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Expression analysis and association of bulbing to *FLOWERING LOCUS T* (*FT*) gene in short day onion (*Allium cepa* L.)

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Abstract

The present study was carried out with the objective of studying the expression of *FLOWERING LOCUS T* (FT) genes responsible for bulbing in onion. Six *AcFT* genes were used for expression studies at prebulbing (45 DAT), bulbing (60 DAT) and post bulbing (75 DAT) stage. Two varieties *viz*., Brown Spanish (long day) and Pusa Riddhi (short day) were used. It was observed that out of the reported six *AcFT* primers, four were able to amplify. Two new primers exhibiting amplification of *AcFT1* and *AcFT6* are reported. In short day onion variety, Pusa Riddhi, expression of five out of six genes studied (*AcFT1, AcFT3, AcFT4, AcFT5 and AcFT6*) were highest at the bulbing stage (60 DAT) suggesting their role in bulbing. In long day variety, Brown Spanish, expression of all the six genes except *AcFT1* was very low at bulbing stage (60 DAT). This signifies that specific photoperiod conditions for gene expression were not achieved under Delhi conditions in long day onion. More studies towards understanding of the genes need to be addressed to understand the basic molecular process of bulbing in short day onion.

Key words: Bulbing, *FLOWERING LOCUS T*, short day onion, *AcFT* genes

Introduction

Onion (*Allium cepa* L.) is a biennial plant and completes its life cycle in two growing seasons i.e., forms bulbs in the first year and flowering and seed production occurs in second year. Bulbing in onion is in response to long day photoperiod (Garner and Allard 1920) and based on the minimum photoperiod requirement for bulbing, onion varieties are categorised as short day, intermediate day and long day. This classification provides an indication about the latitude where a particular variety can be grown. Long day onions are cultivated in temperate regions where bulb formation occurs when the day length reaches at least sixteen hours per day while short day (SD) onions are cultivated successfully in tropical short day climate and require a minimum day length of twelve hours for bulb formation (Brewster 2008). Bulbing in onion is a photoperiodically driven process depicting similar mechanism as the photoperiodic control of flowering in other plant species (Mettananda and Fordham 1997). Bulbing ratio, calculated as a ratio of bulb diameter versus neck diameter, can be used to indicate bulbing initiation. When bulbing ratio reaches to a value greater than two, bulbing is considered to have been initiated (Clark and Heath 1962). Besides day length requirement, temperature is yet another pivotal factor which governs flowering and bulbing in onion. Both of these physiologically antagonistic phenomena are driven by the same group of genes. Taylor et al. (2010) explored the genetics underlying onion flower and bulb development and concluded that *AcG1* and *AcFKF1* are the key genes involved in the photoperiodic control of bulb initiation. Lee et al. (2013) identified six *FT* like genes and suggested that different *FLOWERING LOCUS T* genes regulate flowering and onion bulb formation. They opined that *AcFT1* promotes bulb formation and *AcFT4* prevents upregulation of *AcFT1* and thereby inhibits bulbing. Tagashira and Kaneta (2015) observed that the expression levels of *AcFT4, AcFT5* and *AcFT6* increased as it got closer to a condition in long days in association with the onion bulbing. Manoharan et al. (2016) identified eight *FT* like genes (*AcFT*) encoding PEBP (phosphatidylethanolamine binding protein) domains in onion

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which were identical to previously reported *AcFT1*-6 (Lee et al. 2013). Besides, another gene (*AcFT7*) was also reported which showed highly conserved region with *AcFT6* and yet another (comp106231) with low similarity to MFT protein, but containing a PEBP domain. Rashid et al. (2016) did not detect *AcFT2* and observed that *AcFT3* shared 83% identity with *AcFT5* and concluded that *FT-like protein 1* and *FT-like protein 2* genes, similar to *AcFT6* and *AcFT5*, might have influence on bulb formation. In short day onion, Dalvi et al. (2016) reported that *AcFT6* expression level was high during bulb initiation stage and suggested that it might be involved in bulb initiation. Our aim was to investigate the differential expression of six *FT* genes involved in bulb formation of long day and short day genotypes under tropical Indian conditions.

