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Flowering ability and expression of the shLFY (shallot-LFY) gene in several Indonesian shallot (*Allium cepa*, aggregatum group) varieties

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Abstract. *Irawan J, Dinarti D, Sudarsono, Maharijaya A. 2021. Flowering ability and expression of the shLFY (shallot-LFY) gene in several Indonesian shallot (*Allium cepa*, aggregatum group) varieties. Biodiversitas 22: 5468-5474.* Indonesian shallots *(Allium cepa*, Aggregatum group) have different flowering abilities. The direction of seed development in the form of true seed shallot (TSS) makes the study of shallot flowering important to support its breeding program. Flowering genes' presence and their expression demonstrate their significance in inducing flowering. This study aimed to determine the ability of the shLFY gene expression in four shallots varieties (Bima Brebes, Batu Ijo, Biru Lancor, and Sumenep) and the level of expression in each variety during the formation of flower primordia. The research was arranged in a randomized complete block design (RCBD) with varieties as a single factor, for qRT-PCR relative expression data analysis using ΔΔCT comparative method. The results showed that there were differences in flowering ability and the expression value of the shLFY gene between the tested shallot varieties. These differences also were shown within each variety when the umbels of varying ages were tested. There was a positive correlation between the ability to flower and the shLFY gene expression value. The greater shLFY gene expression will be followed by greater flowering ability. Bima Brebes was the variety with the highest flowering ability (91.66%) and highest shLFY gene expression value between other varieties.

Keywords: Flowering, gene expression, qRT-PCR, shallot, shLFY

INTRODUCTION

The flowering ability of a plant is regulated by several factors, including the agro-climatic and genotypic characteristics of the plant. The initiation of flowering is crucial in the plant's development, as the transition from the vegetative to the generative phase is strongly influenced by the environment and endogenous plants (Zhang et al. 2013a; Li et al. 2014; Liu et al. 2016). Flowering and seed formation in shallots (*Allium cepa*, Aggregatum Group) are heavily influenced by the environment, such as sunlight, temperature, and humidity, as well as internal factors such as genetics, varieties, and the balance of plant endogenous hormones (Bergonzhi and Albani 2011; Liu et al. 2017; Marlin et al. 2018). In general, there are six pathways of flower formation, i.e. photoperiod, autonomous, vernalization, gibberellin induction, temperature, and age (Lee and Lee 2010; Huang et al. 2013; Kim and Sung 2014; Liu et al. 2015; Liu and Cao 2018; Kim 2020; Waheed and Zeng 2020). Several shallot varieties in Indonesia have different abilities in flowering. Bima Brebes, Tajuk, Ilokos, Mentes, Maja Cipanas, Trisula, Pancasona were varieties that required initiation in flowering, Bentanis was the responsive variety in flowering, Sumenep and Palasa not capable of flowering (Idhan et al. 2015; Marlin et al. 2018; Nurdjani and Djufry 2018).

Flowering in plants involves a large number of genes that regulate the process of flower initiation (Huang et al. 2013). In *Arabidopsis*, it has been studied that there are at least 180 genes are involved in regulating flowering time (Fornara et al. 2010). Several flowering genes have been identified in *Arabidopsis* plants and also in other plants such as perennial plants and annuals plants. Genes that play a role in flowering include CO (Constant), SOC1 (Suppression of Constant1) or AGAMOUS LIKE (AGL), gene-box MAD, FKF, FT (Flowering locus T), FD, APETALA1, LFY (LEAFY), and FLC (Flowering Locus C) (Thomas 2006; Kobayashi et al. 2012; Silva et al. 2016; Yang et al. 2016; Yang et al. 2017; Lai et al. 2021).

Indonesia is one of the countries where shallots genotypes with different flowering abilities are developed. There are 21 varieties of shallot that are commonly cultivated in Indonesia (Fairuzia 2021). This becomes particularly interesting to study considering the direction of future development of shallot seeds in the form of True Seed Shallot (TSS). The formation of shallots flowers begins with the formation of inflorescence from the apical meristem tissue, once the inflorescence appears, shoot growth will cease (Brewster 2008). The formation of flowers in plants, especially in shallots, cannot be separated from the role of the flowering gene. The LEAFY homologous genes (LFY) are known to play a function in flower formation. Marlin et al. (2018) reported that five shLFY (shallot-LFY) genes are involved in the shallots

flowering in Indonesia, namely; bm1LFY, bm2LFY, bm3LFY, bm4LFY, and bm5LFY, where the translation of these 5 genes is identical to the FLORICAULA/LFY gene in GeneBank.

