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Photoperiod Effects on Expression of PHYA and PHYB Transcripts in Alfalfa with Different Fall Dormancy

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Abstract — **Short day-length (SD) is the main environment-induced factor leading to fall dormancy (FD) in alfalfa (*Medicago sativa* L.). Although the influence of the photoperiod on dormancy has been extensively reported in many plants that phytochromes (Phys) act in light perception, the difference of phytochromeA (PHYA)and phytochromeB (PHYB)gene expression under the entrainment of natural and artificial photoperiod controlling of fall dormancy have been poorly studied. In this article, PHYA and PHYB transcripts of alfalfa with three different fall-dormancy types were assessed in a SYBR Green-based real-time PCR procedure and PHYA and PHYB transcripts were quantified in alfalfa growing under natural and artificial photoperiod. Our data indicate that short photoperiod was inductive to the synthesis of PHYA and PHYB critically at transcription, photoperiod regulates PHYA and PHYB gene expression in alfalfa(natural and artificial photoperiod conditions), and PHYA and PHYB gene expression differences exist among alfalfa varieties differing of fall dormancy. The expression of PHYA and PHYB transcripts in alfalfa with different fall dormancy had the similar trend; Short natural photoperiod was inductive to the synthesis of PHYA and PHYB critically at transcription under natural and artificial photoperiod conditions at transcription.**

Keywords — **Photoperiod, Fall Dormancy, Alfalfa, PHYA, PHYB**

I. Introduction

Alfalfa (*Medicago sativa* L.) has been gradually concerned as an important forage crop in the world. It has dormant characterizer in fall and winter [1], and fall dormancy (FD) is a kind of growth characteristics of alfalfa as a special way to response the changes of environment, it has long been recognized as the most important single factor determining the adaptation of alfalfa varieties. In general, more dormant varieties tend to have better winter hardiness and persistence than less dormant cultivars; however, they also have less vigor, less late summer and fall growth and lower forage yield potential than the less dormant types[2]. According to different varieties responses of low temperature and day-length, they were classified into three groups, dormant (1–3), semi-dormant(4–6), and non-dormant (7–9) [3]. Dormant types show reduced fall shoot growth or cease to grow and decumbent shoots after cutting. Non-dormant types show rapid shoot regrowth and upright shoots. Semi-dormant types show phenotypes between dormant and non-dormant types [4,5]. The physiological, biochemical and molecular mechanisms causing FD were not understood yet. The study showed that photoperiod or short day (SD) length is the main environmental factor of fall dormancy [4,6,7]. Plants can perceive the change in day-length or photoperiod through photoreceptors, and the expression of most light-induced genes is mediated through phytochromes (Phys)[8]. Phytochromes are the most well studied of all these photoreceptors that can perceive the day-length and regulate the fertility of green plants [9]. There are PHYA, PHYB, PHYC, PHYD, and PHYE genes in the phytochrome family, which may have redundant and overlapping functions [10], and they can function individually but interact together to influence and maintenance of fall dormancy as an interconnected network [11,12]. There was a highly significant correlation between day-length and the expression of phytochromes mRNA [13]. PHYA and PHYB are prominent under normal growth to mediate the establishment of a light-grown program, also referred to as photo morphogenesi [14].Both PHYA and PHYB were identified to play an important role in the control of endodormancy [15].

It has now been well established that phytochromes are involved in the onset and maintenance of dormancy in many plant species, however, the photoperiodic regulation of PHYA and PHYB gene expression has been poorly studied in alfalfa, little information is known about their roles in fall dormancy, the objectives of this research were to determine: quantify expression of PHYA and PHYB transcript in alfalfa with different fall dormancy growing under natural and artificial photoperiod, and highlight the difference between natural and artificial photoperiod in light perception by alfalfa.

II. Materials and Methods

Plant material and treatments

The three standard alfalfa varieties were Maverick, Dupuils, and CUF101, having different fall dormancy type. We can see the plant material variety and fall dormancy class in table1.

Ⅰ. Alfalfa leaves were selected from 2-year-old alfalfa, since the April till October(day-length7~16h,sample dates were 25-Apr, 24-May, 22-Jun, 23-Jul, 23-Aug, 23-Sep, 23-Oct and the day-length of the sample dates were**:** 13h 22min; 14h 12min; 14h 29min; 14h 07min; 13h 13min; 12h 09min; 11h 04min;) in the year 2013.The day-length and temperature are listed in Fig.1. Three replicates were performed for each month. The Experimental Station is in Henan Agricultural University, 30km north of Zhengzhou City. The total sunlight is 2000-2600 h and frost-free period is 220-250days. The soil is a sandy loam with 0.39% total nitrogen, 0.75% organic matter, and 0.10% of total phosphorus. The annual average rainfall is about 600mm. The annual average temperature is 14.3°C with extremes of -20°C in January and 40°C in July.

