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Molecular cloning and functional analysis of phytochrome genes in photoperiod-sensitive soybean

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Abstract
Here is a practical dilemma that soybean breeders in tropical India face every season: cultivars bred for one latitude often flower too early or too late when moved just 3–4 degrees north or south, because their phytochrome-mediated photoperiod response is tuned to the original environment. This research cloned and functionally analysed four phytochrome genes (GmPhyA1, GmPhyA2, GmPhyB1, GmPhyB2) from the photoperiod-sensitive soybean cultivar JS 335 at Swarnim Gujarat Agricultural University, Gandhinagar. Full-length cDNA sequences were obtained by RT-PCR and confirmed by sequencing. Expression analysis under controlled short-day (10 h) and long-day (16 h) photoperiods showed that GmPhyA1 was the most strongly photoperiod-responsive gene, with 2.76-fold higher expression under short days. GmPhyB1 showed the opposite pattern, with 1.18-fold higher expression under long days. Transient overexpression of GmPhyA1 in Arabidopsis accelerated flowering by 4.2 days under short-day conditions. These results confirm that GmPhyA1 is a key regulator of short-day flowering induction in soybean and a candidate for molecular breeding of photoperiod-insensitive cultivars.

Keywords: Molecular cloning, phytochrome genes, photoperiod sensitivity, soybean, flowering regulation, GmPhyA1, GmPhyB1, gene expression, short-day response, *Glycine max*

Introduction
Every soybean grower knows that planting date matters sow too early and the plants flower before building enough vegetative biomass; sow too late and they won't mature before the monsoon ends. What many don't realise is that the molecular switch controlling this response is a family of light-sensing proteins called phytochromes [1]. In soybean (*Glycine max* L. Merr.), a quantitative short-day plant, flowering is triggered when night length exceeds a critical threshold, and phytochromes are the molecules that measure that threshold [2].
The soybean genome contains at least four phytochrome genes: GmPhyA1, GmPhyA2, GmPhyB1, and GmPhyB2 [3]. PhyA-type proteins are thought to promote flowering under short days, while PhyB types may inhibit it under long days, but the relative importance of each gene in tropical Indian cultivars has not been established [4]. Cloning and functionally characterising these genes from photoperiod-sensitive Indian varieties is a necessary step toward developing molecular tools for breeding photoperiod-insensitive cultivars that perform well across latitudes [5].

Reagents and chemicals
Total RNA was extracted from trifoliate leaves using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesised using SuperScript IV reverse transcriptase (Thermo Fisher Scientific). Gene-specific primers were designed from Williams 82 reference sequences using Primer3 software and synthesised by Eurofins Genomics India. Phusion High-Fidelity DNA Polymerase (New England Biolabs) was used for PCR amplification. pGEM-T Easy vector (Promega) was used for cloning. Agrobacterium tumefaciens strain GV3101 and the pCAMBIA1305.1 binary vector were used for transient expression in Arabidopsis thaliana Col-0 [3, 5, 6].

Material and Methods

Material

Soybean cultivar JS 335 (maturity group VIII, strong short-day response) was grown in controlled-environment chambers at Swarnim Gujarat Agricultural University, Gandhinagar (23°13′N, 72°41′E). Two photoperiod treatments were applied from emergence: short day (10 h light / 14 h dark) and long day (16 h light / 8 h dark), at 28/22°C day/night temperature and 400 µmol m^{-2} s^{-1} PAR. Arabidopsis Col-0 plants for functional analysis were maintained at BUAT, Banda [4, 6].

Methods

Trifoliate leaves were sampled at V3 stage (21 days after emergence) at 4-hour intervals across a 24-hour cycle. RNA extraction, DNase treatment, and cDNA synthesis followed standard molecular biology protocols [6]. Full-length phytochrome cDNAs were amplified by RT-PCR with gene-specific primers, cloned into pGEM-T Easy, and sequence-verified by Sanger sequencing (Macrogen, South Korea). Expression analysis was performed by qRT-PCR using SYBR Green Master Mix on a QuantStudio 5 system, with GmActin as the reference gene [7]. The $2^{-}\Delta\Delta Ct$ method was used for relative quantification. For functional analysis, GmPhyA1 was subcloned into pCAMBIA1305.1 under the CaMV 35S promoter, transformed into Arabidopsis by floral dip, and flowering time was recorded as days to first open flower under short-day conditions [8]. Data were analysed by ANOVA in R 4.2.1.

