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An emerging concept of diurnal variation in expression of UDP-Glucose Pyrophosphorylase gene (*StUGPase2*), in potato (*Solanum tuberosum* L.)

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Abstract

Diurnal regulation of expression of the gene encoding the dominant isoform of UDP-Glucose Pyrophosphorylase, *StUGPase2*, was studied in potato leaves at different developmental stages (pre-tuberization, tuber initiation, and tuber bulking). Across the developmental stages, the *StUGPase2* steady-state mRNA level increased gradually from morning (6 AM) to noon (12 PM), which was followed by a transient drop at 2 PM. The mRNA level increased thereafter, and the highest transcript abundance was recorded at 8:00 PM. A sharp decline in *StUGPase2* transcript abundance at 2 PM in all the stages studied is believed to be associated with the previous reports of diurnal behaviour of sucrose concentration and light responsiveness of sucrose synthase enzyme. Moreover, in all three stages, a steady state increase of *StUGPase2* transcript despite a consistent decrease in irradiance after 12 PM, suggests the presence of more complex transcriptional regulation. Comparatively higher *StUGPase2* transcript abundance at the tuberization initiation stage as compared to the pre-and-post-tuberization stages indicates the prominent role of the gene during tuber development. Bioinformatics analysis of the *St*UGPase2 promoter region predicted the presence of various light-responsive *cis*-regulatory nucleotide sequences, which are considered to be associated with the light-mediated diurnal change in *StUGPase2* gene expression. This research will aid in understanding the pattern of UGPase gene regulation, which is ultimately connected to potato tuber yield and bulking attributes.

Key words: Diurnal regulation, UDP-Glucose pyrophosphorylase, *StUGPase2*, gene expression, potato, promoter

Introduction

Diurnal plant metabolic shifts have indeed been researched for a long time (Geiger and Servaites, 1994; Kruger, 1997; Fernie and Willmitzer, 2004), along with the genes responsible for sucrose and starch metabolism, nutrient influx and absorption, and redox control, which are more often responsive to such variations (Blasing *et al.,* 2005). As the plant develops, the distribution of its carbon resources changes considerably, requiring extremely dynamic fine-tuning during the diurnal cycle (Ruan, 2014; Stitt *et al.,* 2012; Wiese *et al.,* 2007). Thus far, leaves are the preferred tissue of investigation to examine carbohydrate metabolism in a diurnal environment. In leaves, starch is produced during the day and broken down at night to fuel crucial biological functions. Triose phosphates are transported from the chloroplast to the cytosolic region of mesophyll cells, where they begin the process of sucrose biosynthesis in the leaf/source tissues (Winter and Huber, 2000). Triose phosphate is transformed into Fructose-6-phosphate (Fru-6-P) in the cytosol by the action of fructose-1,6-bisphosphatase (FBPase), and sucrose is subsequently produced from Fru-6-P and UDP-glucose by the enzymes sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP). UDP-glucose is transformed into starch by the enzymes ADP-glucose pyrophosphorylase (AGPase), Starch Synthase (SSy), and 1,4-α-Glucan Branching Enzyme along the starch and sucrose metabolic pathway (GBE1). UDP-glucose pyrophosphorylase (UGPase) which catalyzes the reversible synthesis of UDP-Glu and pyrophosphate (PPi) from Glc-1-P and UTP, plays a crucial role in carbohydrate metabolism. UGPase is predominantly engaged in the sucrose biosynthesis pathway in young and mature leaves, supplying UDP-Glu for sucrose phosphate synthetase (SPS). However, in other tissues, such as immature apical leaves that partially rely on supplied carbon, UGPase may participate in sucrose breakdown by utilizing UDP-Glu generated by sucrose synthase (SuSy) as its substrate (Rees, 1992; Winter and Huber, 2000; Sergeeva *et al.,* 2022).

