Introduction

Carbon and nitrogen are essential elements for plant growth and development. Plants assimilate carbon dioxide by photosynthesis and synthesize starch in chloroplasts. This temporal storage of starch is degraded in dark period, and sugars as breakdown product of starch move from source to sink organs where they are resynthesized to starch (Smith et al., 2004). On the other hand, acquisition of nitrogen source is dependent on root function that fulfils the uptake of nitrate and ammonium from the soil environment (Crawford and Glass, 1998; von Wirén et al., 2000). Nitrate and ammonium incorporated to roots or those transferred to shoots will enter the pathways of nitrate reduction and amino acid biosynthesis (Stitt, 1999). Connections of carbon and nitrogen metabolisms occur in these metabolic pathways where they receive energy from photosynthesis. Carbon and nitrogen metabolisms are inter-regulated under the circumstances where supply of nitrogen source and activities of photosynthesis and related metabolisms may alter (Scheible et al., 1997a). When both nitrate and carbon source are adequately supplied, the assimilated carbon will be converted to organic acids and used for the synthesis of amino acids. By contrast, carbon flux in starch synthesis will increase when nitrogen is limited.

In this work, public microarray data of *Arabidopsis* was used to analyze relationships of gene expression in starch and nitrogen metabolisms. We focused particularly on diurnal regulations since both carbon and nitrogen metabolisms fluctuate over the day/night cycles. Potential regulators of starch and nitrogen metabolisms were predicted by co-expression analysis, and their functions and regulatory relationships were validated by using T-DNA and transposon insertion mutants.

Methods

Arabidopsis lines and growth condition

Mutant lines of *col* (SALK_061956) and *col7* (CS124499) were obtained from the T-DNA and transposon mutant collections of *Arabidopsis* of The Arabidopsis Biological Resource Center (ABRC) (Alonso et al., 2003) and John Innes Centre (Tissier et al., 1999), respectively. Wild type (Columbia-0) and mutant seeds were vernalized at 4ºC for 3 days under dark condition, and sown on a mixture of vermiculite and peat-based compost. Plants were grown under 12 hr light/12 hr dark (short day) cycles with the light intensity of 100 μmol m^{-2} sec^{-1}, 60% relative humidity, and 22ºC. Plants were watered by sub-irrigation with nutrient solution (Fujiwara et al., 1992). Leaves of 1-month-old plants were harvested at 1, 11, 13, and 23-hour while the first 12-hour is the dark period. Two to three fully expanded leaves from 3 plants were taken for RNA preparation.

Expression analysis by quantitative real-time PCR (qRT-PCR)

Approximately 100 – 200 mg fresh weight of leaf tissues were harvested for expression analysis. Total RNA was extracted by RNeasy plant mini kit (Qiagen) and used to synthesize first strand cDNA. *Ubiquitin2* gene was used as an internal control for qRT-PCR. The primers for qRT-PCR were col7-F:AGGAGCAAGAGTTGTGCGTT and col7-R:CCATGATTGCCTTCTCGACT for *COL7* (At1g73870); col-F:GGGAAGAGAACACATAGGCG and col-R:GGAAGCCTTTTGACACCGTA for *COL* (At2g21320); gbss-F: AGGCACCACAGGTTCTGAAC and gbss-R: TGTAGAC-TCCGCGGGATTGATA for *GBSS* (At1g32900); amt1;2-F: GTTCGCAAGGAAAG-AATACGTTAACGAG and amt1;2-R: TCCGGCTTGTAGGCTCCACTCTTCCAG for *AMT1;2* (At1g64780); UBQ2_144F:CCAAGATCCAGGACAAAGAAGGA and UBQ2_372R:TGGA-GACGAGCATAACACTTGC for *UBQ2* (At2g36170).

Clustering analysis

Hierarchical and k-means clustering were performed by Genesis (Sturn et al., 2002).

