料即使在光下也表现出长下胚轴的表型,表明高表达水平的*PIFs*在光下也具有生物学功能[10]。

*PIFs*受到赤霉素的调控,赤霉素信号途径的阻遏蛋白DELLAs能够与PIFs蛋白发生直接的蛋白互作,并抑制PIFs蛋白结合DNA的活性[13-14]。外源施加GA合成抑制剂能够显著抑制*PIFs*对拟南芥下胚轴生长的促进作用[13]。此外,PIFs的功能还受到油菜素内酯乙烯、生长素、脱落酸等内源激素以及高温等环境因素的调控[2]。

果树中对*PIFs*基因也进行了一定的研究,主要涉及光信号对其表达的影响以及其在花色素苷合成中的调控功能[15-16],但未涉及其在器官伸长中的功能及在果树不同器官中的表达规律。笔者将通过拟南芥转基因株系的构建、亚细胞定位分析,以及酵母双杂交等手段,分析荔枝*LcPIF4*基因的功能,并通过qRT-PCR对*LcPIF4*基因在不同器官及其响应烯效唑的表达规律进行研究,初步揭示烯效唑抑制荔枝花穗生长的分子机制。

## 1 材料和方法

### 1.1 植物材料

以荔枝种质资源圃种植的树龄(16 a)一致、树势较壮的'妃子笑/怀枝'砧穗组合荔枝结果树为取样树,取开花时期的根、叶、雌花、雄花和果实发育时期的果皮等器官。同时以烯效唑(100 μmol·L$^{-1}$)和水喷施花穗材料,并在处理后5 d及10 d进行取样。所有材料均用液氮处理并于-80 ℃保存备用。

### 1.2 *LcPIF4*基因序列的获得及序列分析

从'妃子笑'花穗RNA-seq数据中,筛选获取荔枝*LcPIF4*基因转录本,利用NCBI(https://www.ncbi.nlm.nih.gov/orffinder/)的Open Reading Frame Finder在线软件分析荔枝*LcPIF4*的开放阅读框[1]。将LcPIF4的蛋白序列在Pfam (http://pfam.sanger.ac.uk/)和Prosite (http://prosite.expasy.org/)在线软件中进行分析。

### 1.3 *LcPIF4*表达水平分析

采用联川生物技术公司的Total RNA Purification Kit提取荔枝不同器官的总RNA,用RNase-free DNase消化去除基因组DNA污染,总RNA采用Life technologies公司的M-MLV酶合成第一链cDNA。用Primer Premier 5.0设计定量引物,qRT-PCR反应在ABI 7500Real-Time PCR System上进行,运用$2^{-\triangle\triangle CT}$法进行数据处理。所有试验均设3次重复,内参选用*LcACTIN*基因。所用引物序列如下,PIF4RT1F:CGAGCCTCGAGCAATGGTAT,8995PIF-4RT1R:CCACCAGAAGAGGACGTGAC。

### 1.4 过表达载体及YFP标签载体构建

利用pCAMBIA2300和pCAMBIA2300-YFP载体上的*Sma* Ⅰ酶切位点将载体进行线性化,随后利用重组酶以同源重组的方式构建目的基因的载体,载体构建成功后采用液氮冻融法,将其导入农杆菌

菌株GV3101中[17]。所用载体序列如下：PIF4OEF：tcatttggagaggacagggtacccATGAATCCATGCATCCCTG，IF4OEF：AGGTCGACTCTAGAGGATCCCCTTAACCACTTTGAGGGGCA，PIF4YFPF：tcatttggagaggacagggtacccATGAATCCATGCATCCCTG，PIF4YFPR：CTCACCATTCTAGAGGATCCCCATTAACCACTTTGAGGGGA。

### 1.5　拟南芥转基因与亚细胞定位

采用农杆菌介导的侵染方法，首先将携带有目的基因的载体转入农杆菌，随后将农杆菌侵染拟南芥花序，继续培养直到收获种子。随后将种子于含有20 μg·mL$^{-1}$卡那霉素的1/2MS培养基中进行筛选，挑选阳性苗种植或于激光共聚焦下观察根系荧光信号[18]。