Circadian rhythm governs the transcriptional activity of numerous starch genes' and the amount of sugar in the cellular system (Lu *et al.,* 2005; Smith *et al.,* 2004). Tenorio *et al.* (2003) found that the Myb-related CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and Late Elongated Hypocotyl (LHY) modulate regulation of the Granule-Bound Starch Synthase 1(GBSS1) gene expression in Arabidopsis leaves, whereas two interacting proteins from the MYC and EREBP families were found to govern GBSS activity in rice endosperm (Zhu *et al.,* 2003). Putative regulators of starch biosynthetic pathways have been discovered through co-expression analysis in rice, and successive functional studies revealed that the APETALA2 (AP2)/EREBP type TF acts as a negative modulator of the genes associated with starch biogenesis and are a crucial factor in determining the amount and

composition of starch (Fu *et al.,* 2010). SRF1, a Dof protein, was shown to indirectly promote starch production in sweet potatoes (Tanaka *et al.,* 2009). FLO2 (FLOURY ENDOSPERM2) in rice seeds was also discovered to have an impact on the transcription of the starch genes (She *et al.,* 2010). These instances highlight the importance of transcriptional control for potato starch metabolism, which is still mostly unknown. In potato, the expression of SuSy and AGPase follows the diurnal rhythm in leaves and tubers (Geigenberger and Stitt, 2000). Although UGPase plays a crucial role at the crossroads of sucrose biosynthesis and degradation, it has attracted less attention than the other sucrose metabolism enzymes.

Different numbers of UGPase gene homologs and isoforms have been identified in different plant species. Rice (*Oryza sativa*) possesses two UGPase homologs (Huang and Mu, 2005) while *Arabidopsis thaliana* is known to have three different isoforms of UGPase with different enzymatic kinetics (Meng *et al.,* 2008, Meng *et al.,* 2009). Recently, through genome-wide analysis of starch metabolism genes in potato (*Solanum tuberosum*) two UGPase homologs (*StUGPase1* and *StUGPase2*) have been predicted (Van Harsselaar *et al.,* 2017). A study of the transcriptional behaviour of both homologs across various tuber developmental stages indicated *StUGPase2* as a major homolog involved in the tuberization process (data not given). To the finest of our knowledge, no research has been done so far to examine *StUGPase2's* transcriptional or post-transcriptional expression kinetics in a diurnal setting at different developmental phases of potato plants. Therefore, the present study was designed to study and generate a better understanding of the light-mediated regulation of *StUGPase2* expression during various developmental stages.

Materials and methods

Plant material: Potato cultivar Kufri-Chipsona 1 (KC1) obtained from ICAR-Central Potato Research Institute, Shimla was used in the present study. Plant tissue samples were collected from various developmental stages (Supplementary Fig.1) *viz.* at pre-tuberization (21 days post-germination, S1), tuber initiation (45 days post-germination, S2), and post-tuberization stage (60 days post-germination, S3). Plantlets were grown in pots under natural irradiance in ICAR-IARI, New Delhi with average 11-h photoperiod and day/night temperature of 17-18/28-29ºC [during pre-tuberization (S1 stage)]; 9.5-h photoperiod, and day/night temperature of 13-14/22-24ºC [during tuber initiation (S2 stage)];, and 10.5 h photoperiod with the temperature of 4-5/14-15ºC [during tuber bulking (S3)]. Young fully expanded leaves were collected in triplicate from 6 AM to 8 PM at intervals of two hours (6 AM, 8 AM, 10 AM, 12 PM, 2 PM, 4 PM, 6 PM, and 8 PM), frozen in liquid N_2, and stored at -80°C until used. The observed irradiance has been summarized in Supplementary Table 1 for all three developmental stages. The irradiance increased from 116 µmol (PAR) $m^{-2}s^{-1}$ at 8 AM to a maximum (781 µmol $m^{-2}s^{-1}$) at 12 PM while it decreased to 11 µmol $m^{-2}s^{-1}$ at 6 PM for S1. Similarly, at S2 and S3 the irradiance at 8 AM was 87 µmol $m^{-2}s^{-1}$ and 136 µmol $m^{-2}s^{-1}$ respectively, which reached up to 383 µmol $m^{-2}s^{-1}$ and 1076 µmol $m^{-2}s^{-1}$ at 12 PM, respectively. At 6 PM the irradiance at S2 was recorded 1 µmol $m^{-2}s^{-1}$ while 11 µmol $m^{-2}s^{-1}$ was recorded for S3. In all the developmental stages the irradiance at 6 AM and 8 PM ranged from 0-2 µmol $m^{-2}s^{-1}$.