Results and Discussion

In *Arabidopsis*, 45 genes are annotated to encode enzymes for starch biosynthesis and metabolism. For nitrogen assimilation, 30 genes may encode nitrate and ammonium transporters, and enzymes for glutamine/glutamate and asparagine/aspartate biosynthesis. Expression data of these 45 starch genes and 30 nitrogen genes on 12-hr-day/12-hr-night cycle time-series microarray experiments of *Arabidopsis* leaves (Smith et al., 2004) were excerpted from a public database. Figures 1 and 2 are the overviews of starch and nitrogen genes, respectively, observed by hierarchical clustering of the expression patterns relative to the starting point of dark cycle (0 hr).

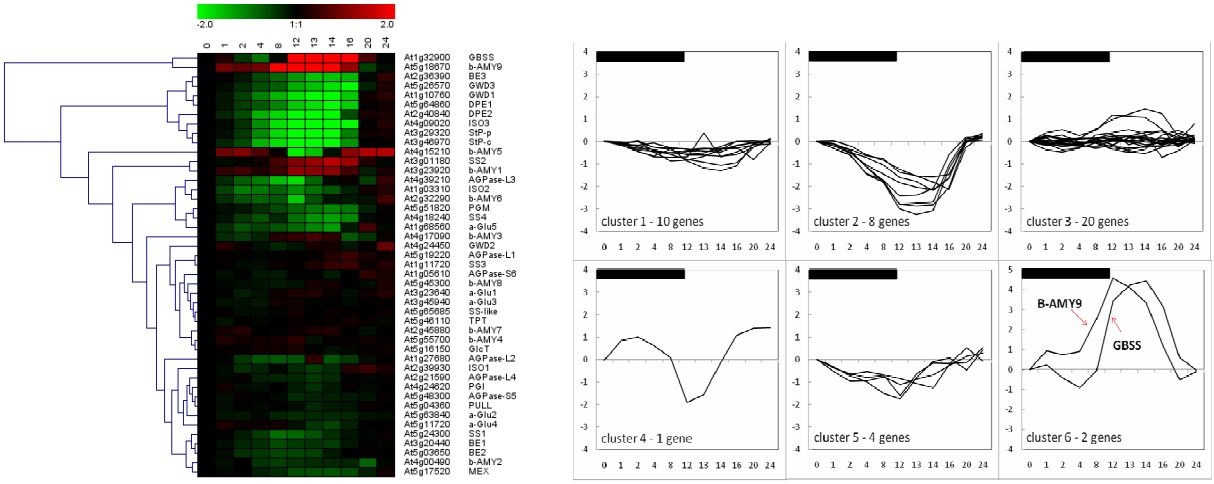

Figure 1. Clustering analysis of starch genes under a day/night cycle. Values derive from transcriptome data (Smith et al., 2004) and are indicated by log2 ratios to time 0 (the beginning of the dark period). Black bars on the right panels indicate the dark period.

Clustering analysis of starch genes

Since starch is synthesized only during the daytime, accumulated in chloroplasts, and consequently degraded in the following night period, the anabolic and catabolic genes should act dominantly during the day and the night period, respectively. However, not many of the starch genes show marked expression profiles over the day/night cycle. Approximately half of the starch genes showed subtle changes under this condition. Some distinctive diurnal patterns were revealed when observed by k-means clustering (Figure 1). A group of 8 starch genes (isoamylase 3 [ISO3]: At4g09020, disproportionating enzyme 1 and 2 [DPE1 and DPE2]: At5g64860 and At2g40840, starch phosphorylase [StP]: At3g29320 and At3g46970, glucan-water dikinase 1 and 3 [GWD1 and GWD3]: At1g10760 and At526570, and branching enzyme 3 [BE3]: At2g36390) showed similar pattern which declines in the night period but restores in 4-8 hours in the day time (cluster 2 in Figure 1). Members in this cluster were reported to play major roles in starch degradation in leaves, except for *BE3* (Zeeman et al., 2007). It is suggested that transcripts of these degradation enzymes accumulate towards the end of the light period, preceding the night cycle where starch remobilization actually occurs. On the other hand, *BE3* is unlikely related

with starch degradation. This enzyme is rather required for normal starch synthesis in leaves and its activity is suggested to be mostly redundant with *BE2* (Dumez et al., 2006).