Materials and methods

Two varieties *viz*., Brown Spanish and Pusa Riddhi were selected for this study. Brown Spanish, a long day (LD) onion, does not form bulbs under Delhi conditions whereas Pusa Riddhi, a short day (SD) onion, develops bulbs. Both the genotypes were grown at Division of Vegetable Science, New Delhi. Under short day conditions, bulb initiation starts at 60 days after transplanting (DAT). Hence, seedlings were transplanted and sampling was done at prebulbing Stage 1 (45 DAT), bulbing Stage II (60 DAT) and post-bulbing Stage III (75 DAT). Whole plants were uprooted carefully and washed with clean running water. Collected samples were wrapped in aluminum foil, kept in liquid nitrogen and finally stored at –80^{o}C for RNA isolation. RNA isolation was done by Trizol reagent method and quantitative estimation was done using spectrophotometer (Nanodrop, USA). Qualitative estimation of RNA was done using 1% agarose gel in TAE buffer. Electrophoresis was performed at 80 V constant voltage for one hour and visualized under UV transilluminator. Two microgram of RNA from each sample was reverse transcribed as per the instructions given in Thermo Scientific Verso cDNA Synthesis Kit #AB-1453/B (Thermo Scientific, USA). Expression analysis of six *FT* genes (*AcFT1* to *6*) was performed to get an insight about the genes involved in bulb formation. The cDNA was diluted 50 fold and 1 µL was taken as a template for qPCR of 10 µl reactions comprising of 5 µl SYBR green, 3 µl nuclease free water and 1 µl of desired gene primer. The $\triangle$Ct of genes was normalized with internal control *AcTubulin.* Three biological replicates were used for each developmental stage studied. Samples from prebulbing stage (45 DAT) were used as calibrator in both short and long day plants studied. Hence, $\triangle\triangle$Ct was calculated by finding the difference between the $\triangle$Ct of test sample and $\triangle$Ct of calibrator ($\triangle$Ct of prebulbing stage). *AcTubulin* was used as a house keeping gene for relative gene expression analysis. Six primers reported by Lee et al. (2013) and Dalvi et al. (2018) were used for amplification. Besides two primer pairs were mined from the NCBI database for *AcFT1* (KC485348) and *AcFT6* (KC485353) gene. A real time PCR amplification was performed with initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 30s, 55°C for 30s, 72°C for 1 min and the final extension at 72°C for 5 minutes using Roche Light cycler (Roche Ltd, USA). Relative gene expression was calculated using $\triangle\triangle$Ct method and fold change in expression of each gene at various time points was recorded.

Results and discussion

The expression of *AcFT1* expression increased at 60 DAT by 1.7 fold in Pusa Riddhi but only 0.37 fold in Brown Spanish in comparison to expression at prebulbing stage, whereas at post bulbing stage (75 DAT) the expression of *AcFT1* decreased in Pusa Riddhi but increased by two fold in Brown Spanish. Expression of *AcFT1* decreased in Pusa Riddhi at 75 DAT as compared to 60 DAT but still the transcript level was more as compared to 45 DAT stage. In long day (LD) variety, *AcFT2* expression was observed to be significantly down regulated at 60 DAT and was slightly up-regulated at 75 DAT but the expression was very low as compared to the control at 45 DAT. In short day (SD) variety i.e., Pusa Riddhi, expression of *AcFT2* was initially decreased by 0.25 fold at 60 DAT and further increased by 0.7 fold at 75 DAT. Expression of *AcFT2* in Pusa Riddhi was higher and kept on increasing till 75 DAT as compared to Brown Spanish. In short day (SD) variety, expression of *AcFT3* increased 0.7 fold at bulb initiation stage (60 DAT) and then the expression remained more or less same at 75 DAT. Whereas in long day (LD) variety, expression of *AcFT3* decreased 0.46 fold at 60 DAT but then again increased by 0.31 fold at post bulbing stage (75 DAT). Expression of *AcFT4* declined by 0.35 fold at 60 DAT and again increased by 0.7 fold at 75 DAT compared to bulbing stage in Brown Spanish. In Pusa Riddhi, expression of *AcFT4* was up-regulated by 1.49 fold at 60 DAT and later decreased by 1.0 fold at post bulbing stage (75 DAT), but this was still higher by 0.55 fold as compared to the expression at prebulbing stage. Expression level of *AcFT5*