RNA isolation and cDNA synthesis: Total RNA was isolated from all the plant samples (S1, S2, and S3 stages) of *S. tuberosum* using PureLink™ RNA Mini Kit (Invitrogen™) following the manufacturer protocol. Isolated RNA samples were treated with DNAseI to remove any contaminating genomic DNA using Ambion TURBO DNA-free™ Kit (Ambion) and then quantified using Nanodrop 1000 spectrophotometer (Thermo Scientific). Approximately 800 ng of total RNA with an absorbance ratio ranging from 1.9 to 2.1 at the 260/280 wavelength were used for cDNA synthesis using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Inc) following the manufacturer's protocol.

Quantitative real-time PCR analysis: For the quantification of diurnal variation in *StUGPase2* expression in leaves of KC1 at various developmental stages, qRT-PCR analysis was performed. qRT-PCR reactions were performed with the Agilent qPCR system using the SYBR Green Master Mix (Takara, China) under the following conditions-initial denaturation at 95ºC for 5 min, followed by 40 cycles of 95˚C for 30 sec, 62˚C for 30 sec and 72˚C for 20 seconds. Real-time PCR analysis of each cDNA sample and internal control was done in technical replicates of three. The primer sequences for *StUGPase2* and the normalizer gene, *StEF1α*, are listed in Table 1. Sequences of *StUGPase2* (NCBI reference: NM_001288019.1) and *StEF1α* (Acc. No. AB061263) were derived from GenBank and used for primer designing through IDT online tool. The relative expression level was calculated using the 2^−ΔΔCt method (Pfaffl, 2004) where Δthe Ct value of S1-6 AM was used as a base. The change in expression from 6 AM-8 PM during all developmental stages has been presented as a log2 value of 2^−ΔΔCt. The specificity of the PCR amplification was checked through melt curve analysis. Two-way ANOVA was done to check the interaction between gene expressions at different sampling times at different developmental stages. Tukey's multiple comparison test of the real-time expression of Log_2 fold change in the StUGPase2 gene expression was performed using the GraphPad Prism tool.

Table 1. Primer set used for Real-Time PCR analysis

Primer name	Sequence (5'-3')
StUGPase2-F	AGATAACTTGGGTGCCATTGTTG
StUGPase2-R	GGTGCCACCTTTGACATCAGCT
StEF1α -F	ATTGGAAACGGATATGCTCCA
StEF1α-R	TCCTTACCTGAACGCCTGTCA

***In-silico* promoter analysis**: The upstream region of *StUGPase2* (~800 bp) was selected for promoter analysis and *cis*-acting regulatory elements. Online web tools PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PlantPan 3.0 (http://PlantPAN.itps.ncku.edu.tw) were used to predict the various motifs sequence involved in the binding of Transcription Factors (TFs). In the present study, only those motifs that were predicted to be responsive to light stimulus were selected and visualized graphically using TBtools (https://github.com/CJ-Chen/TBtools/releases).

Results

Diurnal changes in the PAR level and *StUGPase2* transcript abundance across key developmental stages were analyzed and compared to understand the correlation between them. The qRT-PCR analysis of *StUGPase2* demonstrated a diurnal variation in UGPase transcript level in response to varying PAR (from 6 AM

to 8 PM). Moreover, a significant change in the level of expression at various developmental stages (S1, S2, and S3) of *S. tuberosum* was also observed.

Diurnal variation in *StUGPase2* expression: *StUGPase2* expression during three major developmental stages of potato, where a major shift in carbon flux and metabolism is believed to occur, was studied through Real-Time PCR. *StUGPase2* expression followed a similar pattern across the three potato developmental stages (Fig. 1). The steady-state mRNA level of *StUGPase2* started increasing from 6 AM to 12 PM followed by a sharp decline at 2 PM. Thereafter, a steady increase in mRNA level was observed till 8 PM. The highest level of *StUGPase2* expression was observed at 8 PM in all three developmental stages studied. When compared to 6 AM the expression of *StUGPase2* at 8 PM was 1.54 fold higher at S2 and 0.435 fold higher at S3. The lowest expression in S2 and S3 was observed at 2 PM which was 1.87 and 0.04 fold higher than 2 PM of S1, respectively. Whereas, S1 has the lowest expression at 6 AM, which was 3.23 and 1.16 fold less than the 6 AM values of S2 and S3 respectively.