### 1.6　酵母双杂交

将目的基因构建入pGADT7载体，诱饵基因构建入pGBKT7载体，依据Clontech公司实验手册进行酵母双杂交实验，利用共转的方法进行酵母转化并在SD/-Trp-Leu培养基培养2~3 d(倒置培养)，待板上生长出单克隆后，挑取30个单克隆接种在新的SD/-Trp-Leu板活化，培养2 d左右。将SD/-Trp-Leu板上的菌落划线在SD/-Trp-Leu-His-Ade培养基平板上，如果能够生长，说明2者之间存在蛋白互作；如果不能生长，说明二者之间不存在互作[19]。

### 1.7　数据分析

应用SPSS 20.0对试验数据进行统计分析，利用LSD法进行显著性检验。

## 2　结果与分析

### 2.1　*LcPIF4*基因的鉴定及分离

根据荔枝花穗RNA-seq数据对所得基因序列进行分析注释，鉴定到1个拟南芥*PIF4*的同源基因*LcPIF4*。该基因ORF长1 617 bp，编码539个氨基酸，氨基酸序列与拟南芥PIF4蛋白的相似度为42.7%。在蛋白的N端具有典型的APB结构域而在蛋白的C端具有典型的HLH结构域(图1)。暗示该基因与拟南芥*PIF4*具有类似的生物学功能。