decreased significantly by 0.8 fold in long day variety at 60 DAT whereas it increased almost 0.53 fold in short day onion variety at bulb initiation stage (60 DAT). The expression level of the gene decreased after bulbing stage in both the varieties as compared to the bulb initiation stage by approximately 0.7-0.8 fold. In Brown Spanish, expression of *AcFT6* decreased rapidly at 60 DAT by 0.8 fold whereas in Pusa Riddhi, expression of *AcFT6* increased by 1.5 fold in comparison to the pre bulbing stage. At 75 DAT i.e., stage 3, expression of *AcFT6* increased more than 0.63 fold in Brown Spanish whereas at the same stage, expression of *AcFT6* was almost at par with the expression at prebulbing stage (Fig. 1).

It was observed that out of the six primers reported by Lee et al. (2013), only one primer i.e., *AcFT3* amplified. Out of the other reported six primers (Dalvi et al. 2018), *AcFT2*, *AcFT4* and *AcFT5* amplified in our genotypes. *AcFT1* and *AcFT6* did not amplify by any of the reported primers. Hence, mining of the NCBI database was done and sequences of *AcFT1* (KC485348) and *AcFT6* (KC485353) were used for picking the primers. The primers, so developed, were able to amplify the *AcFT1* (AKF1-5′-ACTTGCATTGGA TGGTGTCA-3′; AKR1-5′-TTAAAGTTTTGCCGCCAG TT-3′) and *AcFT6* (AKF6- 5′-TGGTGACGGACATA CCAGAA-3′; AKR6-5′-TACAGCAGCCACAGGTG AAC-3′) genes in bulb onion. It was observed that in LD variety, expression of *AcFT1* was high from 60-75 DAT which indicated the role of this *FT* gene in bulb formation although no bulbing was observed under short day conditions. Similarly, in case of short day (SD) variety, it was observed that expression of *AcFT1, AcFT3*, *AcFT4*, *AcFT5* and *AcFT6* genes was significantly higher at bulbing stage than their expression at pre or post bulbing stages. Lee et al. (2013) reported that onion bulb formation is regulated by two antagonistic *FT*- like genes and reported that *AcFT1* gene promotes bulb formation whereas *AcFT4* represses *AcFT1* and inhibits bulb formation. In our study, we observed that *AcFT1* was upregulated in bulbing and post bulbing stage in both LD and SD varieties. However, *AcFT4* was downregulated at bulbing stage in long day variety and upregulated in short day variety. In their research on identification of bulbing hormone genes in onion, Tagashira and Kaneta (2015) observed that expression of *AcFT4*, *5* and *6* increased as it got closer to a bulb induction in long day onion and concluded that these genes are possibly the bulbing genes. Our results were contrary to these observations as expression of all these three genes was downregulated in long day variety at bulbing stage. Dalvi et al. (2016) studied the expression of six genes (*AcFT1* to 6) in short day tropical onion. They observed that expression of *AcFT1* gene was low until 70 DAT after which it started to increase indicating that in the short day onion, *AcFT1* may not be responsible for bulb initiation. These results are contrary to our studies where expression of *AcFT1* increased upto stage 2 (60 DAT) but decreased at 75 DAT in short day variety. Dalvi et al. (2016) also observed strong expression of *AcFT2* and *AcFT3* in late bulbing stage and concluded that these genes might be responsible for maintaining vegetative growth instead of bulbing. In comparison to their study, we observed that expression of *AcFT2* decreased by 0.25 fold at bulbing stage but started increasing afterwards till 75DAT. Contrary to their findings, we observed strong expression of *AcFT3* at bulbing stage and it remained more or less steady till 75DAT. In long day onion, expression of *AcFT2* was severely reduced in 60 and 70 DAT but the expression of *AcFT3* exhibited somewhat increased expression in stage 3 (75 DAT) emphasizing that the role of *AcFT3* may be more in maintaining bulb formation. Dalvi et al. (2016) observed that the expression of *AcFT4* remained high till 60 days and then decreased indicating that this gene may be involved in vegetative growth and not in bulbing. In our studies also, we found that *AcFT4* increased in its expression at 60 DAT, which was the stage for bulb initiation, and then started decreasing in expression indicating that this gene may be involved in vegetative growth. In long day variety (Brown Spanish) the expression of *AcFT4* was very low as compared to short day onion indicating that this gene may not be involved in bulbing initiation in LD onion. Expression of *AcFT5* was high in prebulbing stage and increased in expression at bulb initiation stage (stage 2) in short day and then decreased rapidly and these results are in conformity with the results of Dalvi et al. (2016) who also reported that *AcFT5* expression pattern was high in the pre bulbing stage up to 60 days and then decreased thereafter. In case of the expression of *AcFT6*, it was observed that the expression increased rapidly till 60 DAT in short day (SD) onion and then decreased rapidly in 75 days after transplanting. This is in contrast to the findings of Dalvi et al. (2016) who reported that the relative gene expression of *AcFT6* increased from day 50 onwards with a peak at 60 days and remained high thereafter suggesting that this gene may be responsible for bulb induction. But if we look at the expression of *AcFT6* gene in Dalvi et al. (2016), it was observed that the expression of *AcFT6* increased at 60 DAT and then