Stage-specific variation in *StUGPase2* expression: A significant variation in *StUGPase2* expression among the various tuber developmental stages under consideration (S1, S2, and S3 stages) was observed. The least abundance in StUGPase2 transcript level was recorded in S1 under different PAR during which sampling was performed. Whereas, the highest level of *StUGPase2* was recorded in S2 which was 1.4-3.8 fold and 1.1-2.17 fold higher than S1 and S3, respectively. The steady-state level of *StUGPase2* in S2 was around 3.23 fold higher than S1 and 1.16 fold higher than S3 at 6 AM. While at 8 PM this difference was 1.54 and 1.1 fold compared to S1 and S3, respectively.

***in-silico* analysis of the cis-regulatory elements in UGPase2 promoter sequence**: The Plant Promoter Analysis Navigator (PlantPAN) and PlantCARE tools were used here for detecting transcription factor binding sites (TFBSs), corresponding TFs, and other important regulatory elements (CpG islands and tandem repeats) in the promoter region corresponding to 1-800 nt position of *StUGPase2* gene. While PlantCARE directly provided the information about light-regulated *cis*-elements, a manual search for light-responsive motifs was also done by using the motif name/TF ID generated from PlantPAN and using it as a query in TAIR database (https://www.arabidopsis.org/). The important light responsive elements detected were: TCR, TCR; CPP, C2C2, AP2B3; AT-Hook, Homeodomain, bHLH, HD-ZIP, bZIP, CSD, Myb/SANT, Myb-Related, and Dof. The list of TFBSs, various regulatory elements, and corresponding TFs obtained through this *in-silico* analysis has been provided in Supplementary Table 1. However, only those TFBSs predicted to be involved in light-mediated regulation of gene expression were presented graphically in Fig. 2.

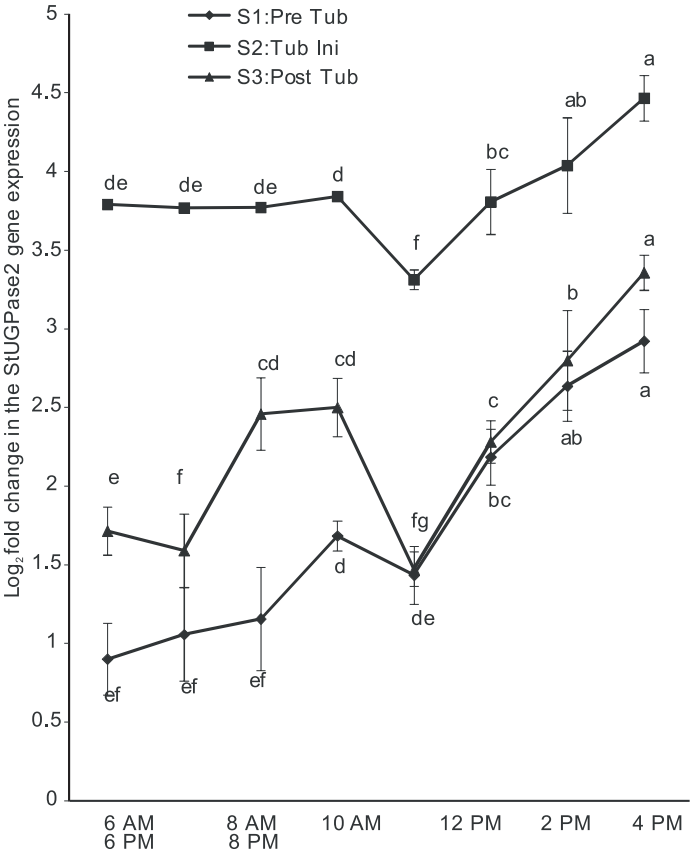

Fig. 1. Diurnal variation in the levels of accumulation of transcript *StUGPase2* in potato leaves at various developmental stages. Plants were grown in natural light from October to February. Leaf samples were harvested from 6 AM to 8 PM at intervals of approximately 2 h for three consecutive days. The graph represents the levels of *StUGPase2* mRNA estimated for each sample with the transcript of Pre-tub at 6 AM taken as base.