Ⅱ. The same alfalfa plants with roots were dug up, transplanted in plastic pots and placed in the experimental rooms. The pots were filled with the soil from Experimental Station. The plants were cultured as follows: day 25℃ /night 15℃ under four different photoperiod conditions: 7/17h (day/night) 10/14h (day/night) 13/11h (day/night) and 16/8h (day/night), provide the light with a minimum of 242 μmol $m^{-2}S^{-1}$ of photo synthetically active radiation at the plant top. The plant was irrigated regularly to maintain relative humidity 65%. After 35 days, the upper-most buds and leaves were sampled, immediately frozen in liquid N_2 and stored at -80℃ until analysis. Three replicates were performed for each treatment.

RNA isolation and cDNA synthesis

RNA was extracted using RNAiso Plus (Takara Biotechnology CO., Dalian, China). DNA-free RNA was obtained according to the manufacturer instructions (RNAiso Plus, Code No. 9108/9109). cDNA was prepared from 2 μg of total RNA by using M-MLV RTase cDNA Synthesis Kit according to the manufacturer instructions (Takara Code No.D6130).

Primer design for PHYA and PHYB

PHYA forward prime:
5'-GAGAGATAGCTTTATGGATGTCTGAGT-3', and the reverse primer was 5'- GCGACCTAAACCAGAAAACTATGT-3';
PHYB forward prime:
5'-GTAGAGGACGCTATGGGGAAGT-3', and the reverse primer was 5'-TGGAGCAAGCATTCACCACTAT-3', these pairs of primers were used to amplify168bp and 146bp fragment.

PCR reactions

Template cDNA 2μl was placed in a final 20μl reaction volume, in which PCR tubes contain 0.4μl of each primer (10mM), 10μl SYBR® Premix Ex Tax (Takara Code No. RR420A), ROX Reference Dye 0.4μl and 6.8μldd H_2O. The PCR programs were carried out as shown in Table 2.

Standard curve

Plasmid DNA standards were constructed by purifying the PCR using Gel Extraction Kit Ver.3.0 according to the manual's instructions(Takara Code No. 9762). The DNA was quantified using a Thermo Nanodrop Spectrophotometer ND-1000. Purified PCR products were transformed into PMD18-T Vector (Takara Code No.D101A). Characteristic white colonies were picked and cultured overnight in LB broth, only positive recombinants obtained after the transformation. The plasmid was prepared using Plasmid Purification Kit Ver.3.0 (Takara Code No. D821A). Standard curves were prepared by amplifying plasmid DNA samples. The Cycle Threshold (Ct) corresponding to the analyzed sample is compared with the other Ct data by the application of a dilution series of standards to generate a standard curve, serial dilutions were performed in DNA grade water over five points: 1011, 1010, 109, 108, 107, 106, 105, 104 copy/μl. The copy numbers of the PHYA and PHYB gene were calculated based on the following formula, in which m is the DNA mass, NA is Avogadro's number (6.02×1023bp/mol) and M is the average molecular weight n=m× NA /M.

III. Results

Standard curves of PHYA and PHYB

Standards absolute quantification of alfalfa leaves using real-time PCR are expressed as copy number. Copy number is recording to the quantity of target gene molecules in every PCR reaction. Copy number is typically prepared using genomic DNA [16-19] Efficiency of PHYA and PHYB PCR application are 95.64% and 102.61% (90%~110%), whereas both the standard curves correlation coefficient(R^2) are higher than 0.998.After the photoperiod treatment, PHYA transcripts copies had the linear regression when the copies were less than 10^7 copy/μl, therefore, it was reasonable to consider that the PHYA expression was higher from the standard curves(Fig. 2).

Artificial photoperiod treatments

As the photoperiod continued to prolong, PHYA transcripts copies of semi-fall dormancy Dupuils and non-fall dormancy CUF101 gradually decreased with the order 7h＞10h＞13h＞16h(Fig. 3). Under the 7h photoperiod treatment, PHYA transcripts copies of Maverick was less than 10h photoperiod treatment but more than 13h and 16h photoperiod treatment, respectively. PHYA transcripts copies of three kinds of alfalfa were in the same order 10h＞13h＞16h, corresponding to their fall dormancy levels (Maveric＞Dupuils＞CUF101). During the transition from SD to LD(long day-length) under artificial photoperiod, the rhythm of PHYA transcripts copies remained relatively similar as natural photoperiod treatments.