In *Arabidopsis*, the LFY gene is required for flower initiation and flower development controller. This gene is expressed throughout its life cycle (Blazquez et al. 1997; Engelhorn et al. 2014; Yamaguchi et al. 2014a). The LFY gene regulates flowering genes involved in the meristem-flowering transition, and has been implicated in the initiation of flower primordia in numerous plants (Moyroud et al. 2010; Pose et al. 2012; Siriwardana and Lamb 2012; Seonghoe 2015; Lai et al. 2018). LFY, for example, induces AGAMOUS/AG expression to form flowers in *Arabidopsis* (Engelhorn et al. 2014). According to Blazquez et al. (1997), the study of the LFY gene became more interesting because its activity was altered in the meristem, as happened in *Arabidopsis*.

The flowering process does not only depend on the flowering gene. There are additional genes involved to support or even has antagonist (anti-florigen) characteristics during the flower initiation or flowering inability of a plant. This study aimed to determine the ability of the shLFY gene expression in four shallots varieties, namely Bima Brebes, Batu Ijo, Biru Lancor, and Sumenep and the level of expression in each variety during the formation of flower primordia.

MATERIALS AND METHODS

Plant and sample preparation

The study was conducted from May 2019 to Maret 2021 at the experimental station of IPB (Bogor Agricultural University) Pasir Sarongge, Cianjur District, West Java, Indonesia at an altitude 1200 m above sea level. Molecular work was conducted in the Molecular Laboratory of Agronomy and Horticulture Department, IPB University and Biotechnology laboratory SEAMEO-BIOTROP, Bogor. The shallots varieties used were Bima Brebes, Batu Ijo, Biru Lancor, and Sumenep as a calibrator variety (not capable of flowering).

All tubers were planted in planting boxes and grouped into 4 varieties. The research was arranged in a randomized complete block design (RCBD) with three replication. The planting media was a mixture of soil, husk charcoal, and manure (4:2:1). The mixture was stirred and then poured into a 95 x 180 cm planting box. Then, the planting media was left for 3 days before planting. Planting was done by immersing shallot bulbs into the ground with the position of the buds facing upward and then covering it thinly with soil with a spacing of 15 x 20 cm. Fertilization was accomplished using NPK (16:16:16) at a dose of 600kg/ha or 102.6g/plot (Rosliani et al. 2013) and a complete liquid organic fertilizer was applied according to label directions. The plants were intensively treated with adequate irrigation and weed control. Control of plant-disturbing organisms was carried out through integrated control, if manual control was not possible, pesticides were used. The shallot plants were treated to produce umbels. The shallots umbels were harvested at 25 days after planting (DAP), 27 DAP, and 29 DAP for each variety as qRT-PCR analysis samples, there were three samples for each age for each variety, whilst the control variety, Sumenep used young leaves as the sample.

Primary design

The primary sequence design for flowering genes was conducted using Primer3Plus software after tracing gene data at NCBI (https://www.ncbi.nlm.nih.gov/). The reference flowering gene was the LFY gene as discovered by Marlin et al. (2018) with accession numbers registered in the GeneBank as KY985382 (bm2LFY), KY985383 (bm4LFY), KY985384 (bm5LFY), KY985385 (bbLFY), KY985386 (bdLFY), and flowering gene on *Allium fistulosum* with accession number as KF270626.1 and KF270625.1. The ACTIN gene, which was a constitutive gene, was employed as an internal control (Wang et al. 2019). The primary sequences used are summarized in Table 1.

RNA isolation and cDNA synthesis

The qRT-PCR analysis was performed on shallot flower umbels (Figure 1) from the varieties of Bima Brebes, Biru Lancor, and Batu Ijo which appeared at the age of 25, 27, and 29 DAP. The Sumenep variety as a calibrator variety (not capable of flowering) used leaves samples. RNA was extracted using the Geneaid RNA kit device in accordance with the manufacture's procedure recommended. All RNA samples were processed with Rnase DNase I for 30 minutes at 37°C to eliminate DNA contamination. The extracted RNA concentration and absorbance values were measured at wavelengths of 260 nm and 280 nm using a Nano Drop 2000 spectrophotometer. The cDNA synthesis was carried out using the Bioline Sensifast cDNA Synthesis kit according to the manufacturer's instructions, and the cDNA reaction was performed using a PCR. The generated cDNA was then diluted to ensure that all samples contained the same concentration of cDNA.

