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POSITIVE REGULATION OF PHYTOCHROME A ON SHADE AVOIDANCE IN MAIZE

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Abstract

Shade avoidance is elongation, apical dominance and some other responses of plants adapting to deficiency light intensity. The spindling plants are susceptible to lodging, resulting in productivity and quality decline. Many studies have indicated the effect of phytochrome B as the predominant photoreceptor for negative regulation of shade avoidance. The role of phytochrome A on shade avoidance has not yet been clarified in detail. In this study, we overexpressed *ZmPhyA1* gene in maize inbred line 18-599. The T-DNA integration site was found out between the 284557834th and the 284557835th bp of chromosome 1 by genome walking amplification. The plant height, ear height, and leaf length under ear of the transgenic line was significantly higher or longer than the receptor line. Leaf angle under ear was significantly less. These phenotypes were concluded as the positive regulation of the *ZmPhyA1* gene to shade avoidance of the receptor line, because no sequence similar to any functional genes, repeated sequences or transposons were found from the flanking sequences from 46 kb upstream to 314 kb downstream of the T-DNA integration site. Therefore, expression suppression of the *ZmPhyA1* gene could be employed for photo-biotechnological improvement for dense planting variety.

Key words: Maize, Phytochrome, Shade avoidance, Transgenic breeding.

Introduction

In modern agriculture, high-density planting has been practiced successfully in increasing crop productivity. However, if planting density is too large, light intensity is deficient in the dense vegetation, and vegetative growth is excessive because of shade avoidance. The spindling plants are more susceptible to lodging, resulting in productivity and quality decline. Shade avoidance is a set of responses that plants compete with their neighbors for light under dense vegetation and deficiency light intensity. It often includes elongation, altered flowering time, increased apical dominance, and altered partitioning of metabolic products (Casal, 2013; Keuskamp *et al.*, 2010; Smith & Whitelam, 1997).

Plants perceive light fluctuations by phytochromes, which are photosensory proteins with crucial roles in photomorphological responses to light (Quail, 2002; Smith & Whitelam, 1997). Genome sequencing shows that there are five members (PhyA-E) of phytochrome family in eudicot, and three (PhyA-C) in monocot (Basu *et al.*, 2000; Franklin & Quail 2010; Sheehan *et al.*, 2004). Although each member performs both distinct and redundant roles: PhyA and PhyB regulate numerous processes of plant growth and development (Chen & Chory, 2011; Kami *et al.*, 2010). PhyA is the primary photoreceptor mediating deetiolation under far-red (FR) light, whereas PhyB predominantly regulates light responses in red light (Goyal *et al.*, 2013; Muller *et al.*, 2014; Sharrock & Clack, 2002; Wang *et al.*, 2011). Many studies has indicated the effect of PhyB as the predominant photoreceptor for negative regulation of shade avoidance (Ciolfi *et al.*, 2013; Dubois *et al.*, 2010; Keller *et al.*, 2011; Wang *et al.*, 2016). However, the role of PhyA on shade avoidance has not yet been clarified in detail (Ciolfi *et al.*, 2013; Goyal *et al.*, 2013; Muller *et al.*, 2014; Su *et al.*, 2015).

Maize is one of the most important cereal crops in the world. Its phytochrome family contains six members (PhyA1, A2, B1, B2, C1 and C2), since its tetraploidization origin (Sheehan *et al.*, 2004; Swigonova *et al.*, 2004). Transcriptome and mutant analysis showed that the photomorphological responses of maize seedlings to the shade avoidance were predominantly mediated by phytochrome B and phytochrome A (Sheehan *et al.*, 2007; Wang *et al.*, 2016), the same as that in *Arabidopsis* (Chen & Chory, 2011; Kami *et al.*, 2010). In this study, it was attempted to elucidate the role of the endogenous *ZmPhyA1* gene on shade avoidance of maize by constitutive expression in transgenic line, and hope to provide information for photo-biotechnological improvement of shade avoidance in maize breeding (Gururani *et al.*, 2015).