Discussion

UGPase is a lesser-known but crucial enzyme involved in the growth and development of plants. In young and mature leaves, it is mainly involved in the sucrose biosynthetic pathways, supplying UDP-Glu for SPS, while in other tissues, such as immature apical leaves and tubers, UGPase may participate in the breakdown of imported sucrose by utilizing UDP-Glu produced by SuSy as substrate (Rees, 1992; Winter and Huber, 2000). That way UGPase creates precursors for crucial biological processes like cell wall biosynthesis, glycolysis, and starch biosynthesis (Kleczkowski and Decker, 2017). Previously, UGPase homologs have also been reported to play diverse and important roles in plant growth and development like floral formation in rice (Woo *et al.,* 2008), and Programmed Cell Death (PCD) signaling (Chivasa *et el*., 2012). In potato, Van Harsellar *et al.* (2017) predicted the presence of two UGPase1 (Chr. 5) and UGPase2 (Chr. 11) through the genome-wide study of genes involved in carbohydrate metabolism. The present revealed *StUGPase2* as a major UGPase homolog involved in the growth and development of potato plants.

The gene encoding for UGPase has previously been cloned and its organizational analysis found the UGPase gene consists of 6.6 kb structural and a 1 kb regulatory region. Analysis of the promoter region revealed the presence of a transcriptional start site at 84 bp and a putative TATA-box at 141 bp upstream of the translation initiation point, respectively. Through stepwise deletion study, researchers have tried to identify the cis-regulatory elements in the UGPase promoter (Sowokinos *et al.,* 1994; Borovkov *et al.,* 1996). The Promoter analysis in our study revealed the presence of several motifs, involved in the circadian rhythm, photo-morphogenesis, photoperiodism, and responses to various light spectrums, in the promoter region of St*UGPase2*, (Fig. 2 and Supplementary Table 1). Therefore, a rapid rise in the

