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FUNCTION ANALYSIS OF PHYTOCHROME B GENE IN MAIZE

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Abstract

ZmPHYB1 and ZmPHYB2 with some other factors and their own interactions were studied using yeast two-hybrid technology. Results showed that interaction signals were detected among different segments. Results speculated that interaction signals between ZmPHYB1 and ZmPHYB2 could be detected under certain lighting condition after a certain period of time. AtSPA1 and AtCOP1 may form complexes with other interaction factors co-interacting ZmPHYB accumulation. Agrobacterium-mediated genetic transformation study of functional *ZmPhyB1* and *ZmPhyB2* into Arabidopsis thaliana showed that genes can complement the corresponding mutant phenotype, while over-expressing transgenic plants gave rise to more obvious phenotypes in photo-morphogenesis. We speculated that endogenous *PhyB* gene interferences were responsible for different of indexes of necessary light-inducing phenotypic conditions between over-expression transform-ants and complementary transform-ants.

Introduction

With rapid development of modern molecular biology, the awareness and understanding of gene structure and functioning reached an unprecedented height(Chen *et al*., 2012; Shen *et al*., 2012; Yang *et al*., 2012), but the functional studies of encoding protein is still at a lower level. It is well known that the features of all organisms are often formed by complex networks, in the presence of multiple proteins within and with constant interactions. Yeast two-hybrid system established by Song and Field (Fields *et al*., 1989) is based on proteins interactions detection *in vivo* in yeast cells and their corresponding genetic systems. This system is an effective means for protein study in living eukaryotic cells. Not only can the system detect interactions in the known proteins but also detect unknown protein and their interaction with known proteins (Yan *et al*., 2003). Plant transformation technology is an effective method for crop improvement. Traditional plant transformation method is time consuming and laborious, requires relatively expensive laboratory equipment. Bechtold *et al*., (1993) proposed a simple new method by using Agrobacterium dip in *Arabidopsis* flower in vacuum, but this method has only been successfully used in Arabidopsis; therefore, the application is very limited. Steven improved many aspects of this transformation method, so that the method can be used in constructing mutant library, map-based cloning and other genetics research, and genetics scientists can do transformation in other species referring to this method (Clough & Bent, 1998). Under light condition, *PhyB* plays an important role in physiological processes, is the most abundant class of phytochrome in plants (Briggs *et al*., 2001). The function, expression pattern and its upstream and downstream protein relationship of *AtPhyB* has been extensively studied (Wang & Deng, 2002). In cereal crops, *PhyB* function and expression patterns in rice have been studied in-depth (Takano *et al*., 2005), structure and expression patterns of a copy of PHYB protein in wheat were preliminarily studied (Li *et al*., 2010). *PhyB1* and *PhyB2* analysis is limited to preliminary studies on its expression pattern in maize (Sheehan *et al*., 2004; Sheehan *et al*., 2007), the functions of encoded proteins have not been reported. Yeast two-hybrid protein interaction technology is an effective technological means in protein function study. Earlier research has shown that interaction happens between AtPHYA and AtSPA1, AtPHYA and AtCOP1. Furthermore, AtSPA1 and AtCOP1 will form a complex, degrading AtPHYA by 26S proteasome pathway (Hoecker *et al*., 1999; Saijo *et al*., 2008), but interactions among AtSPA1, ZmPHYB and AtCOP1 have not been reported. In this study, the relationships between ZmPHYB and other factors were studied by yeast two-hybrid technology, *ZmPhyB* were transferred into Arabidopsis genome by using Agrobacterium flower-dip method. The purpose of this study was to lay some scientific foundation for further functional studies of ZmPHYB1 and ZmPHYB2.

Materials and Methods

Construction of point mutant in *ZmPhyB1*: TA cloning of *ZmPhyB1* in plasmid was extracted using fast plasmid kit (Biomed, Beijing, China). As sequences were distinguished by Bgl and Mfe were in *ZmPhyB1*. To construct vectors for yeast two-hybrid, primers were designed to mutate Bgl and Mfe , respectively (Table 1). PCR amplification was done using LA Taq polymerase (TakaRa, China) with proofreading activity. The temperature cycle was: 4 min at 94°C, 1 min at 94°C, 1 min at 58°C, 6 min at 72°C for 15 cycles, and 10 min at 72°C. After the reaction, following components were added: 1µl Dpn , 6µl 10 × Buffer 4, 3µl sterile water. The product was digested with restriction enzyme by maintaining 1.5 h at 37°C, transferred into E. coli DH5α. The identified positive clone was sequenced to Invitrogen Co. Ltd. (Shanghai, China), its plasmid was template to mutation Bgl . The temperature cycle was: 4 min at 94°C, 1 min at 94°C, 1 min at 60°C, 6 min at 72°C for 15 cycles, and 10 min at 72°C. The following operation was the same as mentioned above. Positive clone was sequenced by Invitrogen Co. Ltd. (Shanghai, China).