Quantitative RT-PCR analysis

The kit used for qRT-PCR reaction was Toyobo Thunderbird SYBR qPCR Mix. The qRT-PCR reactions were conducted in mixtures constituted with 2 µL SYBR qPCR mix, 0.5 µL amplification primer, 0.2 µL ROX solution, 6.3 µL nuclease-free water, and 1 µL cDNA template. Each group of reactions was accomplished with three repetitions. The PCR reaction was performed on the Applied Biosystem StepOnePlus 96 well. The qPCRs were run as follows: Predenaturation at 95ºC for 30 s, followed by 50 cycles of 95ºC for 5 s denaturation, annealing at 54ºC for 10 s, and extension at 60ºC for 10 s. Melting curves of qRT-PCR were performed at 95ºC for 15 s and 60ºC for 1 hour.

Table 1. The shLFY and actin genes and their primary sequences were used in the study

Gene	Primary sequences	Sequence length (bp)	Tm (ºC)	GC (%)
bm2LFY bm4LFY bm5LFY bbLFY bdLFY KF270626.1 KF270625.1	F: ATTCGGATTCAGAGTCGGATGG R: CACTGCTCGTACAGATGAAACAGGTAGT	145	64.6 65	50 46.4
Actin	F: TGCTCTGGATTATGAACAGGAACTTGA R: CAATCATTGAAGGCTGGAACAACACT	146		

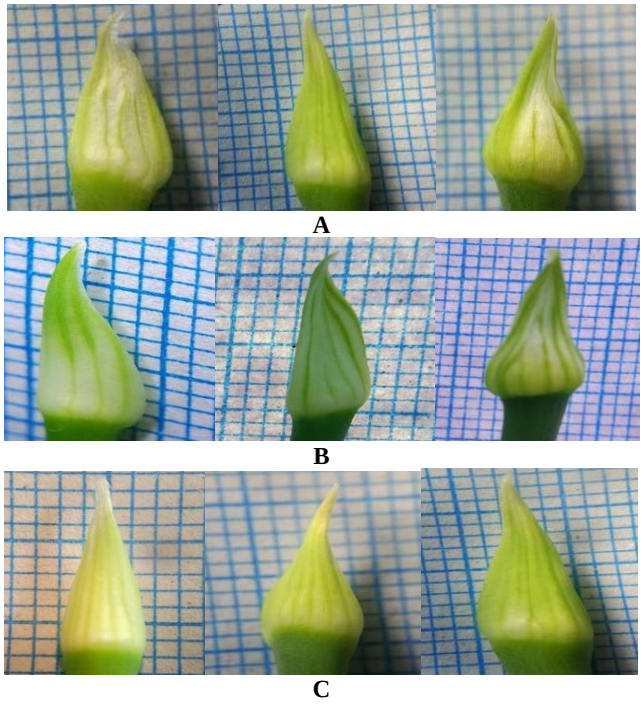

Figure 1. Shallot flower umbels used for qRT-PCR analysis. A. Umbel aged 25, 27, 29 DAP of Bima Brebes variety, B. Umbel aged 25, 27, 29 DAP of Batu Ijo variety, C. Umbel aged 25, 27, 29 DAP of Biru Lancor variety

Data analysis

Observational data on flowering number/umbel parameters were tested with ANOVA at a level of 5%, and further tested using DMRT (Duncan Multiple Range Test) at a level of 5% using the R data analysis program. Flowering ability data was obtained from the counting of umbels on each variety and flower number data was obtained by counting flowers on each umble for each variety. The gene expression data were analyzed using Microsoft Excel with the ΔΔCT comparative method (Livak and Schmittgen 2001) and the actin gene was used as an internal standard. Pearson correlation was used to determine the relationship between relative expression of shLFY and flowering ability.