Materials and Methods

Construction of expression vector: The encoding sequence of maize phytochrome A gene *ZmPhyA1* was amplified from engineering plasmid pMD-18T-*ZmPhyA1* (kindly gifted by Professor Jian Ping Yang from Institute of Crop Sciences, Chinese Academy of Agricultural Sciences) by using primers P1 (5'-CGCGGATCCATGTCTT CCTTGAGGCC-3', the underlined bases are *BamHI* restriction site) / P2 (5'-GGACTAGTTCAATGTCC AGCTGCTGA-3', the underlined bases are *SpeI* restriction site), and High-fidelity La Taq DNA polymerase (TaKaRa, Japan). The product was separated by agarose gel electrophoresis, purified by using DNA Purification Kit Ver.2.0 (TaKaRa, Japan), sequenced for confirmation at Sangon (China), and inserted between multiple cloning sites *BamHI* and *SpeI* of expression vector pCAMBIA1390 by using T4 DNA ligase (TaKaRa, Japan), resulting in expression vector pCAMBIA1390-*Ubi*-*ZmPhyA1*-*T*-*nos* (Fig. S1). The *ZmPhyA1* gene was under the control of monocotyledonous constitutive promoter *Ubiqitin* and terminator *T*-*nos*. The selection maker gene was *hpt* for hygromycin resistance. After transformation of *E. coli* strain DH5α, screening on resistant medium, identification by restriction digestion, the ligated product was used for transformation of *Agrobacterium tumefaciens* strain C58.

Maize transformation: As described by Fu *et al.*, (2011), embryonic calli were separated from immature embryos of maize inbred line '18-599', transformed by the *Agrobacterium* strain containing pCAMBIA1390-*Ubi*-*ZmPhyA1*-*T*-*nos*, screened on hygromycin medium, and regenerated for plantlets. Positive plants were identified in each generation of the transformation by PCR amplification of a 311 bp sequence across the transformed *ZmPhyA1* gene and its engineering promoter *Ubi* with primers (5′-TGCTCTAACCTTGAGTACCTAT-3′ / 5′-CCAACAGG CAACCAAAT-3′) until homozygous transgenic line was obtained.

Amplification of T-DNA flanking sequence: The genomic DNA was extracted from the homozygous transgenic line by the CTAB method (Saghai-Maroof *et al.*, 1984). Three nested primers complementary to the backbone sequence within the left border of the T-DNA (LSP1-3, Table S1), three nested primers complementary to the backbone sequence within the right border of the T-DNA (RSP1-3, Table S1), four long arbitrary degenerate primers (LAP1-4, Table S1), and a short arbitrary degenerate primer (SAP, Table S1) were designed, and used to amplify the flanking sequences of the T-DNA integration sites by three rounds of high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) as described by Liu & Chen, (2007). The product of the third round was cloned into pMD^{TM}19-T vector (TaKaRa, Japan), and sequenced at Sangon (China). After removing the backbone sequences of the pMD^{TM}19-T vector and the expression vector, the result was aligned against the maize genome sequences (http://blast.maizegdb.org/), to find out the T-DNA integration site in the maize genome.

In case that the amplified sequences were complementary to the backbone sequence of the expression vector beyond the left or right borders of the T-DNA, but not to the maize genome sequence, six nested primers complementary to the backbone sequence 100-150, 200-250, and 250-350 bp beyond the left and right borders of the T-DNA (LSP4-6 and RSP4-6, respectively) were designed, and used to amplify the flanking sequences of the T-DNA integration sites by other three rounds of hiTAIL-PCR as described as genome walking by Siebert *et al.*, (1995).

Supplementary material - the following online material is available for this article

Table S1. Nested primers and arbitrary degenerate primers for hiTAIL-PCR amplification.