Another distinct expression pattern was found in cluster 6. Two starch genes, β-amylase 9 (BAM9: At5g18670) and granule-bound starch synthase (GBSS: At1g32900), were included in this cluster. The expression patterns resembled but timing of induction and repression was slightly different as represented by earlier induction and repression of *BAM9* compared with *GBSS* (Figure 1). *BAM9* mRNA accumulated towards the end of the night and started to decrease immediately after the daybreak. This pattern likely synchronizes with starch degradation in leaves. One of the *BAM* genes in *Arabidopsis*, *BAM3*, indeed encodes a major functional enzyme in starch degradation (Scheidig et al., 2002). By contrast, *GBSS* was induced at the daybreak, showed maximal transcript levels during the early phase of the light period, and decreased thereafter towards the end of light period. GBSS is responsible for amylose synthesis and extension of long-chain amylopectin, and has been reported as a circadian regulated gene (Tenorio et al., 2003). Low abundance of its transcript level in the dark period is considered to be related with degradation of starch granules within which GBSS is incorporated (Smith et al., 2004).

Clustering analysis of nitrogen genes

NIA1 (At1g77760) and *NIA2* (At1g37130), the two nitrate reductase genes, showed a clear diurnal variation; the *NIA* transcripts increased along the night period and gradually decreased during the day (Figure 2; cluster 2). Light can induce *NIA* transcripts (Cheng et al., 1991). On the other hand, it is reported that *NIA* transcripts highly accumulate towards the end of the night, which may allow rapid production of NIA enzymes used for nitrate assimilation in the light period (Scheible et al., 1997b). The peaks of *NIA1* and *NIA2* observed around the mid-night period (Figure 2) correspond to the latter mechanism.

Three nitrate and ammonium transporters, *AMT1;2*, *NRT1.3*, and *NRT1.4*, showed a similar diurnal pattern under this condition (Figure 2; cluster 1). Expression of both nitrate transporters continuously increased in the dark period and started to decrease in the second half of the light period. *NRT1.3* and *NRT1.4* are in the family of low-affinity nitrate transporters. *NRT1.3* and *NRT1.4* were expressed higher in shoots than roots and induced by nitrate (Okamoto et al., 2003). Additionally, *NRT1.4* plays a role in nitrate storage in leaf petioles (Chiu et al., 2004). The expression patterns of both genes might be related with nitrate re-absorption by leaf cells and be concurrent with diurnal nitrate uptake by roots.

AMT1;2 showed a quite similar pattern of increase and decrease of transcripts as shown for *GBSS* (Figure 1). The peak of *AMT1;2* appeared 2 hours after the onset of the light (Figure 2). *AMT1;2* is one of high-affinity ammonium transporters (Yuan et al., 2007). The present results suggest AMT1;2 may work as an ammonium transporter in leaves for absorption of ammonium released from xylem or photorespiration. *AMT1;2* showed the highest correlation value, 0.908, to *GBSS* among the starch genes. Based on this significant correlation of expression profiles, we hypothesized *AMT1;2* and *GBSS* might be controlled under the same regulatory process in day/night cycles.