Yeast transformation vector construction: by using Matchmaker LexA Yeast two-Hybrid Yeast two-hybrid system (Serino *et al*., 2003), the AtSPA1, AtCOP1 and

its loss of function in different sections were constructed (Sajio *et al*., 2003; Yang *et al*., 2005; Yang *et al*., 2005), the AtSPA1, AtCOP1 and ZmPHYB1 and ZmPHYB2 interactions *In vitro* analysis were done. Each domain played domain-specific role in the exercise of phytochrome functions. According to AtPHYB, each of ZmPHYB1 and ZmPHYB2 proteins were divided (Usami *et al*., 2007) into N-terminal domain and C-terminal domain (Fig. 1), and the construction of LexA and GAD vectors for ZmPHYB1 and ZmPHYB2 full-lengths and functional deletion segments (ZmPHYB1, ZmPHYB1-NT651, ZmPHYB1-CT548, ZmPHYB2, ZmPHYB2-NT651 and ZmPHYB2-CT548) were done through the following steps: 1) specific primers corresponding segments mention above were designed (Table 2). 2) the target fragments from the cDNA of maize cultivar B73 were amplified and cloned into pMD19-T (Takara, Japan) vector. 3) the fragments were transferred into yeast vectors pEG202 and pJG4-5 by restriction enzyme digestion and ligase, thus LexA and GAD vectors with the corresponding fusion of *ZmPhyB1* and *ZmPhyB2* fragments for yeast two-hybrid test were prepared. The procedure and the required reagents were from Matchmaker LexA Yeast Two-Hybrid, Catalog # K1609-1 (ClonTech, USA) and the product instructions.

Table 1. Primers for mutant Bgl and Mfe sites in *ZmPhyB1*.

Primers name	Sequences
Former primer to Bgl site	5'-CCTTCGCCGCGCGCGAGATATCGCTGCTCAACCC-3'
Reverse primer to Bgl site	5'-GGGTTGAGCAGCGATATCTCGCGCGCGGCGAAGG-3'
Former primer to Mfe site	5'-GAACATGGGGTCAATAGCGTCGCTTGTTATG-3'
Reverse primer to Mfe site	5'-CATAACAAGCGACGCTATTGACCCCATGTTC-3'

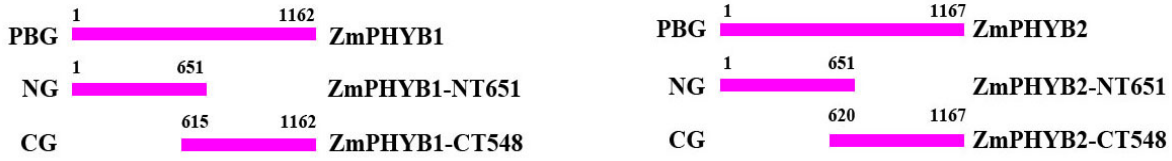

Fig. 1. Domain of ZmPHYB1 and ZmPHYB2 derivatives
A. Domain of ZmPHYB1 derivatives, B. Domain of ZmPHYB2 derivatives

Table 2. Primers for construction expression vectors in yeast two – hybrid.