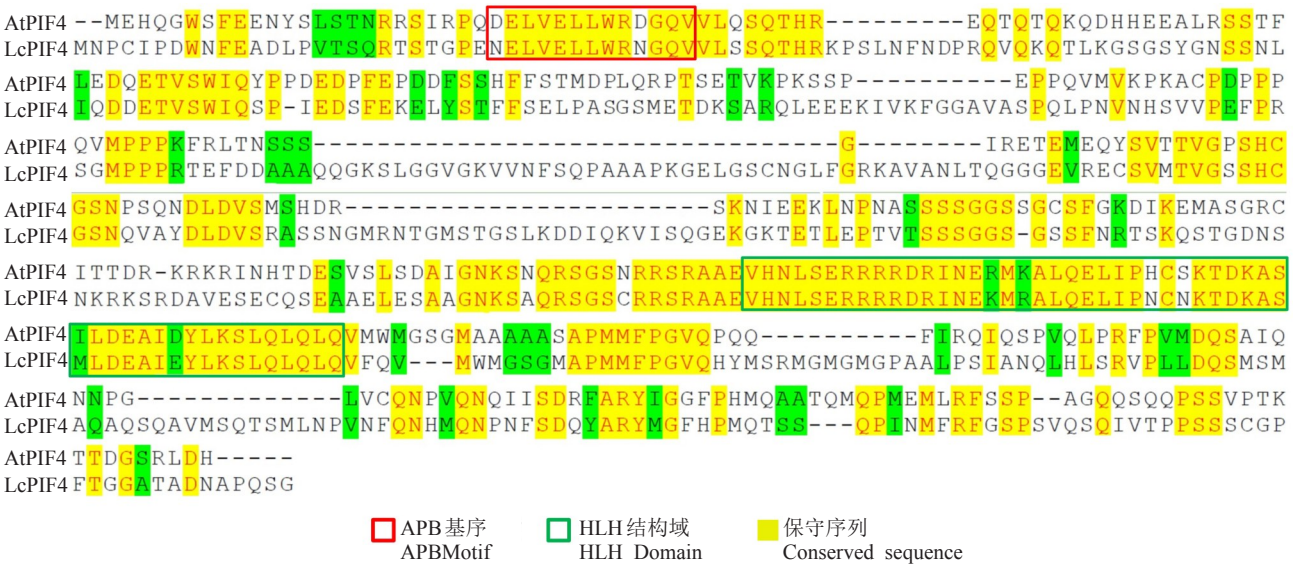



**图1　荔枝LcPIF4蛋白与拟南芥PIF4蛋白的序列比对**

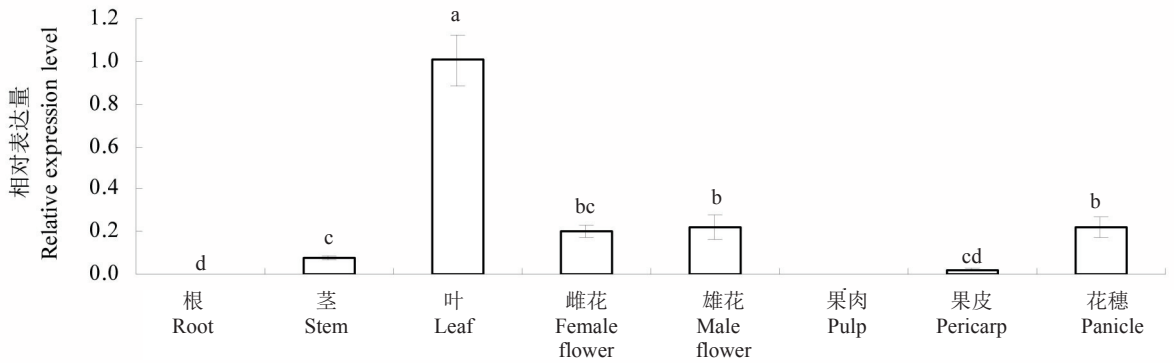
**Fig. 1 Alignments of *Litchi* LcPIF4 and *Arabidopsis* PIF4 protein**

### 2.2　*LcPIF4*基因的表达分析

为了对*LcPIF4*基因的表达特性进行分析，取根、茎、叶及花穗等8个器官的RNA进行qRT-PCR检测。结果显示，*LcPIF4*基因的表达具有明显的器官特异性，在叶片中的表达水平最高，其次为花穗、雄花和雌花，而在根、茎、果肉及果皮中的表达水平较低(图2)，暗示其在叶片、花穗、雄花等器官生长发育过程中具有重要作用。对烯效唑处理花穗材料中*LcPIF4*基因的表达水平进行检测，结果显示烯效唑(200 μmol·L$^{-1}$)处理5 d后*LcPIF4*表达水平降为对照的一半，10 d后*LcPIF4*表达水平仅为对照的1/3(图3)，该结果表明烯效唑可抑制*LcPIF4*基因表达。

### 2.3　*LcPIF4*基因的功能分析

将*LcPIF4*基因构建入过表达载体pCAM-

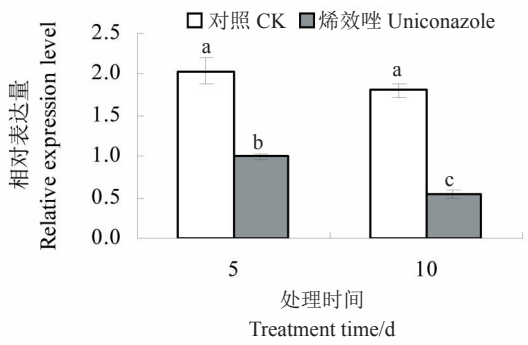



$n$=15。不同小写字母表示在 $p < 0.05$ 差异显著。下同。

$n$=15. Different small letters indicate significant difference at $p < 0.05$。The same below.

