# Flowering gene expression in Indonesian long harvest black rice (*Oryza sativa* L. 'Cempo Ireng')

## Yekti Asih Purwestri[1,2*], Febri Adi Susanto[1], Anisa Nazera Fauzia[1]

[1]Research Center for Biotechnology, Graduate School, Universitas Gadjah Mada, Jl. Teknika Utara, Barek, Sleman, Yogyakarta 55281, Indonesia
[2]Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia

*Corresponding author: yekti@ugm.ac.id

**Abstract**

Many studies have reported the great potency of black rice as functional food for human diet. Cempo Ireng is one of Indonesian black rice cultivars with the highest content of anthocyanin. However, it also suffers from long harvest period. This experiment aims at investigating the behavior of the flowering genes in order to gain basic information to develop this cultivar. We sampled the leaves' blades of Black Rice 'Cempo Ireng' at 48, 55, 68, 81, and 90 DAP then performed RNA isolation and cDNA synthesis, amplification of targeted flowering genes, and a semi-quantitative analysis to see the expression of flowering genes. Our results showed that the flowering genes *Hd3a* and *RFT1* were redundantly up-regulated to induce flowering in black rice Cempo Ireng under a neutral day condition in a tropical region. We also noted that the patterns of FT-like genes and flowering regulatory genes including *FT-L5, FT-L6, FT-L9, FT-L10* and *Hd1, OsCOL4* were expressed together with two major flowering genes. *FT*-like genes were temporally co-expressed with two flowering genes *Hd3a* and *RFT1*, whereas the *Hd1* had a unique expression pattern. Meanwhile, *OsCOL4* as the flowering repressor was only detected in the early stage when the flowering gene *Hd3a* began to express. The results suggest that black rice Cempo Ireng has similar and conserved flowering pathway under a neutral day condition as indicated in the common rice flowering models.

**Keywords:** black rice, Cempo Ireng, flowering, gene expression, long harvest.
 **Abbreviations:** CRISPR_ Clustered regularly interspaced short palindromic repeats, DAP_ Days after planting, DTH7/8_ Days to , Heading 7/8, Ehd1_ Early heading date 1, FAC_ Florigen activation complex, FT_ Flowering locus T, GF14c_ 14-3-3 isoform protein c, *Ghd7_ Grain height date 7, Hd1_ Heading date 1, Hd3a_ Heading date 3 a*, LD_ Long day, *MADS_ MCM1, Agamous*, deficiens, SRF, ND_ Neutral day, *OsCOL4_ Oryza sativa CONSTANS-like 4, OsFD1_ Oryza sativa flowering locus D-like 1, RFT1_ Rice flowering locus T 1*, SAM_ Shoot apical meristem,  SD_ Short day.

## Introduction

Functional food has been extensively studied and become an interesting topic due to the direct impact on human health. The exploration and development of functional food, especially from primary food crops, are the best ways to increase human nutrition intake. Rice has been consumed by more than half of the world's population (Dogara et al., 2014). However, rice consumption leads to nutrient deficiency due to low iron, zinc, essential amino acid, and vitamin A. Pigmented rice, especially black rice, is the most suitable candidate for increasing the nutrition of traditional staple food which most people take in. Black rice was reported to contain higher nutrition (anthocyanin, fibers, and iron) compared to red/brown rice and white rice (Pratiwi and Purwestri, 2017). In addition, the enriched taste and its aroma give black rice the potency to be an established functional food.

Many researchers have also reported the benefit of black rice. Black rice consumption in rats affected lipid parameters and hepatic enzyme activities, and those researchers concluded that black rice had a cardioprotective effect (Kim et al., 2006). Another research reported that the

anthocyanin content of black rice peonidin 3-glucoside and cyanidin 3-glucoside could prevent the occurrence of tumor metastasis by inhibiting DNA binding activity and nuclear translocation of AP-1, which means consumption of black rice served as an anticancer (Chen at al., 2006). It was also reported that black rice had the ability of anti-hypercholesterolemic (lipidemia) (Zawitoski et al., 2009; Salgado et al., 2007), increased fecal cholesterol excretion (Goufo and Trindade, 2017), cytotoxicity on human promyelocytic leukemia (HL-60) (Vichit and Seawan, 2015), reduced 50% of atherosclerotic plaque (anti-atherogenic) (Ling et al., 2001), plaque stabilization (Xia et al., 2006), improved antioxidant and anti-inflammatory status in patients with coronary heart disease (Wang et al., 2007), and prevented degenerative diseases and cancer (Pratiwi et al., 2014). These findings suggested that black rice potentially becomes one of the healthy food crops.