RESULTS AND DISCUSSION

Flowering ability

The study results showed that each shallot variety had different flowering abilities, with the Bima Brebes variety exhibiting the greatest flowering ability (Table 2). Statistical analysis showed a significant difference in the number of flowers per umbel variables in the Bima Brebes variety compared to the Biru Lancor variety and a very significant difference when compared to Batu Ijo variety. Bima Brebes variety was able to produce 192 flowers in each umbel.

Expression of the shLFY gene

Differences in the relative expression of shLFY were found between shallot varieties. The relative expression of shLFY in the Bima Brebes variety was 2.59 times higher than the Sumenep variety (Figure 2), the Batu Ijo variety was 1.35 times greater than the Sumenep variety, and the Biru Lancor variety was 1.65 times higher than the Sumenep variety. The shLFY gene expression in the Bima Brebes variety at the highest value compared to other varieties, as in the field the Bima Brebes variety showed had greater ability to produce umbel in high numbers compared to the other varieties (Table 2). The flowering ability of each shallot variety had a positive correlation with the shLFY expression value; the higher shLFY expression, the higher correlation value (Table 3).

Differences in shLFY expression did not only occur between varieties, but also within samples of the same variety. In the Bima Brebes variety, sample B1 showed the highest shLFY expression value when this sample had the first umbel to appear (25 DAP) and this was the beginning of the formation of shallot primordia (Figure 3), and then the expression value decreases as the flower bud develop. The highest expression of shLFY in the Biru Lancor variety (Figure 4) was found in sample L2, which was in the form of umbel 27 DAP; this sample consisted of flower primordia that had matured into flower buds. The Batu Ijo variety had poorer flowering ability compared to Bima Brebes and Biru Lancor, which had its highest shLFY expression value in sample i2 (Figure 5) which was the beginning of the creation of shallot primordia. Similar to Bima Brebes, the expression value continued to decrease along with the development of flower primordia.

Table 2. Flowering ability and number of flowers on each variety of shallots

Variety	Flowering ability (%)	Flower number/umbel
Bima Brebes	91.66	192a
Biru Lancor	80	151b
Batu Ijo	11.66	80.67c

Note: a,b,c value followed by the same letter in the same column are not significantly different based on DMRT (p =0.05)

Table 3. Pearson correlation relative expression shLFY towards flowering ability in shallot

Variety	Expression value	Flowering ability (%)
Bima Brebes	2.59	91.66
Biru Lancor	1.65	80
Batu Ijo	1.35	11.66
Correlation Coefficient		0.77

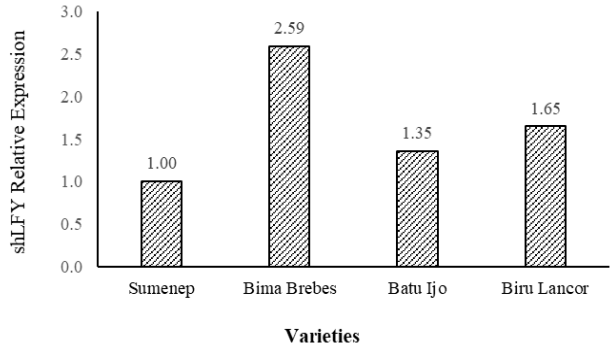

Figure 2. Graph of the relative expression of shLFY in several tested shallot varieties

Discussion

The highland where the research was conducted (1200 m asl.) supports these varieties for flowering, when ambient temperature range from 20-25ºC. Highlands are influenced in inducing flower at shallots where low temperatures are able to induce flowering in shallot plants (Hantari et al. 2019). Some cultivated plants, including shallot require an ideal temperature for flowering, and temperature plays a very important role in the induction of flowering (Ha et al. 2013; Capovilla et al. 2015). Khodorova and Boitel-Conti (2013) suggested that the optimal temperature in inducing and forming flowering meristems ranges from 9 to 25ºC. Differences in flowering ability appear in three varieties, where the Bima Brebes variety has the ability to produce flowers better than other varieties, and plant genetic has a role here.