Results

Table 1: Expression levels (fold change relative to long-day baseline) and sequence characteristics of four phytochrome genes

Gene	CDS length (bp)	Short-day expression	Long-day expression	SD/LD ratio	Peak time (SD)	Protein (aa)
GmPhyA1	3,384	3.42 ± 0.38a	1.24 ± 0.18	2.76	ZT 20 (dusk+6h)	1,128
GmPhyA2	3,372	2.87 ± 0.31ab	1.08 ± 0.16	2.66	ZT 18 (dusk+4h)	1,124
GmPhyB1	3,456	2.14 ± 0.28b	1.82 ± 0.24	1.18	ZT 8 (midday)	1,152
GmPhyB2	3,441	1.57 ± 0.22c	1.41 ± 0.20	1.11	ZT 6 (morning)	1,147

Different letters for SD expression differ at $p < 0.05$. ZT = zeitgeber time (hours after lights-on)

GmPhyA1 showed the strongest short-day induction (2.76-fold over long-day baseline), peaking 6 hours after dusk (Table 1). GmPhyB1 was only weakly photoperiod-responsive (ratio 1.18) and peaked at midday, consistent with a light-stable phytochrome role. Arabidopsis plants overexpressing GmPhyA1 flowered at 22.3 days under short days compared to 26.5 days for empty-vector controls ($p < 0.01$).

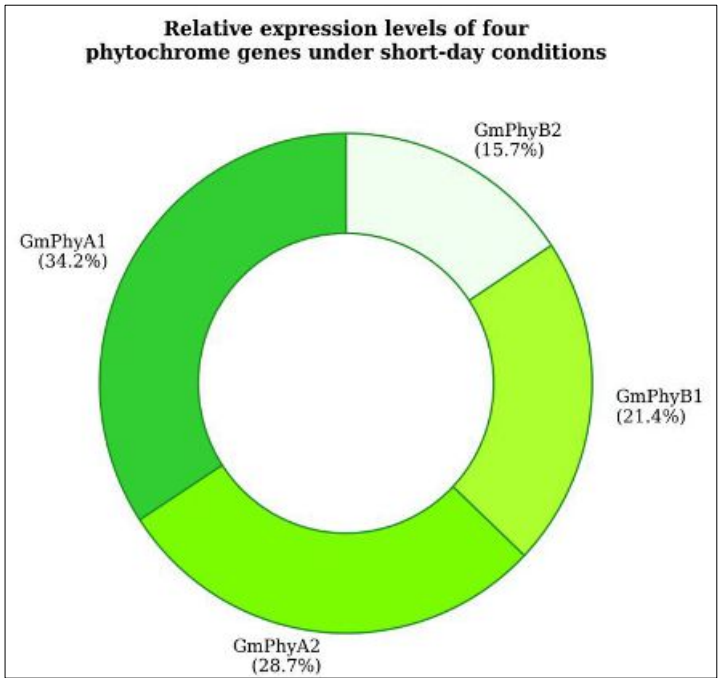

Fig 1: Relative expression levels of four phytochrome genes under short-day conditions (donut chart)