Primers name	Sequences
Former primer to ZmPHYB1	5'-AGATCTGGTACCCAATTGATGGCGTCGGGCAGCCGCGCCAC-3'
Reverse primer to ZmPHYB1	5'-CAATTGACTAGTTCAGACGATTTCTCTACCAGCTGCTG-3'
Former primer to ZmPHYB1-NT651	5'-AGATCTGGTACCCAATTGATGGCGTCGGGCAGCCGCGCCAC-3'
Reverse primer to ZmPHYB1-NT651	5'-GTCGACACTAGTTCAAAATGCCTTGAATGACGACCGTG-3'
Former primer to ZmPHYB1-CT548	5'-AGATCTGGTACCCAATTGATGGCATCACCCTGAGGATAAGG-3'
Reverse primer to ZmPHYB1-CT548	5'-CAATTGACTAGTTCAGACGATTTCTCTACCAGCTGCTG-3'
Former primer to ZmPHYB2	5'-GTCGACTCGGTACCAGATCTGAATTCATGGCGTCGGACAGTCGCCCCCCCAA-3'
Reverse primer to ZmPHYB2	5'-GTCGACACTAGTTTAACATATCAGCTGATTTTCTCTAC-3'
Former primer to ZmPHYB2-NT651	5'-TCTGGTACCGAATTCATGGCGTCGGACAGTCGCCCCCCCAA-3'
Reverse primer to ZmPHYB2-NT651	5'-GACACTAGTTCAAAATGCCTTGAATGATGATGACCGTGG-3'
Former primer to ZmPHYB2-CT548	5'-AGATCTGGTACCGAATTCATGCATCACCCTGAGGATAAGG-3'
Reverse primer to ZmPHYB2-CT548	5'-GAGACTAGTTTAACATATCAGCTGATTTTCTCTAC-3'

Arabidopsis transformation: The restriction enzyme digested and PCR identified expression plasmid vectors were transferred into competent cells of Agrobacterium strain GV3101 by electroporation, positive transform-ants were selected by PCR identification. The transform-ants were incubated in LB liquid medium containing the appropriate antibiotic (rifampicin 20 μg/ml, trough amphotericin 25 μg/ml, kanamycin 50 μg/ml) for activation, the activated bacillus were inoculated in accordance with the ratio of 1:500 fresh LB antibiotics plus for enlargement culturing. Shook the bacillus overnight at 28°C, then centrifuged 5000r/min for 5 min at room temperature, removed the supernatant was removed and freshly prepared Arabidopsis transformation culture solution for precipitation suspension was added. The suspension was poured into a large glass dish, Arabidopsis flowers of Col-0 wild-type and mutant *PhyB-9* were placed upside down, immersed into transformation liquid as possible, put them aside for 1 min, laid flat on a black tray with pre-wet filter paper in it, covered and kept

moist overnight, Arabidopsis was upright after 24 h, slightly added water with normal management. The transformation solution for Arabidopsis was prepared as follows: 25 g sucrose, 100 µl silwetL-77, 5 µl BA, 1.1 g MS salts, with the PH meter for adjusting the PH value to 5.7-5.8, added water to make up the total volume up to 500 ml.

Screening and identification of Arabidopsis

Transform-ants: Mature Arabidopsis seeds of transform-ants were collected in a 1ml centrifuge tube, fully dried and sterilized centrifuge tube. The seeds were germinated on a filter plate with nutrient solution in the presence of antibiotics for resistance screening. The positive transgenic plants were screened under certain light and their phonotypic characters were observed.

Results

Yeast two-hybrid test: According to ZmPHYB1 and ZmPHYB2 functional domains' conjecture, the full-length and functional sections of ZmPHYB1 and ZmPHYB2 were constructed into yeast hybrid vectors, and physical interactions of ZmPHYB1, ZmPHYB2, ZmPHYA1, ZmPHYA2, AtCOP1 and AtSPA1 were tested using yeast two-hybrid test. JG-4-5 empty vector could not self-activate the expression of EG vector, and strong self-activation signal was detected in EG-ZmPHYA2. EG-202 can self-activate JG-ZmPHYB1, and strong self-activating signal was detected with JG-ZmPHYB2. Strong interaction signals were detected among ZmPHYB1 full length, ZmPHYA1 C-terminus, ZmPHYA2 C-terminal and ZmPHYA2 full length. EG-202 strongly self-interacted with and JG-ZmPHYB2 full lengths, but weak interaction was detected between ZmPHYA1 C-terminal and ZmPHYB1 N-terminal, no interaction signals were detected among other protein domains sections (Fig. 2A and C). In order to study the interactions between ZmPHYB and AtSPA1, ZmPHYB and AtCOP1, in vivo interaction tests were carried out. Results showed that the EG-202 can self-activate JG-AtSPA1 full length, JG-AtSPA1 N-terminal and JG-AtCOP1 the N-terminal to varying degrees. Strong interaction signals were detected between AtCOP1 N-terminal and ZmPHYB1 full-length, while interaction between AtCOP1 N-terminal and ZmPHYB1 N-terminal was false-positive (Fig. 2B). Strong interaction signal was detected between ZmPHYB2 C-terminal and AtSPA1 full-length (Fig. 2D).