Primers	Sequences
LSP1	5'-AAGTACTCGCCGATAGTGGAAACCGAC-3'
LSP2	5'-ACGATGGACTCCAGTCCGGCCTCGTCCGAGGGCAAAGAAATAGAGTAGA-3'
LSP3	5'-GGGTTCCTATAGGGTTTCGCTCATGTG-3'
LSP4	5'-GCCGTCTGAAACGAGTACAATGGCTAC-3'
LSP5	5'-ACGATGGACTCCAGTCCGGCCCTAGAGCGCCTCCCATCGTACAACTAAC-3'
LSP6	5'-CCGAGGCAGCTATGATACAATATGCGGT-3'
RSP1	5'-ATTACAGGTGACCAGCTCGAATTTCCC-3'
RSP2	5'-ACGATGGACTCCAGTCCGGCCTGGGTTTTTATGATTAGAGTCCCGCAA-3'
RSP3	5'-GCGGTGTCATCTATGTTACTAGATCGGGA-3'
RSp4	5'-ATAGTGCAGTCGGCTTCTGACGTTCAGT-3'
RSP5	5'-ACGATGGACTCCAGTCCGGCCGGCGTTTTCTTGTCGCGTGTTTTAGT-3'
RSP6	5'-CGGAGACATTACGCCATGAACAAGAG-3'
LAP1	5'-ACGATGGACTCCAGAGCGGCCGCVNVNNNGGAA-3'
LAP2	5'-ACGATGGACTCCAGAGCGGCCGCBNBNNNGGTT-3'
LAP3	5'-ACGATGGACTCCAGAGCGGCCGCVVNVNNNCCAA-3'
LAP4	5'-ACGATGGACTCCAGAGCGGCCGCBDNBNNNCGGT-3'
SAP	5'-ACGATGGACTCCAGAG-3'

Note: V=G/A/C, B=G/T/C, D=G/A/T, N=A/T/C/G.

Quantitative real-time PCR: The seeds of the T_8 line and the receptor line 18-599 were surface sterilized with 1% NaClO, washed with sterile water, and germinated in petri plates at 28˚C. The seedlings were transferred onto plastic foam boards for aquaculture in Hoagland complete medium at 28˚C and 60% relative humidity with a 14 h light (30˚C) and 10 h dark (28˚C) period (Hoagland & Arnon 1950). Half seedlings of each line was incubated under 12 h light of 400 μmol·s^{-1}·m^{-2} and 12 h dark, and the other under 24 h dark, with three replicates, respectively. At the third day, the leaves were sampled, and used for total RNA isolation with RNAiso Plus (TaKaRa, Japan). After detection of concentration and integrity, the total RNA samples were reversely transcribed into cDNA with PrimeScript™ Ⅱ 1st Strand cDNA synthesis Kit (TaKaRa, Japan), and used as templates for quantitative real-time PCR (qRT-PCR).

A pair of specific primers (5'-GGTGATCGACAGG ATGCTCCTT-3' / 5'-GACGCCATCTGCATTCAC TTTT-3') were designed by the Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) to amplify a 217 bp fragment of the *ZmPhyA1* gene. As described by Lovdal & Lillo (2009), the *ZmGAPDH* gene was used as internal control. Each reaction contained 10 μL of SYBR® Premix Ex Taq^{TM} II (Tli RNaseH Plus) (Takara, Japan), 2 μL cDNA, and 0.8 μL of 20 μmol L^{-1} specific primers in a final volume of 20 μL. Three technical replicates were performed for each of the three biological replicates. The thermal cycles were conducted in CFX96^{TM} Real-Time System (Bio-Rad, USA) as follows: 95˚C for 30 s; 36 cycles of 95˚C for 5 s, 58.7˚C for 30 s; 95˚C for 10 s. Then, the temperature was increased by 0.5˚C s^{-1} to 95˚C, so that a melting curve could be calculated and used to differentiate between specific and non-specific

amplicons. The $2^{-\Delta\Delta C}$T method of the CFX Manger™ software version 2.0 (Bio-Rad, USA) was used to normalize the differential gene expression among the multiple internal controls and the target genes (Livak & Schmittgen, 2001). The statistical significance among the lines and illumination recipes was tested by analysis of variance with IBM-SPSS software (http://www.ibm.com/analytics/ us/en/ technology/spss/).