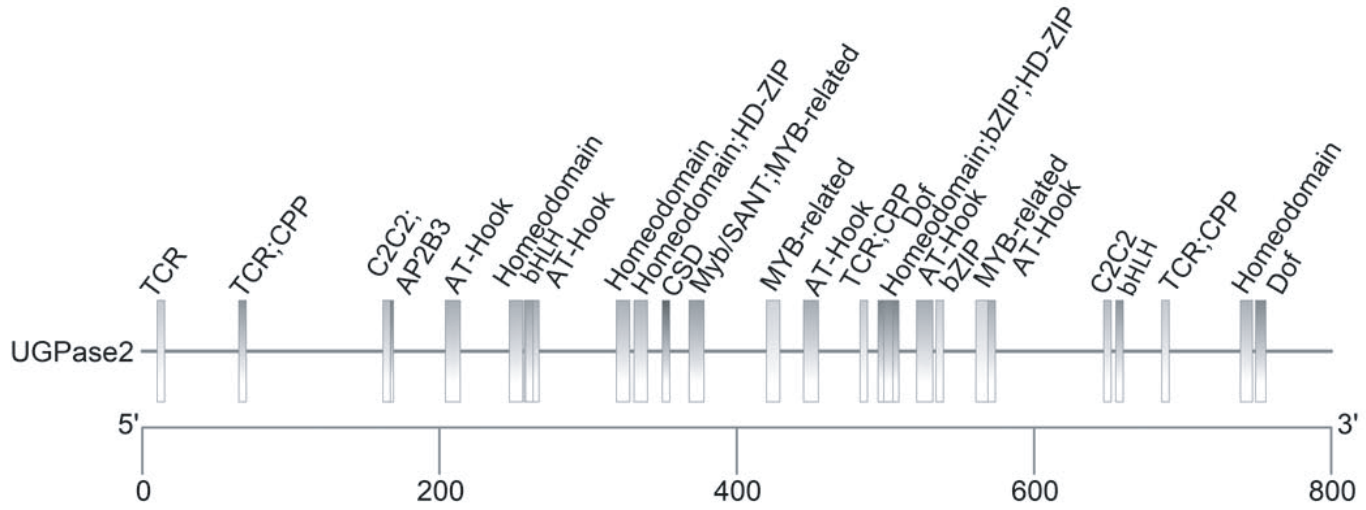

Fig. 2. Position of light-responsive *cis*-regulatory motif sequences across the UGPase2 promoter region.

StUGPase2 transcript abundance after sunrise could be explained by the presence of light-responsive cis-regulatory elements in the promoter region.

The present study describes diurnal changes in *StUGPase2* transcript abundance in potato leaves during various developmental stages in response to varying irradiance at different time points of the day. The photosynthetic rate in leaves increases with maturity peaks only when leaves are completely extended, and then rapidly declines as the plant ages (Olesinski *et al.,* 1989). Like the majority of the annual plants, the rate of accumulation of the amount of sucrose, reducing sugars, and starch increases during day time and is almost completely remobilized by nightfall (Blasing *et al.,* 2005; Fondy and Geiger, 1985; Stitt *et al.,* 1987; Geiger and Servaites, 1994; Matt *et al.,* 1998; Zeeman *et al.,* 1998). UGPase enzyme plays an important role in the starch-sucrose inter-conversion and mobilization of stored starch from the source to sink tissue. It had been demonstrated in Arabidopsis leaves that sucrose significantly up-regulated transcription of the gene encoding UGPase, and the sucrose effect was, to a large extent, mimicked by exposing leaves to light (Ciereszko *et al.,* 2001). Ciereszko *et al.* (2005) demonstrated that light stimulates UGPase expression transiently. Therefore, the diurnal behaviour of the *StUGPase2* expression in the current study could be due to an increase in sucrose level. Sucrose is known to induce UGPase expression (Geigenberger and Stitt, 2000). Spychalla 1994 reported that *UGPase* transcript accumulation pattern correlates well with diurnal variation in UGPase enzyme activity in the stem, leaflets, flowers, root, developing tubers, and tubers stored at 4°C for 10 months. Their research findings linked diurnal variation in the *StUGPase2* expression pattern and its enzymatic activity in potato leaves. Interestingly, the diurnal variation in sucrose content reported by Blasing *et al.* (2005) and Lu *et al.* (2005) correlated well with the diurnal behaviour of *StUGPase2* observed during the present study. However, a steady state increase of St*UGPase2* transcript with a transient decline at 2 PM despite a consistent decline in irradiance after a peak at 12 PM in all the development stages, indicates the presence of more complex transcriptional regulation of UGPase2 expression.

Tuber induction is a highly metabolically active, energy-demanding stage, involving a major shift in the source-sink relationship which is associated with increased expression of carbohydrate metabolizing genes, hence, a higher expression of *UGPase2* is expected (Van Harsselaar *et al.,* 2017). In our preliminary study (data not given), the *UGPase2* expression is highest at the initial phase of tuber development (from stolon swelling to bulking initiation stage) as compared to the pre-tuberization and post-tuberization stages. In the present study, many fold higher transcript abundance of *UGPase2* during the tuberization initiation stage when compared with pre-tuberization and post-tuberization stages indicated the critical importance of *UGPase2* activity during tuberization induction. It is important to mention that despite diurnal as well as stage-specific variation in the *UGPase2* gene expression, a corresponding change in the activity of the encoded enzyme cannot be ascertained. Previous research has uncovered several genes that exhibit diurnal fluctuations in transcript abundance without concomitant changes in protein level (Smith *et al.,* 2004; Turner *et al.,* 1993). This suggests that several diurnally regulating genes are predominantly post-translationally controlled in terms of enzymatic turnover and/or activities, likely since daily protein breakdown and regeneration would be prohibitively expensive for the plant in terms of energy expenditure (Blasing *et al.,* 2005). As mentioned earlier, a high transcript level may only occur during the hours of the day when more protein has to be produced (Okada and Brennicke, 2006). For certain genes, a lower amount of transcripts (and thus less degradation and turnover) during particular times of the day may be a means of cost-saving.