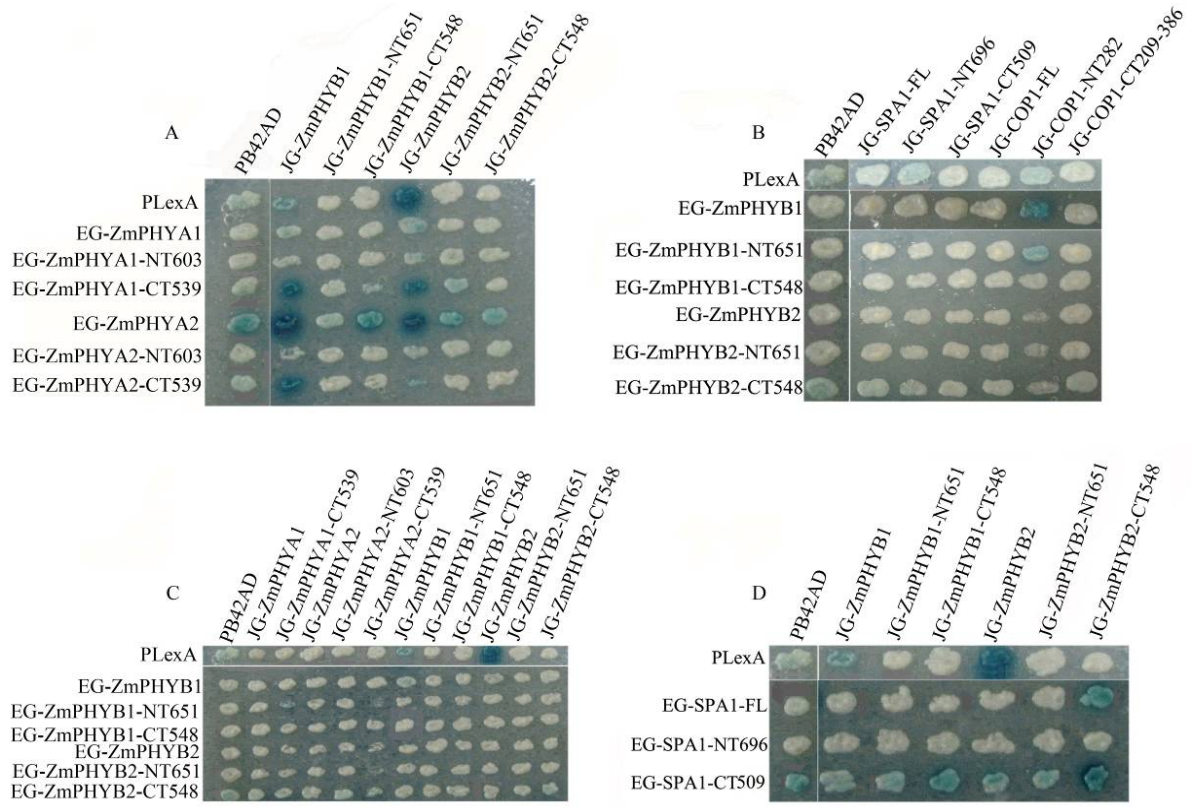

Fig. 2. Function of ZmPHYB1 and ZmPHYB2 *In vivo*
A, C. Function of ZmPHYB1 and ZmPHYB2 *In vivo*
B. D. Function of ZmPHYB1, ZmPHYB2 and AtCOP1, AtSPA1 *In vivo*
Note: In the figure, the first row from left to right and the first line from the top to the bottom are controls in the experiment. If there were function between protein factors, the plate that is cover by X-gal will turn blue. Furthermore, depth of color shows strength of functional signal. Deciding on whether function appear between two factors or not, we need compare functional signal with its control that lies in row and line.