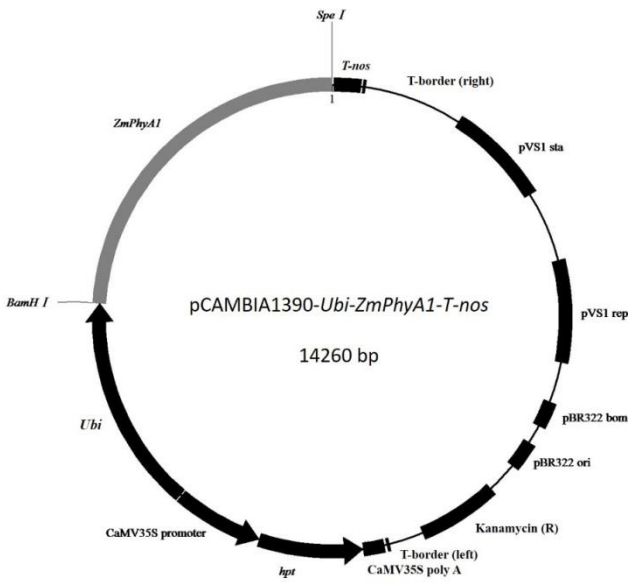

Fig. S1. Constitutive expression vector of *ZmPhyA1* gene for monocot plant. The *ZmPhyA1* gene is under the control of constitutive promoter *Ubi* and terminator *T-nos*. The selection maker gene is *hpt* for hygromycin resistance.

Phenotyping of transgenic line: After preliminary observation for the plant type in a pot experiment in greenhouse, the T_8 line and the receptor line (18-599) were planted under densities of 52500, 67500 and 75000 plant/hm^2 with a random block design of three replicates at Ya'an, Sichuan Province, where is known for rainy and cloudy weather. Silking stage (d), plant height (cm), ear height (cm), leaf length under ear (cm), leaf width under ear (cm) and leaf angle under ear (º) were investigated for every plant, and calculated for means of each plot. Analysis of variance was conducted to show the significance among the lines and densities by using IBM-SPSS software (http://www.ibm.com/analytics/us/en/ technology/spss/).

Results

Homozygous transgenic line: After screening on hygromycin medium, 53 T_0 plantlets were regenerated from the positive calli. Five of them were identified as positive by the PCR amplification, and self-pollinated (Fig. 1). Because of the infection of banded leaf and sheath blight in the rainy weather, only one plant produced T_1 seeds. This transgenic line was continuously self-pollinated and identified by the PCR amplification till T_8 generation, when all the plants were homozygous (Fig. 1).

T-DNA integration site: While the hiTAIL-PCR at the right border of the T-DNA failed to amplify a specific fragment, a specific fragment was amplified by the hiTAIL-PCR with the nested primers LSP1-3 and the arbitrary degenerate primers LAP1-4 and SAP at the left border of the T-DNA. However, the result of sequencing and alignment showed that this fragment was perfectly complementary to the backbone sequence of the expression vector beyond the left border of the T-DNA, but not to the maize genome sequence. Therefore, genome walking was conducted by other three rounds of hiTAIL-PCR with the nested primers LSP4-6 and the arbitrary degenerate primers LAP1-4 and SAP. A specific fragment was separated from the third round of the hiTAIL-PCR amplification (Fig. 2). The sequencing and alignment showed that the sequences of the left and right borders remained intact, and their flanking sequence was 97.7% similar to the sequence from the 284559005th to the 284557834th bp of the maize chromosome 1 (Fig. 3). Therefore, the integration site of the T-DNA was concluded to be between the 284557834th and the 284557835th bp of the maize chromosome 1. No sequences similar to any functional genes, repeated sequences or transposons were found from the flanking sequences from 46 kb upstream to 314 kb downstream of the T-DNA integration.