The effects of artificial photoperiod treatments on the PHYB expression differed from PHYA. The PHYA transcripts copies of three different dormant alfalfa varieties were still higher than PHYB expression under the artificial photoperiod treatments. PHYB transcripts copies of fall dormancy Maverick were in the order of 7h＞13h>10h＞16h. However, under artificial photoperiod, the rhythmic expression of PHYB transcripts oscillated with rhythms different minimum value (Maverick 16h, Dupuils 13h, CUF101 10h) and same peaking (7h). Under the artificial photoperiod treatment, PHYB transcripts weren't in the order of Maverick＞CUF101＞Dupuils with no corresponding to their fall dormancy levels.

Natural photoperiod treatments

The effects of natural photoperiod treatments on the PHYA and PHYB expression of alfalfa were observed. The natural photoperiod effects on PHYA and PHYB transcripts copies in three alfalfa varieties differing in degree of fall dormancy(Fig. 4). It was found that PHYA and PHYB transcripts copies increased with the day-length getting down. PHYA and PHYB transcripts copies of fall-dormant alfalfa were higher than that semi-dormant and non-dormant alfalfa in July, August, September and October(Maverick＞Dupuils＞CUF101). In other words, the stronger the fall dormancy, the higher were the PHYA and PHYB transcripts copies. Under the natural photoperiod, the PHYA transcripts copies of three different dormant alfalfa varieties was higher than PHYB expression, PHYA and PHYB transcripts copies were in the similar trend, corresponding to their fall dormancy levels. There were highly significant differences of PHYA and PHYB transcripts copies in alfalfa among different natural photoperiod treatments.

As day-length continued to shorten, PHYA and PHYB transcripts expression of the three alfalfa varieties gradually increased. The results indicated that short natural photoperiod was inductive to the synthesis of PHYA and PHYB.

IV. Discussion

In this study, standards for absolute quantification PHYA and PHYB gene expression of three standard fall dormancy alfalfa varieties leaves using real-time PCR are expressed as 'copy number'. Copy number unit corresponds to the quantity of target molecules initially present in the PCR reaction. Quantitative real-time PCR has been widely used in many research,such as the simultaneous amplification, detection and quantification of nucleic acids and diagnostic applications. The Cycle Threshold (Ct) corresponding to the analysed sample is compared with the other Ct data by the amplification of a dilution series of alfalfa PHYA and PHYB plasmid DNA samples to generate standard curves, which subsequently allows determination of the initial concentration of target DNA in a sample.

Fall dormancy (endo-dormancy), which is probably the most important single factor determining the adaptation of alfalfa varieties, is a response to reduced day-length in late summer and fall. Plants can be divided into three groups based on photoperiod response: short-day plants, day-neutral plants and long-day plants[20-22]. Alfalfa is an absolute long-day plant and the critical day-length of alfalfa effects on PHYA and PHYB expression under different photoperiod conditions. Day-length obviously helps entrain the regulators of the circadian clock, but day-length also appears to more directly alter dormacy through the action of the red light photoreceptor PHYA. Alterations in PHYA expression disrupt the circadian clock under low light intensities [23]. PHYA and PHYB gene expression (mRNA levels)play an important role in light-signaling pathway of plants, in which 96% of light-induced genes in Arabidopsis are mediate by PHYA and PHYB [8]. In Fig. 5, the kegg and pathway analysis of PHYA and PHYB, we can see PHYA and PHYB have the same phytochrome-interacting factor (PIF), PIF3. Both phyA and phyB redundantly induce this PIF3 phosphorylation, as well as nuclear speckle formation and degradation, by direct interaction with PIF3 via separate binding sites[24,25]. This system has been dubbed the "circadian clock," and both light and temperature influence the timing and impact of circadian clock gene expression. Many of the circadian clock genes were discovered due to their impact on flower time regulation. Because perception of day-length appears to play a role in endodormancy induction in some plants, the genes and processes responsible for perceiving and disseminating these day-length signals likely control both these developmental processes. There is a photoperiod reaction in the phytochromes of light-grown green plants. In other words, the photoperiod reaction is a response of phytochromes to the periodic switches between light and darkness [7]. It has been reported that the difference exists between natural and artificial photoperiod in light perception by plants [26], contrast with the results regarding the phase of PHYA and PHYB transcript accumulation in tomato grown under natural and artificial photoperiod [27].

The difference between natural and artificial photoperiod must be relevant for light perception of plants; therefore, our study was carried out under natural[28] photoperiod and artificial photoperiod conditions. Under artificial photoperiod, twilight periods are absent, and transitions from dark to light or vice versa are abrupt. Whereas, under natural photoperiod, it is not only the extension of the light period, but also the extension of the twilight period varies with changing photoperiod. And twilight periods varies seasonally, and in some instances it may constitute an important part of the daily cycle[13].