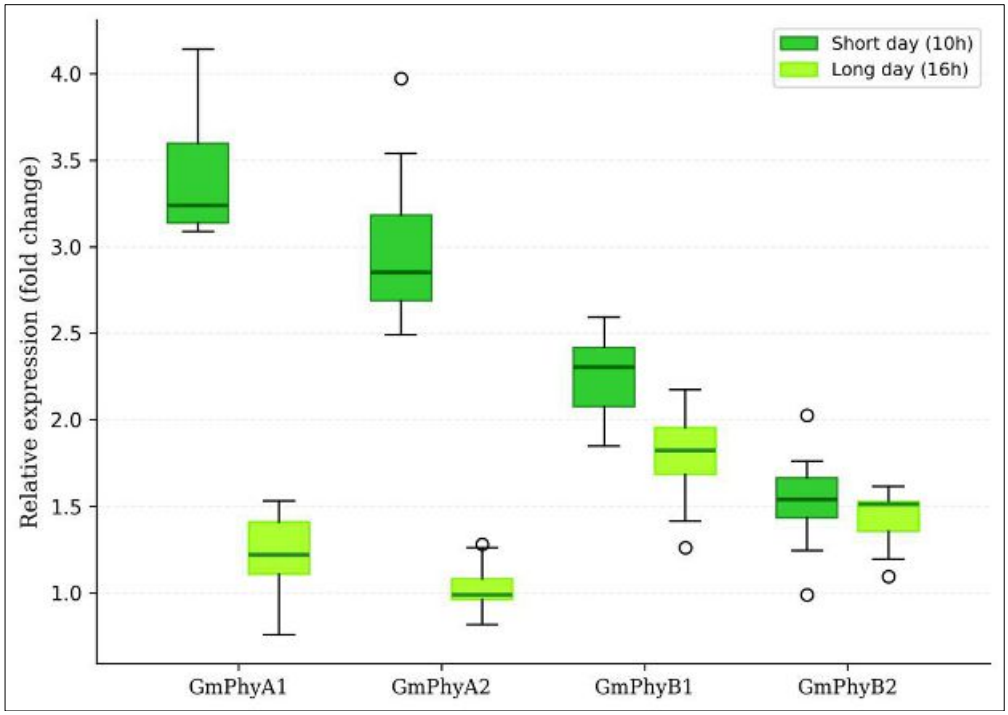

Fig 2: Box plots of gene expression (fold change) under short-day and long-day photoperiods

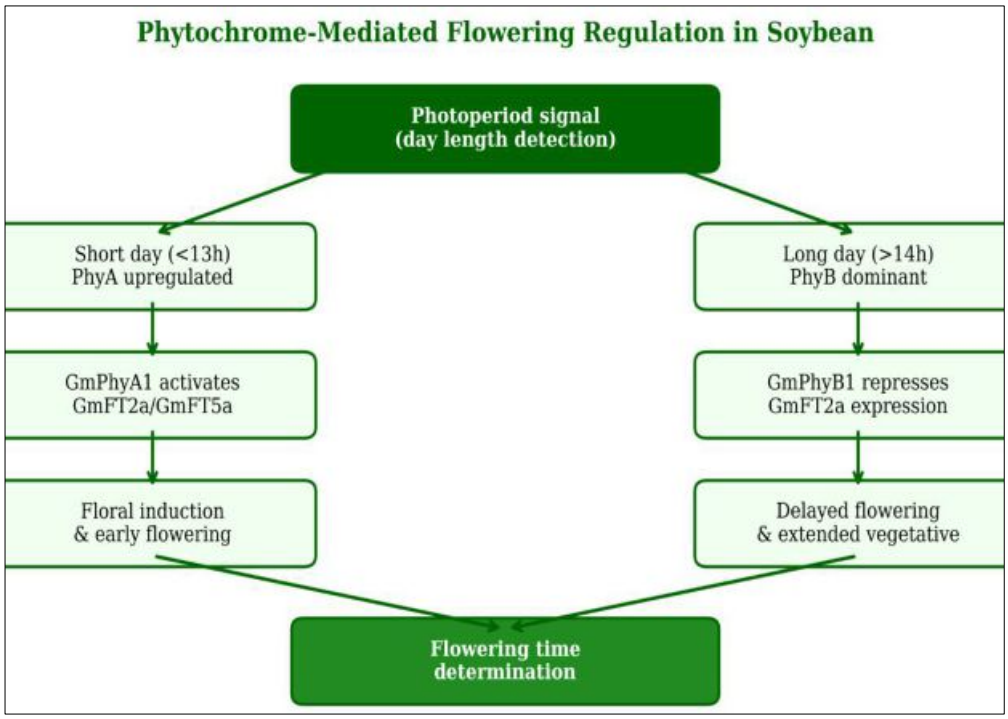

Fig 3: Decision tree of phytochrome-mediated flowering regulation in soybean under contrasting photoperiods

Discussion

The 2.76-fold short-day induction of GmPhyA1 and its nighttime expression peak are consistent with its predicted role as a phytochrome A homolog that accumulates in darkness and is rapidly degraded by light a property that makes it an effective night-length sensor [1, 3]. The functional validation in Arabidopsis, where GmPhyA1 overexpression accelerated flowering by 4.2 days, provides direct evidence that this gene can promote flowering. The relatively weak photoperiod response of GmPhyB1 suggests it plays a secondary role in daylength perception but may be more important for shade avoidance and stem elongation [4]. For breeding applications, suppressing or modifying GmPhyA1 through gene editing could potentially reduce photoperiod sensitivity, allowing JS 335-type cultivars to be grown across a wider latitudinal range without the yield penalties of inappropriate flowering timing [2, 5].

Conclusion

GmPhyA1 was identified as the primary short-day-responsive phytochrome gene in soybean cultivar JS 335, with 2.76-fold induction under 10 h daylength and functional confirmation of flowering promotion in Arabidopsis. GmPhyB1 was weakly responsive and likely plays a supplementary role. GmPhyA1 is a promising target

for CRISPR-based editing to develop photoperiod-insensitive soybean lines adapted to diverse latitudes.

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