The problem of black rice development as functional food arises from the production to fill up its demand. One of local black rice cultivars in Yogyakarta namely 'Cempo Ireng' has very special characteristics including enriched taste, aroma,

and high nutrition content (Kristamtini et al., 2014). Moreover, it has also been reported to have resistance against Xanthomonas oryzae pv. oryzae, one of major and important disease in the rice cultivation around the world (Sutrisno et al., 2018). However, it has long harvest period (6-8 months from transplanted seedling to harvest) and low productivity. Thus, farmers were not interested in cultivating black rice Cempo Ireng. Research to shorten the flowering period and to enhance the production which could lead to the improvement of this cultivar needs to be carried out. Therefore, in this study the preliminary research was to investigate and understand the patterns of flowering gene expression. In rice, flowering is the term used for the emergence of panicle called heading which is subsequently followed by anthesis. Rice flowering mechanism has been extensively studied. However, the mechanism of rice flowering which grows in tropical conditions has not been fully understood. A study which used temperate japonica cultivar grown in sub-tropical condition revealed that the flowering was controlled by day length (Taoka et al., 2013). In general, plants are divided into three classes regarding its sensitivity to day length. SD (short day) plants are flowers induced and produced under short day conditions (14 hours dark period/8 hours light period), and LD (long day) plants flower under long day conditions (8d/14l), whereas ND (neutral day) plants flowers regardless of the day length (Ishikawa et al., 2005). Rice is a short day plant and the gene that is responsible for the flowering is known as a member of *FT (FLOWERING LOCUS T)* family. *FT* was originally characterized in *Arabidopsis thaliana*, and studies confirm that *FT* can be found across the plant taxa. Rice has 13 members of FT-like gene ortholog (*FTL/FT-L1, Heading date 3 a (Hd3a)/FT-L2, RICE FLOWERING LOCUS T 1 (RFT1)/FT-L3, OsFT/FT-L4, FT-L5, FT-L6, FT-L7, FT-L8, FT-L9, FT-L10) (Izawa et al., 2003).* Rice *FT-like 2* ortholog, *Hd3a,* was reported to be a graft-transmissible signal which was called florigen and interacted with GF14c (14-3-3 protein) and *OsFD1* forming FAC-complex (Florigen Activation Complex) to induce the flower meristem identity genes (*OsMADS14* and *OsMADS15*) in the shoot apical meristem (SAM) (Taoka et al., 2013).

*Hd3a* normally expressed under SD condition. Interestingly, when the rice plant was grown under LD condition, the flower was induced by another member of *FT*-like gene *RFT1,* which indicated that rice had both flowering pathways. The flowering under SD or LD condition requires the activation of the respective genes and its regulator which can function either in both pathways or one specialized pathway. Therefore, the members of *FT*-like genes were also characterized by their expression in this study. *FT-like* members were also proposed as floral integrators in the flowering pathways (Izawa et al., 2003).

Cempo Ireng is mainly cultivated under neutral day conditions, and there is no information on whether black rice Cempo Ireng has the LD or SD pathways, or whether it is not sensitive to photoperiod. Therefore, in order to shorten the heading date of black rice Cempo Ireng, the understanding of conserved flowering pathway in the tropical region (ND) needs to be gained. In this paper, we reported on the flowering genes expressions and its flowering regulators of Cempo Ireng under neutral day conditions in the tropical region.

## Results

The black rice Cempo Ireng was heading at 112 DAP, and we examined the major flowering genes *Hd3a* and RFT1, some *FT*-like genes family members *(FT-L5, FT-L6, FT-L9, FT-L10)* as well as the regulator genes for flowering, *Hd1 and OsCOL4*. The *Hd1* is known to promote flowering under SD conditions, and *OsCOL4* is known as the novelly identified gene for the repression of the flowering under SD and LD.

### Expression Patterns of Hd3a and RFT1

*Hd3a* was expressed in all samples examined, indicating the important function not only for flowering but also for another developmental stage such as branching (Tsuji et al., 2015). *Hd3a* and *RFT1* showed similar patterns, and they both expressed at 55 until 90 DAP (Fig 1).