**图 2 *LcPIF4* 在不同器官中的表达**

**Fig. 2 Expression profiles of *LcPIF4* in different organs**

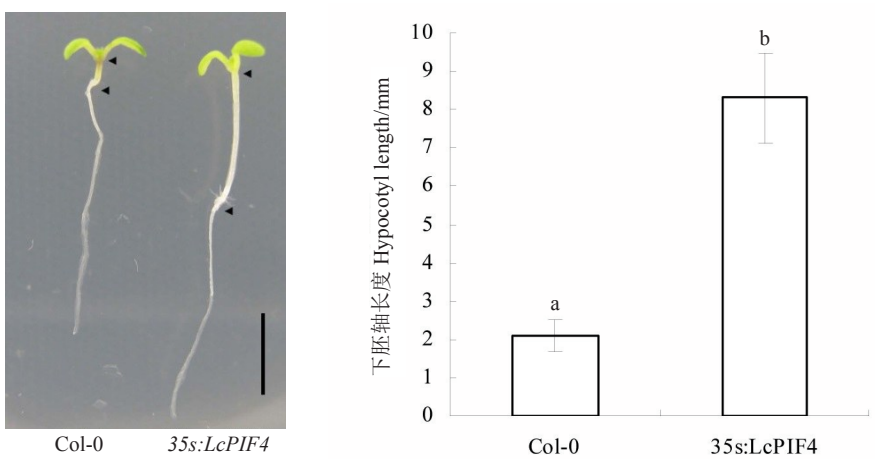



$n$=3。

**图 3 烯效唑处理对 *LcPIF4* 表达的影响**

**Fig. 3 The effect of uniconazole on *LcPIF4* expression**

BIA2300中，并命名为*35S:LcPIF4*。将该载体转化野生型拟南芥Col-0，通过对阳性植株的表型鉴定及统计，分析*LcPIF4*基因的功能。经卡那霉素筛选和PCR检测，获得22株转化植株。转基因后代群体均挑选3个插入位点为单拷贝的株系作为重复，并进行后续的功能分析。选择T3代纯合株系种子进行播种，观察幼苗表型。从图4可以看出，相比野生型，*35S:LcPIF4*转基因植株的下胚轴显著增长，下胚轴的平均长度由野生型的2.1 mm增加至转基因株系的8.3 mm，表明过表达*LcPIF4*可促进下胚轴的生长。




Bar =5 mm。 $n$=5。

**图 4 拟南芥转基因株系的表型分析**

**Fig. 4 The phenotype of transgenic *Arabidopsis***

### 2.4 LcPIF4蛋白的亚细胞定位分析

将*LcPIF4*基因构建入带有YFP标签的过表达载体pCAMBIA2300-YFP中，并命名为*35S:LcPIF4-YFP*，随后进行转化鉴定等步骤，获取纯合阳性苗。挑取萌发7 d的阳性苗在激光共聚焦下进行根系荧光信号观察。结果如图5所示，可在细胞核位

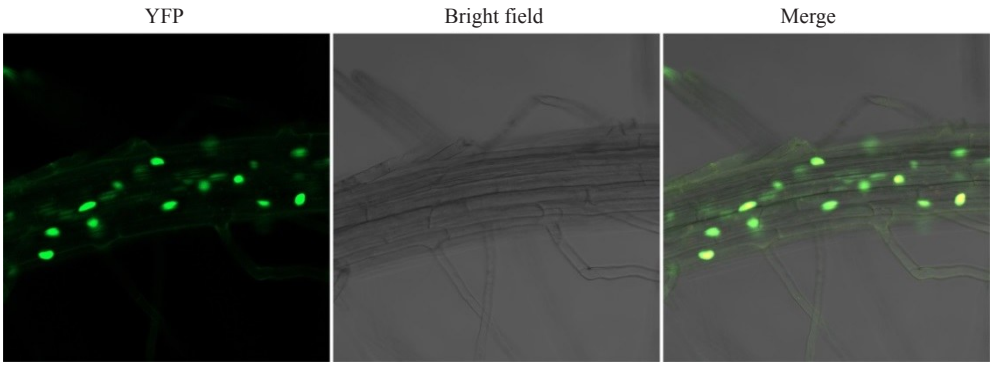



**图5　LcPIF4蛋白的亚细胞定位**

**Fig. 5 Subcellular localization of LcPIF4**

置观察到荧光信号，表明LcPIF4蛋白定位于细胞核。该结果与拟南芥PIF4蛋白的亚细胞定位结果一致。

### 2.5　LcPIF4蛋白与LcDELLA-1的互作分析

在拟南芥中的研究表明，PIF4蛋白通过与DELLA蛋白的互作参与GA信号，并调控下胚轴的生长，那么在荔枝中LcPIF4是否与DELLA蛋白也存在互作？为了回答这一问题，将*LcPIF4*构建入pGADT7载体，LcDELLA-1构建入pGBKT7载体，进行酵母双杂交实验。结果如图6所示，共转入LcPIF4和LcDELLA-1两个载体的酵母菌株能够在四缺培养基（-Ade/-His/-Leu/-Trp）上生长，而对照却无法生长，即LcPIF4和LcDELLA-1在酵母中存在直接的蛋白互作，该结果也表明GA信号途径可通过LcDELLA-1蛋白调控LcPIF4蛋白的功能。