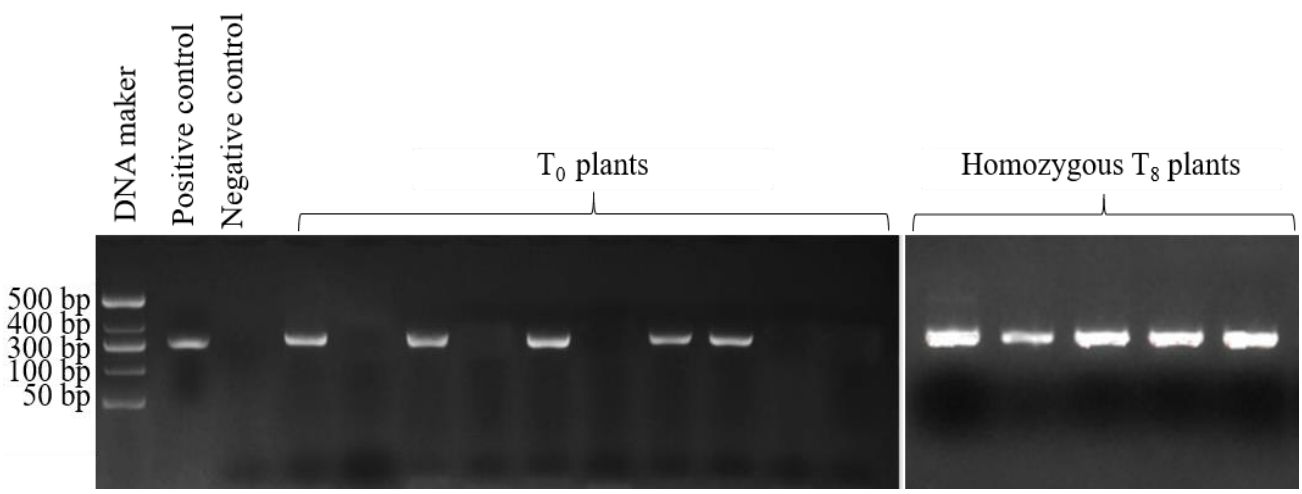

Fig. 1. PCR amplified products of the 311 bp sequence across the transformed *ZmPhyA1* gene and its engineering promoter *Ubi* from the T_0 and T_8 plants, and separated by 1.5% agarose gel electrophoresis. The positive control was the expression vector pCAMBIA1390-*Ubi-ZmPhyA1-T-nos*. The negative control was the receptor inbred line '18-599'.

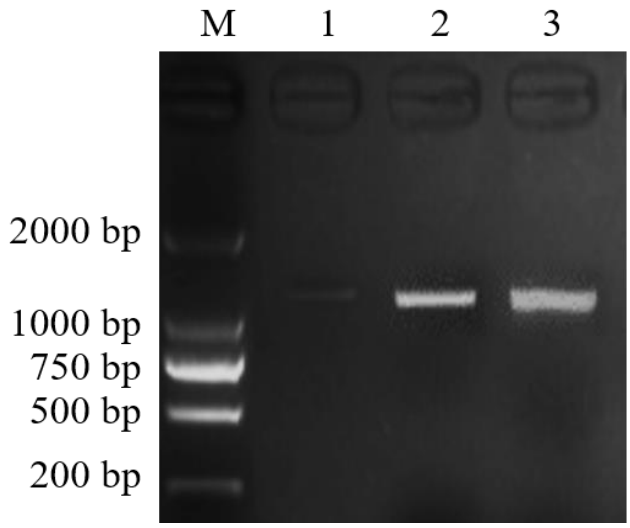

Fig. 2. Fragments amplified from the T_8 line by hiTAIL-PCR with the nested primers LSP4-6 and the arbitrary degenerate primers LAP1-4 and SAP. M: DNA maker; 1: the fragment of the first round amplification with the nested primer LAP1 and the arbitrary degenerate primers; 2: the fragment of the second round amplification with the nested primer LAP2 and the arbitrary degenerate primers; 3: the fragment of the third round amplification with the nested primer LAP3 and the arbitrary degenerate primers.

Table 1. Plant type traits of T_8 and receptor lines under different planting densities.

Line	Density (plant/hm^2)	Plant height (cm)	Ear height (cm)	Leaf length (cm)	Leaf width (cm)	Leaf angle (^{o})
T_8 line	75000	177.7**	65.7**	80.7**	6.4	22**
	67500	188.3**	70.4**	84.3**	6.8	23**
	52500	173.8**	60.2**	79.8**	6.3*	27**
18-599	75000	129.2	58.7	71.1	6.8	31
	67500	134.4	60.2	76.1	7.0	33
	52500	119.7	51.0	70.9	7.0	35

Note: * and ** represent significance and great significance between the T_8 and the receptor lines on possibility levels of 0.05 and 0.01

Expression of transformed *ZmPhyA1* gene: Analysis of variance showed that the relative expression levels of the *ZmPhyA1* gene between the T_8 line and the receptor line 18-599 was significant under dark but not significant under the light (Fig. 4). This result confirmed the overexpression of the transformed *ZmPhyA1* gene in the transgenic line. However, the incomprehensible significance will be discussed later.