The semi-quantitative analysis presented in Fig. 2 revealed that the Hd3a expression was at a high level at 68 DAP. The high level of *Hd3a* transcripts may trigger the transition from vegetative to generative phase. *Hd3a* was barely detected at 90 DAP indicating that the function was to promote flowering, whereas *RFT1* was expressed almost at the same level and detected at a high level at 90 DAP. Interestingly, we found that the expressions of *Hd3a* and *RFT1* decreased at 55 DAP and then increased rapidly. Depending on the respective pathways, the expression of both genes was either synergistic or independent to induce flowering and may be regulated in the different pathways under neutral day conditions.

### Expression Patterns of FT-like Genes in Black Rice Cempo Ireng

Other *FT*-like gene family members including FT-like gene family members *FT-L5, FT-L6, FT-L9,* and *FT-L10* were also examined. Those genes were expressed with similar pattern as two major flowering genes *Hd3a* and *RFT1*. The *FT-L5 and FT-L6* were known to belong to the clade three of the *FT*-like genes family phylogeny tree, whereas *FT-L9* and *FT-L10* belonged to the clade one (Izawa et al., 2003). Those *FT*-like genes were examined to understand their functions regarding their relationship with *Hd3a* and *RFT1* as major flowering genes in rice, which also belonged to clade one to induce flowering. At 68 DAP all of the *FT*-like genes examined were expressed at the highest level, suggesting they were also involved to trigger the flowering. Interestingly, all of which were not detected at 90 DAP even *Hd3a* and *RFT1* were detected, that indicated that they reach the maximum expression at 68 DAP.)

### Expression Patterns of Flowering Regulator Genes in Black Rice Cempo Ireng

The regulatory gene *Hd1*, an ortholog of *Arabidopsis thaliana CONSTANS (CO),* was expressed at 55, 68, and 81 DAP. Hd1 induced the expression of *Hd3a* under SD condition and repressed under LD condition (Kojima et al., 2002). Here, we found that the *Hd3a* expression was still detected at 90 DAP, whereas *Hd1* was not, indicating *Hd1* expression as the upstream of *Hd3a*. Remarkably, *Hd1* was also not detected at 48 DAP and instead detected at a low level at 55 DAP. This finding suggests that *Hd3*a may be

regulated not only by *Hd1* but also by another gene. The potential candidate to regulate *Hd3a* was *Ehd1* (Early heading date 1). Ehd1 is a specific floral inducer which might play an important role to maintain the expression level of the flowering genes under neutral condition (Fauzia and Purwestri, 2013). We also examined the regulator gene which functioned as a repressor of flowering in black rice Cempo Ireng. *OsCOL4*, which belongs to the *CO*-like genes family with 16 members in rice, was reported as a flowering repressor under SD and LD conditions (Lee et al., 2010). *OsCOL4* was only detected in the low level at 48 DAP, indicating the expression was strictly opposite to flowering genes. *OsCOL4* has been proposed as the regulatory gene to maintain the vegetative phase. The finding in black rice Cempo Ireng also supports the *OsCOL4* role as a flowering repressor.

## Discussion

Rice-based diet on humans has affected health conditions, especially in developing countries due to low nutrition contained in white rice. Black rice with known benefits such as increasing humans nutrition intake and preventing degenerative diseases such as diabetes and colon cancer can be used as an alternative. Since developing black rice as functional food faces problems like long harvest period, here we investigate to understand the flowering gene expression patterns in order to increase black rice production. Moreover, studies of heading dates for the adaptation to southern areas of rice cultivation especially in Indonesia are rare and are poorly understood. Rice is a short day plant (SD). Therefore, the flowering is controlled by day length (Taoka et al., 2013). However, when rice is planted in the tropical regions, there is no information about the flowering pathways, and whether or not the rice is affected by day length, especially Indonesian black rice. The cultivated rice in northeastern Asia which requires earlier flowering to set the grain before the cold season arrives has already been understood (Komiya et al., 2009; Naranjo et al., 2014). *RFT1* was the responsible gene for the regional adaptation (Fujino et al., 2013). Meanwhile, the gene responsible for black rice Cempo Ireng flowering is proposed to be regulated by both of *Hd3a* and *RFT1.* In this study, we found the different patterns of major rice flowering genes. In a previous study with japonica background, it was reported that the *Hd3a* and *RFT1* had similar expression pattern (Lee et al., 2010). In this study, we showed that the expression pattern was slightly different. *Hd3a* was expressed in all samples and reached the peak while it was triggering the flowering transition. While RFT1 also had similar pattern, the expression of *Hd3a* was always found higher than *RFT1* except at 90 DAP, which suggests that the expression could function redundantly or substitute each other to induce flowering in black rice Cempo Ireng. Similar pattern was also reported in japonica rice background with normal harvest period (3 - 4 months). Under the LD conditions, it was reported that the expression level of *Hd3a* and RFT1 was barely detected in any developmental stages (Komiya et al., 2008). Here, we could detect the expression of *Hd3a* and *RFT1* under neutral conditions. The expression of flowering genes was maintained and may be regulated in independent pathways under neutral day conditions. However, the flowering pathway of black rice Cempo Ireng was still conserved. Based on our results, the flowering genes were expressed regardless of day length. Thus, black rice Cempo Ireng was considered to be photoperiod insensitive. Moreover, further study in SD and LD conditions is required to ensure if the flowering pathway is affected by day length or not.