The Bima Brebes variety was able to produce flowers when cultivated in lowlands and highlands, and the flowering ability increase up to 90.84% in highland (Hantari et al. 2019). This research showed that internal factors played a major role in causing differences in flowering ability between shallot varieties, and it was abundantly clear that the expression of the shLFY gene affected the flowering of three shallots varieties. Bima Brebes was the higher relative expression of shLFY between other varieties. The LFY was known to play an important role in the initiation of flowering in a plant (Liu et al. 2017). LFY encodes a unique transcription which results in the formation of floral meristems (Moyroud et al. 2010). LFY acts as a gene regulator and has been well characterized (Chandler 2012; Sayou et al. 2016; Jin et al. 2021; Lai et al. 2021). LFY involves several genes in the regulation of flowering time and also flower formation (Winter et al. 2011). In relation with flowering time, LFY involves five genes including FD (Amasino 2010), FDP (FD PARALOG) (Michaels 2009), SPL 11(SQUA, PROMOTER BINDING PROTEIN-LIKE 11) (Yant et al. 2009), TOE3 (TARGET OF EAT1 3) and TEM1 (TEMPRANILLO 1) (Winter et al. 2011). In terms of flower development, LFY involves at least 12 genes, including ANT (AINTEGUMENTA) (Irish 2010), AIL6 (AINTEGUMENTA-LIKE 6), SAP (STERILE APETALA), AN3 (ANGUSTIFOLIA 3) GRF5 (GROWTH REGULATING FACTOR 5), CUC2 (CUP-SHAPED COTYLEDON2), STY2 (STYLISH 2), YUC4 (YUCCA4), KNA2 (KNOTTED-LIKE FROM HOME OBOX GENE 2), ER (ERECTA), ERL1 (ERECTA-LIKE 1), ERL2 (ERECTA-LIKE 2) (Winter et al. 2011). Additionally, LFY regulates flower formation and development through phytohormonal pathways such as auxins and gibberellins (Chahtane et al. 2013; Li et al. 2013; Yamaguchi et al. 2013).

The difference in LFY gene expression observed in shallots is similar to that observed in strawberry plants *(fragaria* x *ananassa),* where the expression of ejLFY-1 increases as flower primordia is formed, and then decreases with flower formation (Liu et al. 2017). Other studies have demonstrated that the cnFL expression in *Chrysanthemum Nankingense* (Ma et al. 2013), MsLFY in *Medicago sativa* (Zhang et al. 2013b), and JcLFY in *Jatropa curcas* (Tang et al. 2016) which are the homolog of the LFY gene is increased during the formation of flower. According to Liu et al. (2017), LFY expression was initiated by competition between FLOWERING LOCUS T and TFL1 to prevent binding of TERMINAL FLOWER 1 (TFL1)-FD COMPLEX to the LFY locus. The TFL1 is an anti-florigen that suppresses LFY expression in the vegetative period (Hanano and Goto 2011; Liu et al. 2017). LFY is transcribed and bound to nucleosomes in the chromatin section, then LFY opens chromatin which is mediated by histone linker1 transfer and recruitment of SWI/NF chromatin-remodeling complex. Following LFY induction, LFY transfers the H1 linker at the APETALA1/AP1 locus, where LFY interacts with SYD and BRM to re-open chromatin and activate AP1, which will eventually form flower primordia (Pastore et al. 2011; Yamaguchi et al. 2014b; Jin et al. 2021). The activation of AP1 by LFY is intended to transform the vegetative apical meristem into a flowering meristem (Mayroud et al. 2010; Winter et al. 2011; Thouet et al. 2012; Yang et al. 2017; Pasriga et al. 2019).

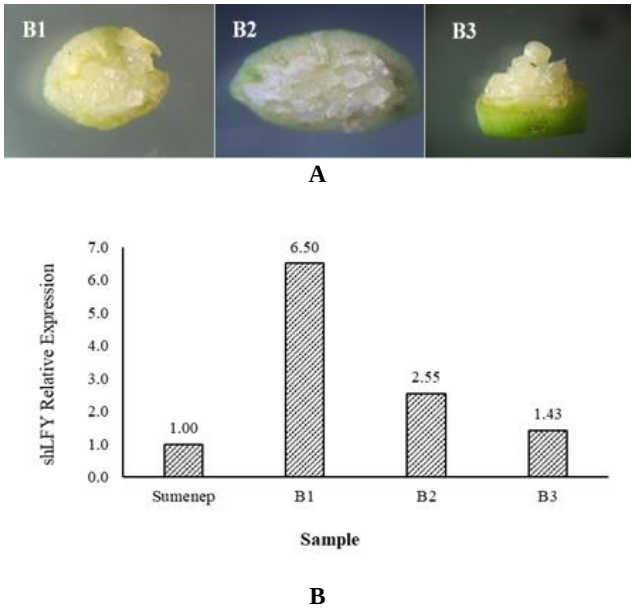

Figure 3. The shLFY gene expression of Bima Brebes variety in different samples. A. Sample of flower primordia Bima Brebes B1 (25 DAP), B2 (27 DAP), B3(29 DAP) on binocular microscope with 10 x magnificient. B. The shLFY relative expression on each sample