The photoperiod effects on PHYA and PHYB expression in three alfalfa varieties differing in degree of fall dormancy and the correlation between natural and artificial photoperiod conditions in regulating fall dormancy have been examined. The results of our study showed that short natural photoperiod was inductive to the synthesis of PHYA and PHYB critically at transcription, and photoperiod regulates PHYA and PHYB gene expression in alfalfa(natural and artificial photoperiod conditions), contrast with those reported in many plants that PHYA and PHYB gene expression is regulated by the circadian clock [29-31] and PHYA and PHYB gene expression differences exist among alfalfa varieties differing in fall dormancy. Plants perceive light through photoreceptors, and the expression of most light-induced genes is mediated through phytochromes (Phys) [8,10,32,33]. As the dormancy and development of alfalfa were regulated by PHYA and PHYB, it is possible to predict the dormancy habit of unknown genotypes by measuring the PHYA and PHYB gene expression.

V. Conclusion

The expression of PHYA and PHYB transcripts in alfalfa with different fall dormancy had the similar trend under natural and artificial photoperiod conditions at transcription. Short natural photoperiod was inductive to the synthesis of PHYA and PHYB critically at transcription, and photoperiod regulates PHYA and PHYB gene expression in alfalfa(natural and artificial photoperiod conditions). PHYA and PHYB gene expression differences exist among alfalfa varieties differing of fall dormancy.

Acknowledgment

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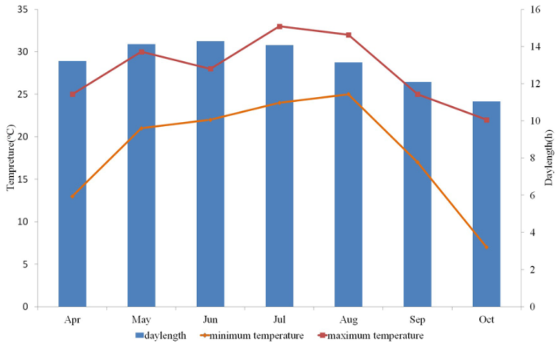

Fig. 1. Temperature and day-length under natural photoperiod.
The maximum and minimum temperatures from April to October were listed with the day-length of sampling stage

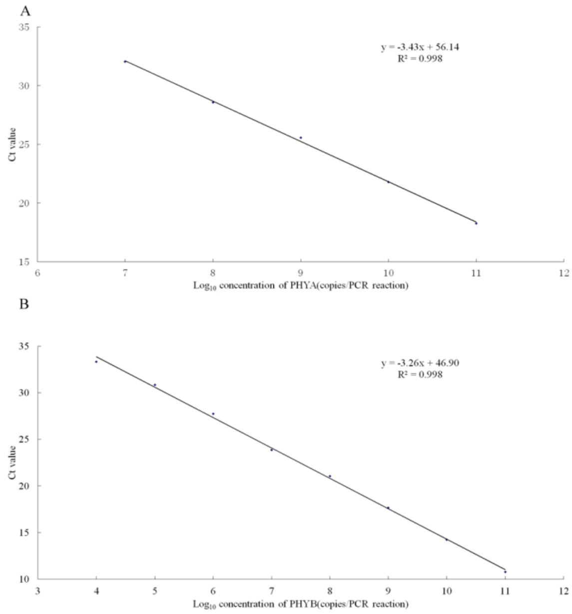

Fig. 2. Standard curves of PHYA and PHYB showing Log_{10} concentration of PHYA and PHYB transcripts Copy numbers.
Log_{10} (copy numbers of PCR reaction) were used in standard curves; copy number is typically prepared using genomic DNA.