In addition, the *Hd3a*, *RFT1*, and other flowering gene regulators which were related to photoperiod pathways were confirmed to have a diurnal expression (Kojima et al., 2002). The diurnal expression was also proposed to be unique characteristics of flowering genes. In our previous study, we confirmed that the *Hd3a* and RFT1 had diurnal expression in black rice Cempo Ireng (Malika and Purwestri, 2014; Yuliani and Purwestri, 2014), thus indicating that the flowering pathway was conserved.

The two regulator flowering genes *Hd1* and *OsCOL4* which belong to *CO*-like gene family were also examined in this study. *Hd1* was the flowering promoter, and its expression was repressed by phytochrome (Lee et al., 2010). In this study, we found the *Hd1* expression at 48 DAP might not be involved in the induction of *Hd3a* due to the expression level which was not detected even when the downstream gene was upregulated. We suggest that the expression of the flowering genes might be regulated by another/other gene(s). *Ehd1*, the floral inducer under SD and LD conditions was proposed to play a role in these circumstances. Our previous study successfully detected and confirmed the expression of *Ehd1* related to the expression of the flowering genes (Fauzia and Purwestri, 2013). The *OsCOL4,* the other regulator, was also observed as the flowering repressor under both SD and LD conditions. *Os*COL4 expression was reported at a high level at 5 weeks after germination and rapidly declined in both SD or LD (Lee et al., 2010). Furthermore, the *OsCOL4* mutant showed early flowering phenotype, and the expression of flowering genes *Hd3a* and *RFT1* increased (Lee et al., 2010). In black rice Cempo Ireng, we found that *OsCOL4* was only detected in a very low level at 48 DAP when the *Hd3a* started to be expressed, thus indicating they both antagonized in the regulation of flowering. Our results also support the proposed OsCOL4 as a novel flowering regulator which maintained the vegetative phase. Further investigation to elucidate the *OsCOL4* expression in early DAP is needed to ensure its role as black rice Cempo Ireng flowering repressor. Another investigation in the *FT*-like gene family in this study also showed a similar pattern with two major flowering genes, indicating they were involved in flowering promotion (Izawa et al., 2003). Another study also revealed that the expression of *FT-L4, FT-L5, FT-L6, and FT-L12* were similar with the wild-type plants (Komiya et al., 2008). However, based on our data the expression was only detected at a particular time and suggested it was not involved directly in the black rice Cempo Ireng flowering regulation. Interestingly, a new finding in a knockout mutant of FT-like gene family using CRISPR/CAS9 system showed a premature leaf senescence phenotype instead of flowering (Ma et al., 2015). The finding strongly suggested that the function of the *FT-like* gene family was not involved in flowering but other developmental stages. In Fig. 3 we reconstructed possible conserved flowering pathways in black rice Cempo Ireng under short day and long day conditions. Under the neutral day condition*, Hd3a* and *RFT1* may be regulated in the independent pathway. However, both of which could be transported to the shoot apical meristem (SAM) and interacted with OsFD1 through