UGPase2 is a metabolically crucial enzyme involved in starch-sucrose metabolism and thus plays a very important role during photo-assimilation in leaves and its remobilization to sink tissues in plants. A large number of studies have been conducted to determine the diurnal behaviour of various genes involved in carbohydrate metabolism in various plants. However, less attention has been given to determining the light-mediated/ diurnal change in the expression pattern of UGPase across various development phases of potato. In our study, we found that the transcript abundance of UGPase2 shows a steady state increase after sunrise and reaches its highest level after sunset. Interestingly, a sharp decline in gene expression at 2 PM across all the developmental phases is in conformity with the diurnal behaviour of sucrose, as studied earlier. Through bioinformatics analysis of the UGPase2 promoter, many nucleotide sequence motifs involved in the bindings of various light-activated TFs

and regulatory molecules have been identified. This information adds to the pieces of evidence related to the diurnal fluctuation in *UGPase2* gene expression and the presence of some putative transcriptional regulation. The highest expression of *UGPase2* was found to be at the tuberization initiation stage followed by the post- tuberization stage and pre-tuberization stage, confirming the tuber initiation stage as the most energy-demanding and metabolically active stage. Since the *StUGPase* gene plays an important role in plants' growth and development, an improved understanding of the diurnal pattern of *StUGPase* gene regulation could ultimately help to improve the potato tuber yield and bulking attributes.

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Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Supplementary Table 1. Irradiance (PPFD; µmol $m^{-2}s^{-1}$) recorded throughout the sampling period

Time	Pre tuberization (S1)	Tuber induction (S2)	Post tuberization (S3)
6 AM	1.08	0.15	2.04
8 AM	116.7	85.5	135.38
10 AM	348.11	264.4	727.33
12 PM	779.44	282.9	1072
2 PM	682.44	346.7	926
4 PM	232.22	200.3	475.9
6 PM	12.11	1.1	16.47
8 PM	0.15	0.146	0.29

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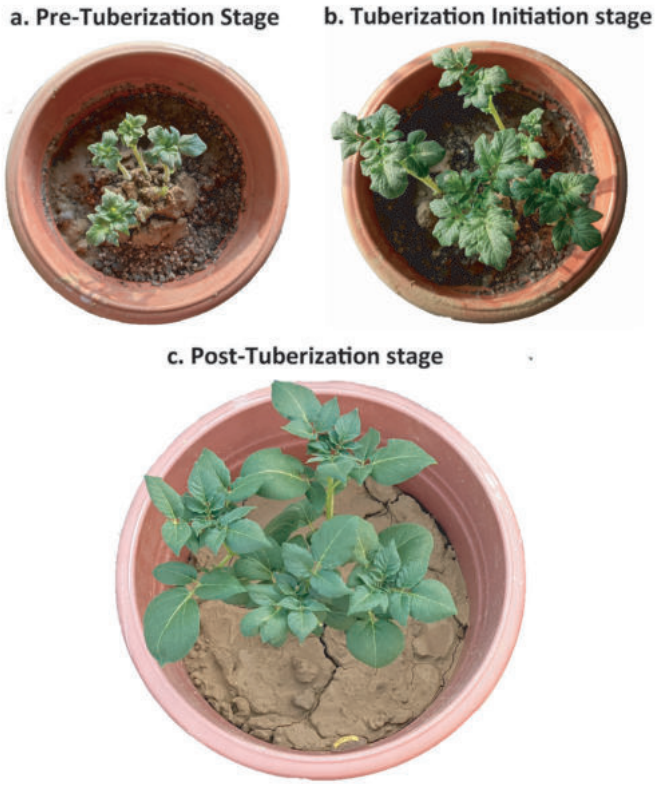

Supplementary Fig.1. Different developmental stages of potato used for studying the diurnal variation in UGPase expression.