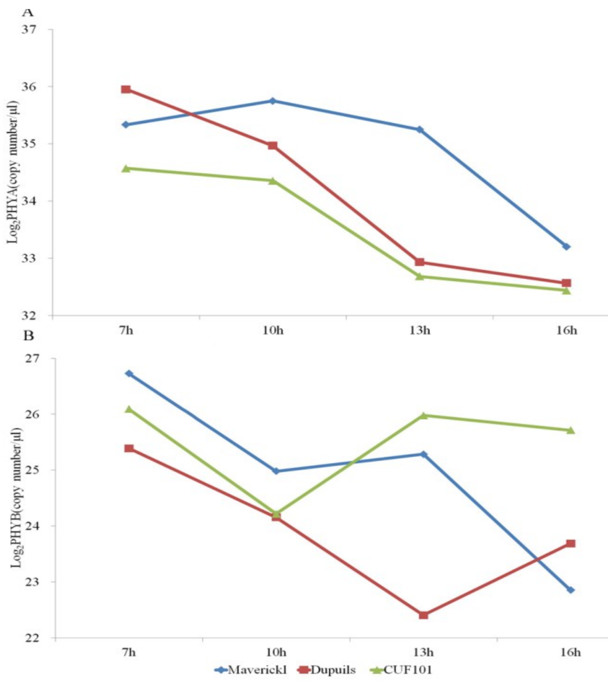

Fig. 3. Copy numbers of PHYA and PHYB transcripts under artificial photoperiod.
Log_2 (copy numbers of PHYA PCR reaction) of three different dormant alfalfa varieties.

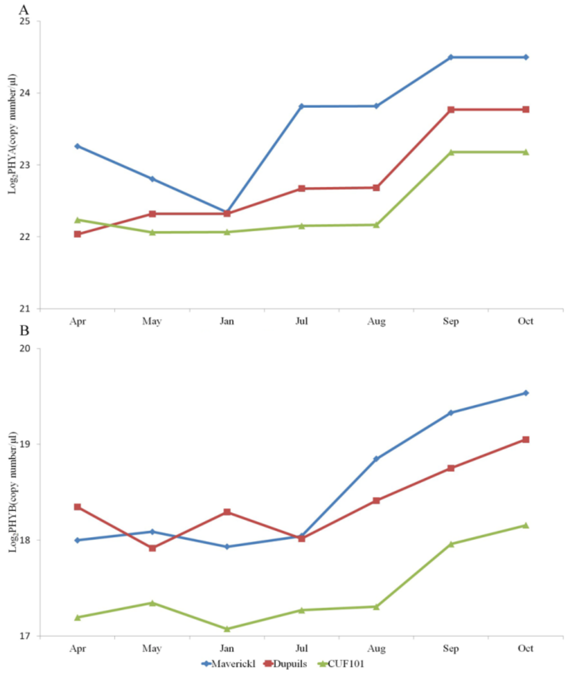

Fig. 4. Copy numbers of PHYA and PHYB transcripts under natural photoperiod. Log2 (copy numbers of PHYB PCR reaction) of three different dormant alfalfa varieties

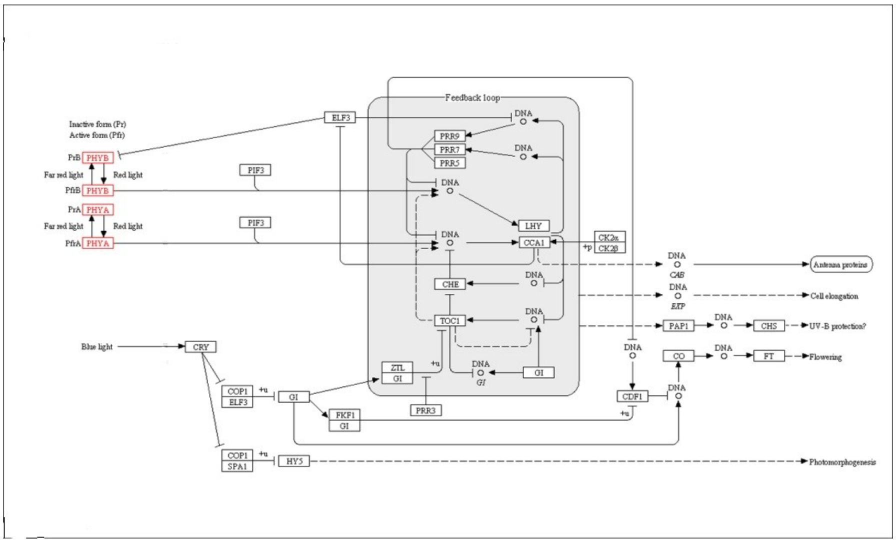

Fig. 5. The kegg and pathway analysis of PHYA and PHYB.

Table 1. Alfalfa varieties, fall dormancy classes and types

Variety	Fall dormancy class	Fall dormancy type
Maverick	FD class 1	fall dormant
Dupuils	FD class 5	semi-fall dormant
CUF101	FD class 9	non-fall dormant

Table 2. Real-time PCR conditions using PHYA and PHYB primers set

Program	SYBR Green-based real-time PCR		
Pre-incubation	95℃, 2min		
Application	40 cycles	Melting	95℃, 2min
	95℃, 10 s		72℃, 1min
	60℃, 15 s		95℃, 30 s
	72℃, 30 s		0.5℃/s
Cooling	30℃, 1min		(Continuous)