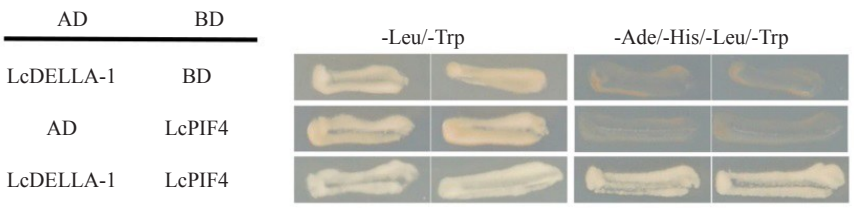



**图6　LcPIF4与LcDELLA-1的互作**

**Fig. 6 The interaction between LcPIF4 and LcDELLA-1**

## 3　讨　论

*PIFs*作为调控植物生长发育的重要组分，整合赤霉素、油菜素内酯、脱落酸、生长素、乙烯等内源信号以及光、高温等外界信号[2,20-21]，使得植物能够感知外界的变化，调控自身的生长发育以适应环境。目前*PIFs*在拟南芥、水稻[22]等模式中已有大量研究，但在果树中的研究还非常少。笔者在对烯效唑抑制荔枝花穗生长提高坐果率的研究中发现，*LcPIF4*的表达水平显著被烯效唑抑制，说明*LcPIF4*在烯效唑抑制花穗生长的过程中发挥重要作用。

为了对*LcPIF4*的功能进行深入分析，首先利用RNA-seq数据对*LcPIF4*基因的ORF及蛋白序列进行预测。结果显示荔枝*LcPIF4*的ORF长1 617 bp，所编码蛋白含有538个氨基酸，比拟南芥PIF4蛋白略大。虽然LcPIF4与PIF4蛋白序列相似度并不是非常高，为42.7%，但LcPIF4蛋白含有PIFs经典的APB结构域和HLH结构域，暗示LcPIF4与PIF4具有类似的功能。

基因的表达部位与其功能存在密切的关系，笔者对荔枝不同组织中的表达情况和烯效唑对其表达的影响进行了分析。结果显示，*LcPIF4*在花穗、叶片、果皮、雌蕊和雄蕊中的表达水平较高，而在根、茎和果肉等部位的表达水平较低。

*PIF4*参与拟南芥下胚轴的伸长[6]，那么*PIF4*是否具有相同的功能？通过农杆菌介导的转基因方法获得了过表达LG*PIF4*的拟南芥转基因株系，结果显示萌发7 d的转基因株系小苗的下胚轴长度达到8.3 mm，显著高于野生型的2.1 mm。该结果表明*LcPIF4*与拟南芥*PIF4*具有类似的功能。蛋白的亚细胞定位决定着蛋白的生物学功能，笔者还对

LcPIF4蛋白的亚细胞定位进行了研究。通过转基因获得了过表达LcPIF4-YFP融合蛋白的拟南芥转基因材料，并对其幼苗根系进行荧光信号观察。结果显示，在细胞核的位置可以看到非常强的荧光信号，而在其他细胞器并未观察到荧光信号，这表明LcPIF4蛋白定位于细胞核，与其转录因子的功能相吻合。

笔者课题组之前的研究已发现*LcPIF4*的表达水平受赤霉素合成抑制剂烯效唑的强烈抑制，表明该基因的转录水平受到赤霉素的调控。事实上在拟南芥中的研究已发现*LcPIF4*基因的蛋白水平也受到赤霉素途径的调控，赤霉素途径的阻遏蛋白DELLA能够与PIF4发生直接的蛋白相互作用，并抑制PIF4的转录激活功能[13]。本研究通过构建LcPIF4和LcDELLA-1的酵母载体，在酵母中发现LcPIF4和LcDELLA-1也存在直接的蛋白互作，表明*LcPIF4*不仅转录水平与赤霉素含量有关，蛋白水平也可能受到赤霉素信号的调控。通过对*LcPIF4*基因的鉴定、亚细胞定位、转基因及表达分析等方面对该基因的生物学功能及其在烯效唑抑制花穗发育中的作用进行了初步的研究，为进一步探讨烯效唑抑制花穗生长的分子机制奠定了良好的基础。

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