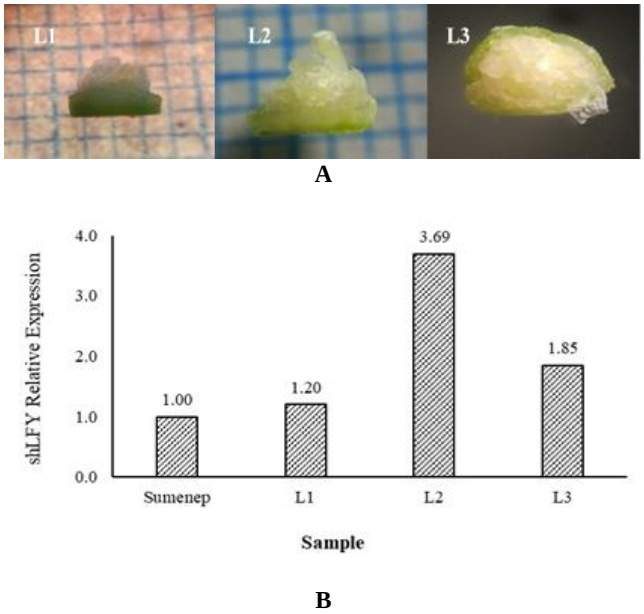

Figure 4. The shLFY gene expression of Biru Lancor variety in different samples. A. Sample of Biru Lancor L1 (25 DAP), L2 (27 DAP), L3(29 DAP) on binocular microscope with 10 x magnificent. B. The shLFY relative expression on each sample

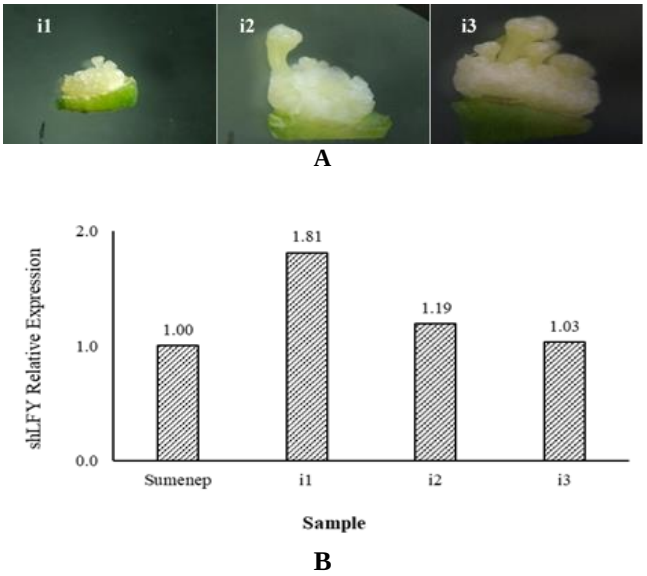

Figure 5. The shLFY gene expression of Batu Ijo variety in different samples. A. Sample of Batu Ijo i1 (25 DAP), i2 (27 DAP), i3(29 DAP) on binocular microscope with 10 x magnificent. B. The shLFY relative expression on each sample

In conclusion, the shLFY played a role in the initiation of flower primordia on shallots, thereby affecting the flowering ability between shallots varieties. The Bima Brebes was the highest flowering ability as well as shLFY relative expression than Biru Lancor and Batu Ijo. The Bima Brebes was also the variety with the highest number of flowers per umbel (192 flowers/umbels). The relative expression of shLFY in shallots increased during the early formation of flower primordia, and that high or low shLFY expression was also influenced by shallot varieties. A more in-depth study of shLFY in terms of genetic material transfer is needed. This would be a very valuable accomplishment if the transgenic method utilizing the shLFY gene can induce flowering in Indonesian shallot varieties that are unable to flower.

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