**Table 1.** PCR primers used for the amplification of black rice flowering and regulator genes.

| NCBI Accesion Number | Primer Name | Nucleotide sequence |
|---|---|---|
| AB052942 | *Hd3a* | F: 5'-CACAAGAA GGTGAAGCTCGC-3'<br>R: 5'-GCCTTCTGGTTGTAGACGTAGG-3' |
| AB426873 | *RFT1* | F: 5'-TGACCTAGATTCAAAGTCTAATCCTT-3'<br>R: 5'-TGCCGGCCATGTCAAATTAATAAC-3' |
| AB041837 | *Hd1* | F: 5'-GTTTGCAGAGAAGGAAGGGAGCGAGTG-3'<br>R: 5'-TCTGGAATTTGGCATATCTATC-3' |
| <u>AP004124</u> | *FT-L5* | F: 5'-GGTTGGTGACGGACATTCCA-3'<br>R: 5'-ACCGGTGGTCCTAGGTTGTA-3' |
| JC624473 | *FT-L6* | F: 5'-CAAGGGACCCACTTGTCGTA-3'<br>R: 5'-GCGAGTCAGGATCCACCATC-3' |
| <u>AP003076</u> | *FT-L9* | F: 5'-TCCAGATGCTCCAAGCCCAAGCAAC-3'<br>R: 5'-AACCTTCTTCCACCAGAGCCGGCTTC-3' |
| <u>AC130603</u> | *FT-L10* | F: 5'-AGCAAGCCACAGGTTGATGT-3'<br>R: 5'-GGCGGAACAGCACAAATACC-3' |
| AK100097 | *OsCOL4* | F: 5'-ATCCACTCGGCGAACCCGCT-3'<br>R: 5'-CGCTTCTCCCTGTACCGCAT-3' |
| AK241233 | *Ubiquitin* | F: 5'-AACCAGCTGAGGCCCAAGA-3'<br>R: 5'-ACGATTGATTTAACCAGTCCATG-3' |
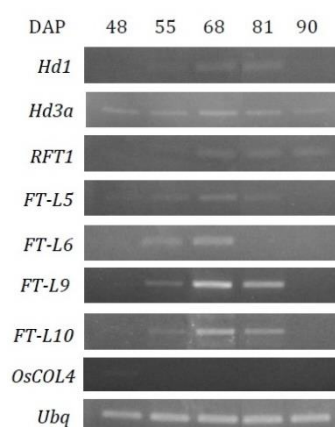


**Fig 1.** The amplicon of expressed flowering and regulator genes in various development stages of black rice Cempo Ireng from the vegetative phase to the generative phase. DAP: days after planting.
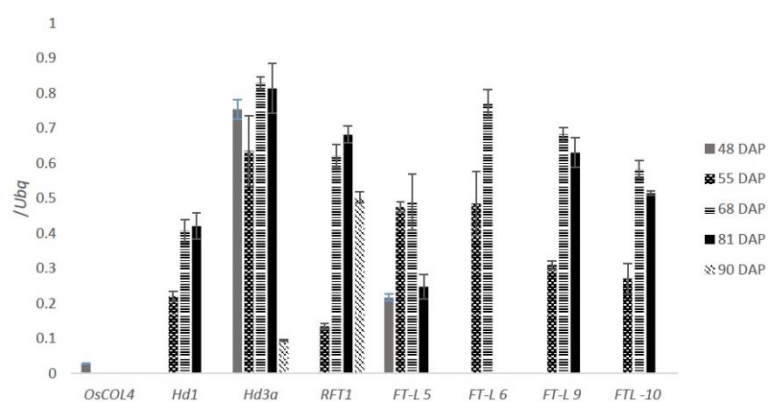


**Fig 2.** Semi-quantitative analysis of the expressed flowering and regulatory genes. All detected genes were normalized with *Ubq* and shown in relative fold changes.

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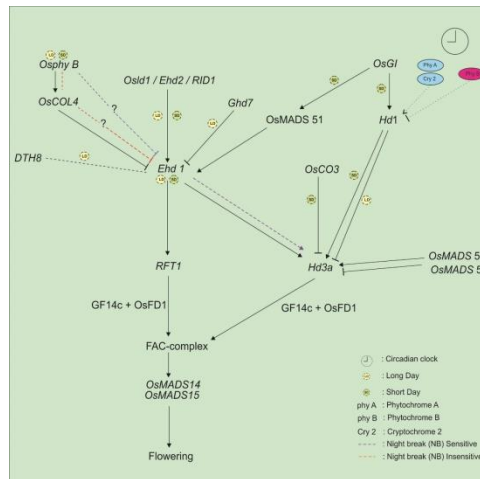
**Fig 3.** The proposed possible conserved flowering pathway in black rice Cempo Ireng under neutral day condition. Flowering genes expressed under short day and long day conditions to induce flowering. *OsCOL4* proposed to be the regulator for maintaining the vegetative phase and its expression is opposite to the expressed flowering genes of black rice Cempo Ireng.