Phenotype observation and identification of transgenic plants: Since phytochrome is the receptor to accept the light signal, the *ZmPhyB1* and *ZmPhyB2* of transgenic plant were activated in particular spectrums of light. The activated structures promoted specific transcription factors binding, thus started a series of downstream reactions, pushing the plant's developmental photomorphogenesis. The PjIM19-Hyg-HA-*ZmPhyB1* T1 transform-ants of *PhyB-9* mutant had shorter hypocotyls than non-transform-ants under 4 days of 50% blue light irradiation (the light intensity 0.06 μmol/s/m), no other significant phenotypic characteristics was observed; the T1 transform-ants of the wild-type Col-0 showed significantly increased surface areas of cotyledons under white light (light intensity 12.61 μmol/s/m) irradiation treatment for 4 days, and the angles between cotyledons increased, but no significant change was observed in hypocotyls lengths (Fig. 3A and B). The PjIM19-Kana-myc-*ZmPhyB2* T1 transform-ants of *PhyB-9* mutant had significant elongated petioles, showed significantly shorter under 4 days of 50% blue light (light intensity 0.06 μmol/s/m) irradiation treatment, the average hypocotyls length was about two-thirds of the length of *PhyB-9* mutant itself, even shorter than that of wild-type Col-0 hypocotyls length. The T1 transform-ants of wild-type Col-0 plants showed openied cotyledons, larger cotyledons angles and dramatically shortened hypocotyls under 4 days of weak white light (light intensity 1.11 μmol/s/m) treatment, which is about one-third of that of the Col-0 wild-type (Fig. 3C and D).

Fig. 3. Phenotype of T1 generation plants of *ZmPhyB1* and *ZmPhyB2* transgenic plants
A. PjIM19-Hyg-HA-*ZmPhyB1* transformed mutant *PhyB-9*
B. PjIM19-Hyg-HA-*ZmPhyB1* transformed wild type Col-0
C. PjIM19-Kana-myc-*ZmPhyB2* transformed mutant *PhyB-9*
D. PjIM19-Kana-myc-*ZmPhyB2* transformed wild type Col-0
Note: scale bar, 1mm

Discussion

Previous research showed that *AtPhyA* and *AtPhyB* can mutually suppress each other in the process of growth and development (Janoudi *et al*., 1997; Whitelam *et al*., 1997; Folta *et al*., 2001; Tepperman *et al*., 2004), and the mutual promotion and inhibition is based on the premise that they interact. *In vivo* yeast two-hybrid results detected strong interaction signals between ZmPHYA2 ZmPHYB1 full length, ZmPHYA1 C-terminus and ZmPHYA2 C-terminus; and weaker interactions were detected between ZmPHYB1 N-terminal and ZmPHYA1 C-terminal. Results showed that ZmPHYB1 performed its biological function through formation of heterodimers with ZmPHYA1 and ZmPHYA2, thus transmit the light signal downstream. No interaction signal was detected between ZmPHYB1 and the N-terminal of ZmPHYA1 or ZmPHYA2 in this study, indicating that the interaction of ZmPHYB1 and ZmPHYA N-terminal is not necessary for the formation of heterodimer of ZmPHYB1 and