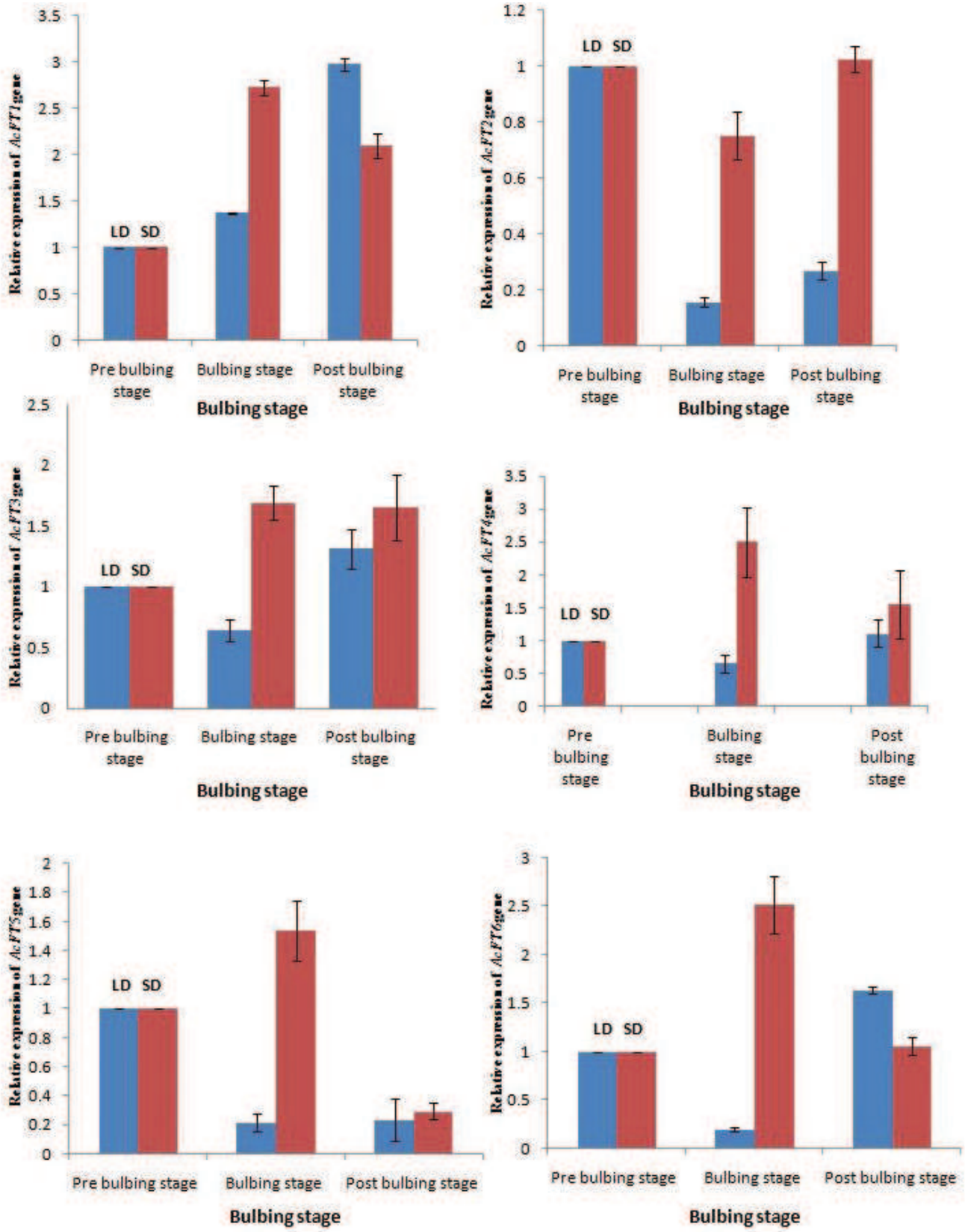

Fig. 1. Relative expression of the six *FT* genes in long day (LD) Brown Spanish and short day (SD) onion Pusa Riddhi against Acβ-tubulin as a reference gene

decreased, was static for 10 days (70-80 days) and again increased from 80-90 days when the bulb attains maturity. This means that *AcFT6* also does not have much role in bulbing in short day tropical onions. Manoharan et al. (2016) suggested that *AcFT4* and *AcFT7* are possibly involved in bulb formation in short day onion lines which is in contrast to our findings. They also observed that *AcFT3*, *AcFT5* and *AcFT6* did not exhibit any significant difference in their expression during plant development but concluded that they may still have different functions since the expressions are largely affected by their interaction with 14-3-3 protein, which together with FD transcription factor forms the complex florigen to initiate flowering (Taoka et al. 2011). However, Manoharan et al. (2016) observed that *AcFT2* showed difference in expression pattern in the lines when grown in short day and long day conditions as compared to the normal greenhouse conditions. They reported that *AcFT2* is strongly affected by the day length conditions, but the expression patterns confirmed the differences in bulb formation under different environmental conditions. In long day variety, Brown Spanish, expression of *AcFT5* and *AcFT6* was very low at bulbing stage but expression levels of *AcFT6* increased 1.4 fold at 75 DAT which suggests a possible role of this gene in bulb development of long day onion.

To conclude, it can be said that in SD onion variety Pusa Riddhi, expression of five of the six genes studied (*AcFT1, AcFT3, AcFT4, AcFT5* and *AcFT6*) was highest at the bulbing stage (60 DAT) suggesting their role in bulbing in SD onions. The expression of *AcFT2* was lowest at bulbing stage indicating that the downregulation of this gene promotes bulbing in short day variety. Contrary to observations made in short day variety, expression of all the six genes tested under study was comparatively very low at bulbing stage (60 DAT) in Brown Spanish. The inference can be made that the specific photoperiod conditions required for induction of these genes and in turn bulb formation in long day onion were not achieved under Delhi conditions. It can be concluded that a beginning towards understanding the genes involved in bulb formation in short day Indian onion has been initiated and more number of genes need to be studied to understand the mechanism of bulb formation under short day conditions.

Author's contribution

Conceptualization of research (AK, MM, ABG, NT); Designing of the experiments (AK, MM, ABG, NT); Contribution of experimental materials (AK, FL); Execution of field/lab experiments and data collection (FL, AK, MM, ABG); Analysis of data and interpretation (MM, ABG, AK); Preparation of manuscript (AK, FL, MM).

Declaration

The authors declare no conflict of interest

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