14-3-3 proteins (GF14c) to form FAC and induce the expression of flowering meristem identity genes to start flowering. The proposed mechanism indicated the conserved model of rice flowering pathway (Zhao et al., 2015; Taoka et al., 2011).

The adaptation of rice and its flowering in tropical condition, especially in black rice Cempo Ireng which has long harvest period, was poorly understood. In this study, we provide the basis for the behavior of flowering genes , and it could be used as the information for developing this cultivar. The long harvest period of black rice Cempo Ireng may be caused by the allelic polymorphisms of *Hd3a* and *RFT1* or epigenetic modification which also exists in the determination of the flowering time (Zhao et al., 2015). Thus, further investigation is required. Other potential genes like *Ghd7, DTH7, and DTH8* which correlate with grain numbers, plant height, and heading date (Wei et al., 2010) seem to be good candidates to study since less productive and excessive plant height of black rice Cempo Ireng also exists. Further investigation to elucidate the flowering pathways and their correlation with productivity becomes an interesting topic to study in the future.

**Materials and Methods**

*Plant materials*

The black rice Cempo Ireng seeds were soaked overnight in tap water before being sown in the soil (with soil to compost fertilizer ratio of 3:1), and only the denser seeds were used to the germinating process. The 21 old-day seedlings were planted on soil with compost, organic fertilizer, and maintained properly in a greenhouse under normal tropical condition (neutral days) or 12h photoperiod (12 hours of dark period and 12 hours of light period). The leaf blades were sampled in three biological replications at 48, 55, 68, 81, and 90 days after planting (DAP) and then used for the RNA isolation.

*RNA isolation and cDNA synthesis*

RNA isolation was conducted using Qiagen RNeasy Plant Mini kit (Qiagen) according to the manufacturer's

instruction. Leaf blades composite of black rice plant at 48, 55, 68, 81, and 90 days after planting (DAP) were used as samples for RNA isolation. The determination of quality and quantity of isolated RNA samples was performed by using gel agarose electrophoresis and spectrophotometer at Å260 and Å280 nm. The 0,5 µg of RNA was used for cDNA synthesis of each sample using Superscript II cDNA synthesis kit (*Invitrogen*) according to the manufacturer's instruction with modification in the incubation period following 30°C 10 min for activation, 42 C° for reverse transcription, and 95 C° for reaction termination using thermal cycler machine (*Applied biosystem*). The successful cDNA synthesis reaction product was checked by amplification of housekeeping gene (*Ubq*).

*PCR amplification*

The cDNA was used as a template for targeted flowering gene detection with a thermal cycler (*Applied Biosystem*) for polymerase chain reaction using Ex Taq PCR kit (Takara). The 20 µL reaction mixture was set as follow; 2 µL 10x buffer, 2 µ 2 mM dNTPs, 1 µL 10 µM primer of each primer (forward and reverse), 0,1 µL (5U/µ L) Ex Taq DNA polymerase and ddH2O µL and 1 µL cDNA of each sample. The primers used in this study are listed in Table 1. The following program of PCR reaction is 3 minutes at 94°C followed by 30 cycles of 1 minute at 94°C, 45 seconds at specific primer annealing temperature, 1 minute at 72°C followed by one cycle of 5 minutes at 72°C. The specific primers temperature are also listed in Table 1. Amplified bands were separated by electrophoresis gel agarose 2% (w/v) (Sigma chemical) and visualized by ethidium bromide staining under UV transilluminator, and the photograph was taken using a digital camera (Canon Ixus 115 HS ).

*Semi-quantitative analysis*

The amplification product of detected genes from the previous step was resolved in agarose by electrophoresis gel agarose 2% (w/v) (*Sigma chemical*) and visualized by ethidium bromide staining under UV transilluminator, and the photograph was captured with a digital camera (*Canon Ixus 115 HS*). *Ubq* was used for normalization of the

detected genes of each sample. The semi-quantitative analysis was conducted using *Image-J* software for Windows 32-bit. The analysis was performed in three replications then they were visualized in a graph with standard deviation and later served in relative fold changes.

## Conclusion

The development of black rice Cempo Ireng as functional food can be achieved by understanding its flowering behaviors. Cempo Ireng grown under neutral day conditions has flowering pathways similar to the conserved rice flowering models grown under SD/LD conditions. The flowering genes and its regulators which were expressed under neutral day conditions were found to be unique and in general followed the conserved model, but some of which showed slightly different expression patterns.

## Acknowledgments

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