Phenotype of transgenic line: The phenotypic difference of plant height and leaf angle was apparently observed between the T_8 line and the receptor line (18-599) at eight-leaf stage in the pot experiment (Fig. 5A), and jointing and silking stages in the field evaluation (Fig. 5B, C). The variance analysis indicated that plant height, ear height, and leaf length under ear of the T_8 line transformed by *ZmPhyA1* was significantly higher or longer than the receptor line (18-599) under each of the three densities (Table 1). Leaf angle under ear was significantly less. The difference of leaf width between the T_8 and the receptor lines was significant only under the density of 52500 plant/hm^2. The differences of these traits were not significant among the three densities.

Discussion

The relative expression level of the *ZmPhyA1* gene in the T_8 line was significantly higher than the receptor line under dark (Fig. 4). This can be explained by the normalization between the phytochrome and its encoding transcripts (Sharrock & Clack, 2002). It is well known that the light-labile PhyA is synthesized in its inactive Pr conformation, converts to its active Pfr conformation upon light irradiation, and mediates signal transduction (Goyal *et al.,* 2013; Muller *et al.,* 2014; Sharrock & Clack, 2002; Wang *et al.,* 2011). In addition, it may function as light-regulated serine/threonine kinases, and can phosphorylate several substrates, including itself *In vitro*. Furthermore, PhyA is also a phosphoprotein, and can be dephosphorylated by some protein phosphatases (Li *et al.,* 2011). The photoactivated PhyA regulates the expression of light-responsive genes together with transcript factors such as COP1 (Constitutive photomorphogenesis 1), HY5 (Elongated hypocotyl 5) and HFR1 (Long hypocotyl in far-red) (Ciolfi *et al.,* 2013; Pancin *et al.,* 2016; Sawers *et al.,* 2005; Viczian *et al.,* 2012).

In some studies, overexpression of exogenous or endogenous *PhyA* genes in transgenic tobacco, rice, potato, and tomato exhibited dwarfism phenotypes, and even improved productivity (Boylan & Quail, 1989; Ganesan *et al.,* 2012; Garg *et al.,* 2006; Halliday *et al.,* 1997; Heyer *et al.,* 1995; Jordan *et al.,* 1995; Kong *et al.,* 2004; Pierik *et al.,* 2004; Robson *et al.,* 1996). In some other reports, overexpression of *PhyA* gene failed to result in any significant phenotypic improvement in transgenic rice and wheat (Clough *et al.,* 1995; Shlumukov *et al.,* 2001). This was explained by the interaction of PhyA with other proteins such as transcript factors COP1 (Constitutive photomorphogenesis 1), HY5 (Elongated hypocotyl 5) and HFR1 (Long hypocotyl in far-red) in the signaling pathways (Ciolfi *et al.,* 2013; Pancin *et al.,* 2016; Sawers *et al.,* 2005; Viczian *et al.,* 2012). However, the suppression of endogenous *PhyA* gene was declared to have promotion effect on tuberization frequency in transgenic potato (Yanovsky *et al.,* 2000). The shade avoidance of an *Arabidopsis* mutant was strongly suppressed by ectopic expression of oat *PhyA* gene with a serine to alanine substitution at position 599 (i.e. S599A-*PhyA*). This mutation is key to the phosphorylation of phytochrome A, and its interaction with signal transducers (Ryu *et al.,* 2005). Transgenic lines of sweet potato, cassava, and turfgrass ectopically expressing the mutant S599A-*PhyA* gene were also improved for their shade avoidance and agronomic traits (Ganesan *et al.,* 2012; Kim *et al.,* 2004, 2009). These results were explained by the positive regulation of phytochrome A on shade avoidance (Ciolfi *et al.,* 2013). A better elucidation of phytochrome signaling mechanisms is helpful for improving shade avoidance of crops (Sawers *et al.,* 2005).