ZmPHYA1. Different degrees of interaction signals were detected between ZmPHYB1 full length, ZmPHYB1 N-terminal and ZmPHYA while no interaction signal was detected between ZmPHYB1 C-terminal and ZmPHYA, indicating that achievement of ZmPHYB1 biological function is mainly through the action of its N-terminal domain. Although the C-terminal domain do not directly participate the biological function, it contains some signal motifs like kinase domain which may amplifying the signals in the biological functioning of N-terminal domain, thereby contributing to ZmPHYB1 function. Different intensities of interaction signals were detected between ZmPHYB2 N-terminal length and full length, C-terminal domain and ZmPHYA1 C-terminal and ZmPHYA2 full-length, but because ZmPHYA2 expression can be self-activated by JG-4-5 and ZmPHYB2 expression can be strongly self-activated by EG-202, so the interaction between ZmPHYB2 N-terminal and full length, ZmPHYA1 C-terminal and its C-terminal domain and among ZmPHYA2 full lengths was considered to be non-existent. As a means of protein interaction study, false positives occur occasionally in yeast two-hybrid protein interaction detection. So in addition to the detection of β-galactosidase relative activity, *In vitro* protein interaction experiments such as co-immunoprecipitation and pull-down test are necessary for reliable evidences in determining the interaction between the protein factors mentioned above. No interaction signal was detected between ZmPHYB1 and ZmPHYB2 in this study. Former research has shown that AtPHYB transmit light signals as homodimer for its biological function (Wang & Deng, 2002), and the interaction signals between AtPHYB and other protein factors could not be detected before certain light condition was given for sometime (Elise *et al*., 2009), so we presumably inferred that the interaction signal between ZmPHYB1 and ZmPHYB2 may be detected after certain light treatment for some time. Previous studies further showed that AtPHYA and AtSPA1, AtPHYA and AtCOP1 interact, and AtSPA1 and AtCOP1 could form a complex, and could depredate AtPHYA though 26S proteasome pathway (Hoecker *et al*., 1999; Saijo *et al*., 2008). Interaction signals were observed between AtCOP1 N-terminal and ZmPHYB1 full-length, AtSPA1 full length and ZmPHYB2 C-terminal, suggesting that the AtSPA1-AtCOP1 complexes may affect ZmPHYB1 and ZmPHYB2 accumulation in different ways. Because no interaction signal was detected among AtSPA1, ZmPHYB1 and ZmPHYB2, AtCOP1, ZmPHYB1 and ZmPHYB2, suggesting AtSPA1 and AtCOP1 may affect the accumulation of ZmPHYB as complexes formed with other factors.

As one of the model organisms for modern molecular biology research, Arabidopsis has drawn wide attention and application. Functional unknown genes need to be transferred into Arabidopsis for their phenotypic impact, and molecular biology detections were followed for further verification. The T1 plants of PjIM19-Hyg-HA-*ZmPhyB1* transform-ants of *PhyB-9* mutant showed shortened hypocotyls, opening cotyledon, and suggested that the mutated *PhyB-9* phenotype has been successfully complemented by *ZmPhyB1*; the T1 plants of PjIM19-Kana-myc-*ZmPhyB2* transform-ants of *PhyB-9* not only showed shortened hypocotyl, cotyledon opening, but also petiole elongation, indicating that *ZmPhyB2* also successfully complemented the *PhyB-9* mutated phenotype. The over-expression transform-ants of PjIM19-Hyg-HA-*ZmPhyB1* into wild-type Col-0 plants showed larger cotyledon area, larger cotyledons angle, indicating reinforced photo-morphogenesis characteristics than that of wild-type Col-0 plants, the over-expression transform-ants of PjIM19-Kana-myc-*ZmPhyB2* into wild-type Col-0 showed the same performance as opened cotyledons, increased cotyledons angle, dramatically shortened hypocotyls, reinforced photo-morphogenesis characteristics compared with their wild-type Col-0 plants. By comparing and analyzing the phenotype of wild-type Col-0 and *PhyB-9* mutant plants. It can be concluded that phenotype changes of the transgenic plants were the results of the integration and expression of *ZmPhyB1* and *ZmPhyB2*. The complementary experiments of PjIM19-Hyg-HA-*ZmPhyB1* transform-ants from *PhyB-9* mutant plants and PjIM19-Kana-myc-*ZmPhyB2* transform-ants from mutant *PhyB-9* were both conducted with 4 days of 50% blue light treatments. The transform-ants showed strong features of photo-morphogenesis; and the over-expression transform-ants of PjIM19-Hyg-HA-*ZmPhyB1* into wild-type Col-0 and transform-ants of PjIM19-Kana-myc-*ZmPhyB2* into wild-type Col-0 was dealt with white light and weak white light for 4 days, respectively. The transform-ants showed different degrees of photo-morphogenesis characteristics. *PhyB-9* is a missense mutation, without endogenous *PhyB* interference, so the changed phenotype can directly reflect the function of transformed exogenous gene, while the interference of endogenous *PhyB* cannot be eliminated in transformation of wild-type *Arabidopsis thaliana* Col-0. Studies have shown that there were many uncertainties, such as the foreign gene copy number, structural integrity, insertion sites and integration pattern, could affect the expression and functions of foreign genes (Wei *et al*., 2001). It is speculated that the endogenous *AtPhyB* affected the insertion site targeting and integration formation of exogenous *ZmPhyB* in over-expression transgenic plants genome, thus affecting *ZmPhyB* expression, leading to different light-inducing conditions in photo-type between over-expression transgenic plants and complementary transgenic plants.

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