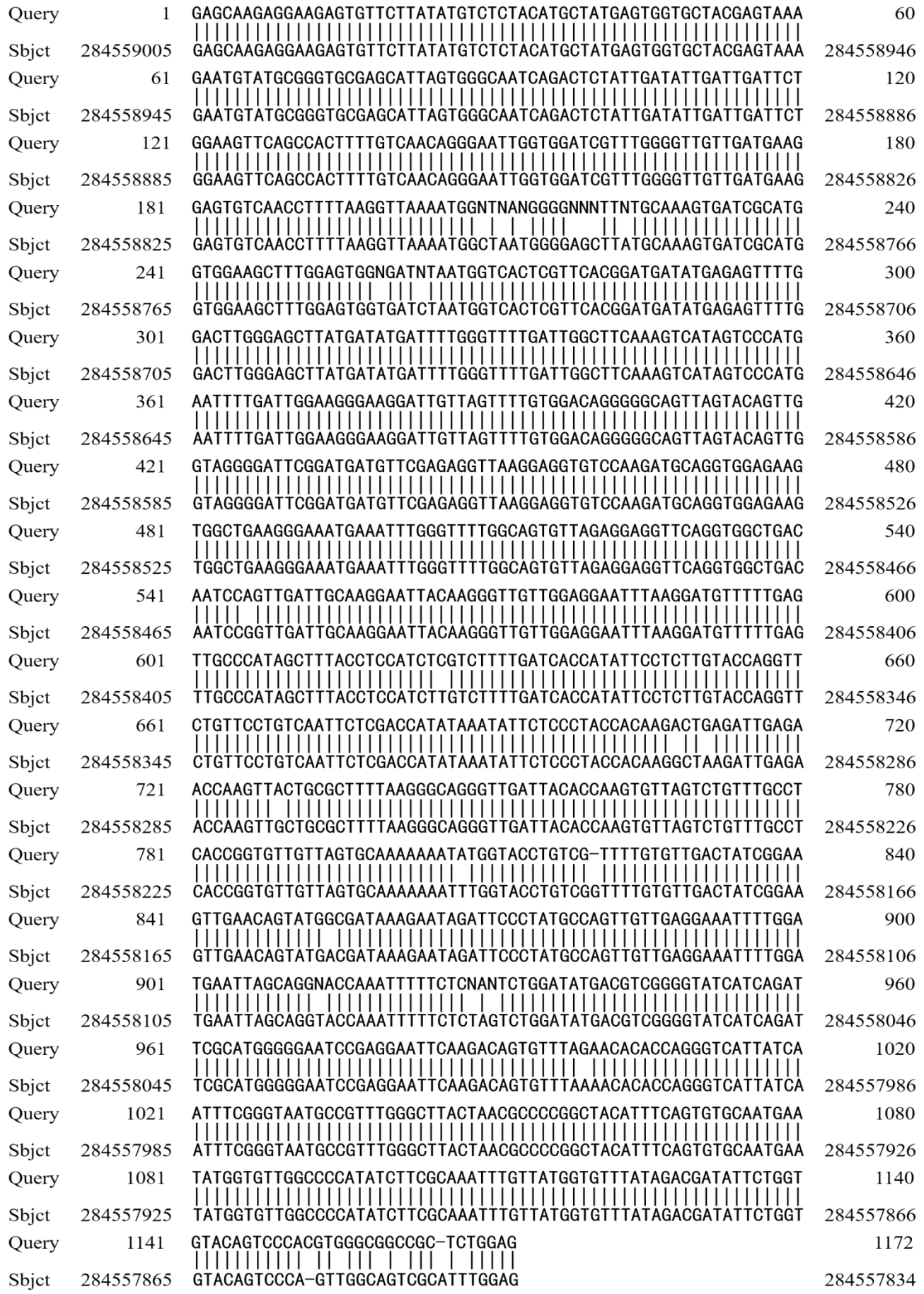

Fig. 3. Alignment between the flanking sequence beyond the left border of the T-DNA and the sequence from 284559005 to 284557834 bp of maize chromosome 1. The similarity between these two sequences was 97.7%.

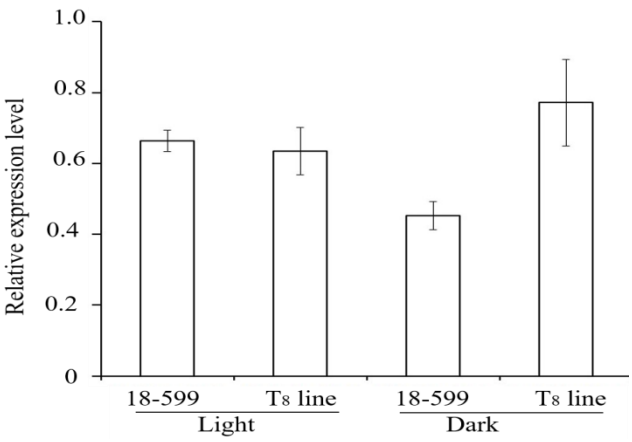

Fig. 4. Relative expression levels of the transformed *ZmPhyA1* gene between the T_8 line and the receptor line 18-599 under light and dark.

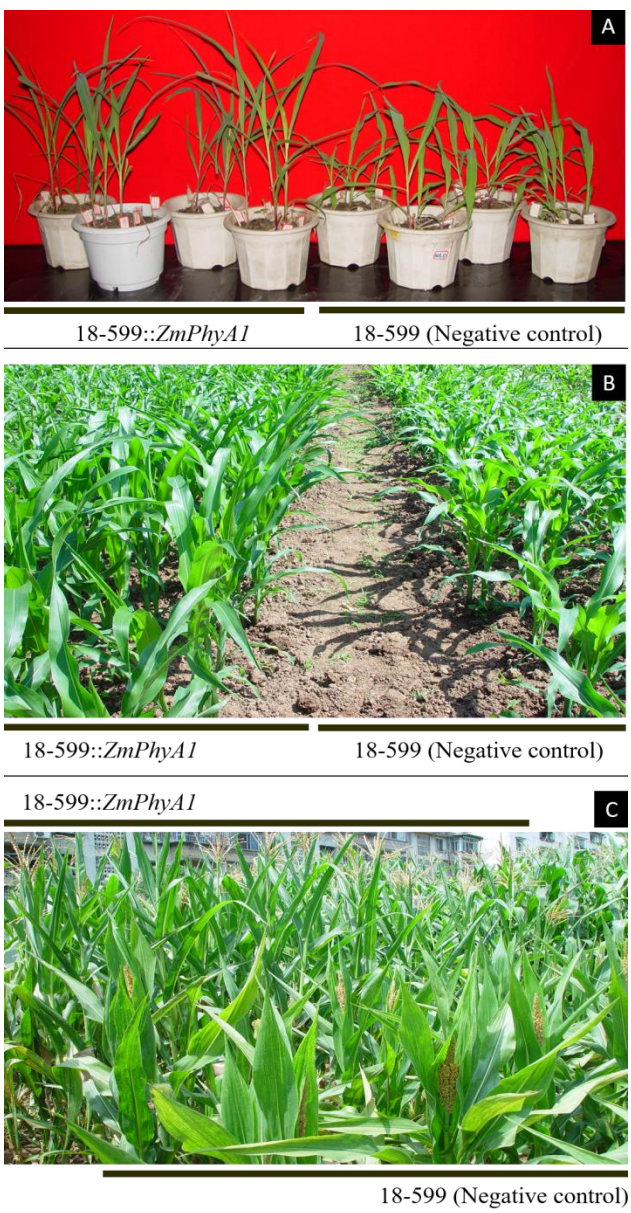

Fig. 5. Phenotype of the T_8 and the receptor lines. The plants of the T_8 line (18-599::*ZmPhyA1*) were apparently higher than the receptor line (18-599) at eight-leaf stage (A), jointing stage (B), and silking stage (C).

In some transgenic events, the expression of the endogenous genes was frequently influenced by T-DNA integration at their flanking sites (Costa *et al.,* 2014). So that T-DNA integration has emerged as an effective technique to induce mutagenesis for mapping and functional identification of unknown genes (Jeon *et al.*, 2000; Larmande *et al.,* 2008; Ruiz-Rojas *et al.,* 2010; Stephan *et al.,* 2016). In the present study, however, no sequence similar to any functional genes, repeated sequences or transposons was found from the flanking sequences from 46 kb upstream to 314 kb downstream of the T-DNA integration site.

Therefore, the higher plant height and ear height, longer leaf, and less leaf angle of the T_8 line in the present study can be concluded as the positive regulation of the overexpression of the transformed *ZmPhyA1* gene to shade avoidance of the receptor line (Fig. 5A, B, C, Table 1). Expression suppression of the *ZmPHyA1* gene, by CRISPR/Cas, RNA interference and other strategies (Kamthan *et al.,* 2015; Khatodia *et al.,* 2016; Saurabh *et al.,* 2014; Zhang *et al.,* 2016), is suggested for photo-biotechnological improvement for dense planting variety.

Acknowledgments

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