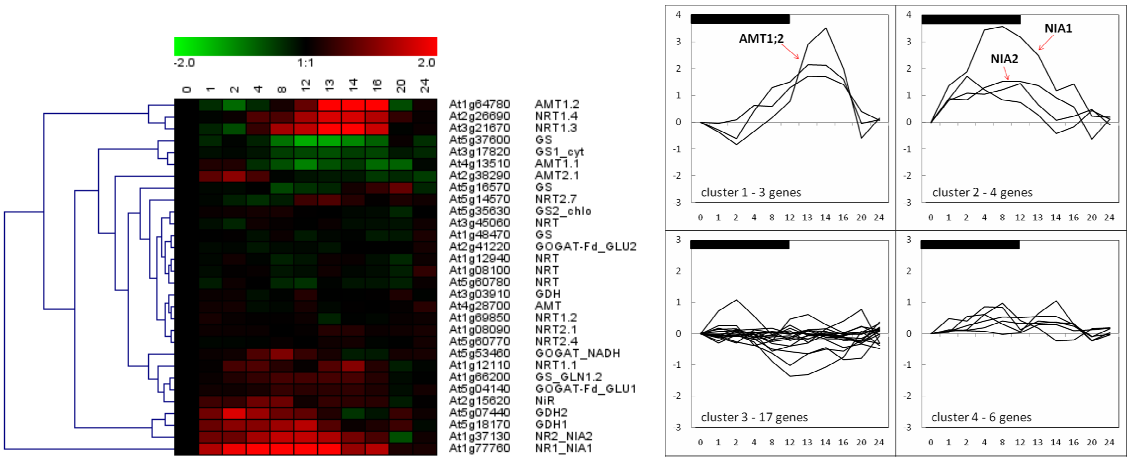

Figure 2. Clustering analysis of nitrogen genes under a day/night cycle. Values derive from transcriptome data (Smith et al., 2004) and are indicated by log2 ratios to time 0 (the beginning of the dark period). Black bars on the right panels indicate the dark period.

CONSTANS-LIKE genes control *GBSS* and *AMT1;2*

We first looked for transcription factors coexpressed with *GBSS* under a day/night cycle. Two *CONSTANS-LIKE* transcription factors, *COL7* (At1g73870) and *COL* (At2g21320), showed the highest correlation values, 0.987 and 0.978, respectively, with the expression pattern of *GBSS* (Figure 3). These transcription factors were also co-expressed with *GBSS* under various experimental conditions. The condition-independent correlation analysis was performed by ATTED-II (*Arabidopsis thaliana* trans-factor and *cis*-element prediction database: http://atted.jp/) which contains publicly available microarray data (58 experiments, 1388 slides) collected by AtGenExpress and identifies co-expressed genes based on weighted Pearson's correlation coefficients calculation (Obayashi et al., 2009). Correlation coefficients of *COL7* and *COL* to *GBSS* were 0.609 and 0.608, respectively. From the expression analysis, both transcription factors were hypothesized to be regulators of *GBSS* expression at transcription levels.

Arabidopsis mutant lines with T-DNA or transposon insertions in *COL* and *COL7* were used to investigate the regulation of *GBSS* expression. Since *AMT1;2* showed similar expression pattern to *GBSS* and might be controlled by both *COL* genes (Figure 3), its expression was also determined in the mutants. The levels of *GBSS* and *AMT1;2* transcripts in the mutants and wild-type plants were measured by qRT-PCR at 4 time points along a 12-hr-day/12-hr-night cycle where the leaves were harvested 1 hour before and after the onset of light and dark period. Significant reduction of *GBSS* and *AMT1;2* was observed in *col* and *col7* mutants (Figure 4). These results indicated that *COL* and *COL7* regulate the *GBSS* and *AMT1;2* transcripts under a diurnal condition. In *Arabidopsis*, GBSS is reported to be controlled by 2 main clock transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1: At2g46830) and LATE ELONGATED HYPOCOTYL (LHY: At1g01060) (Tenorio et al., 2003). In addition, *CONSTANS* is regulated by the circadian clock, although there is no information of circadian regulation of its homologues, *COL* and *COL7*. It might be possible that *COL* and *COL7* are also controlled by the clock and participate in diurnal regulation of *GBSS* and *AMT1;2* as intermediate regulators between the clock and downstream genes.

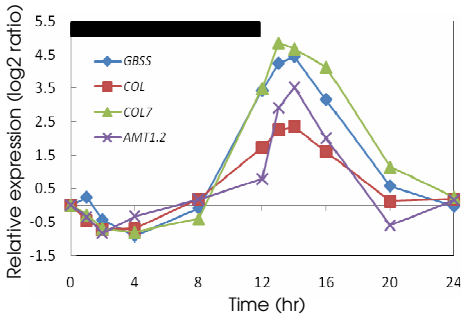

Figure 3. Expression patterns of *GBSS*, *AMT1;2*, and their predicted regulators, *COL* and *COL7* genes under a diurnal cycle. Source data is same as in Figures 1 and 2.

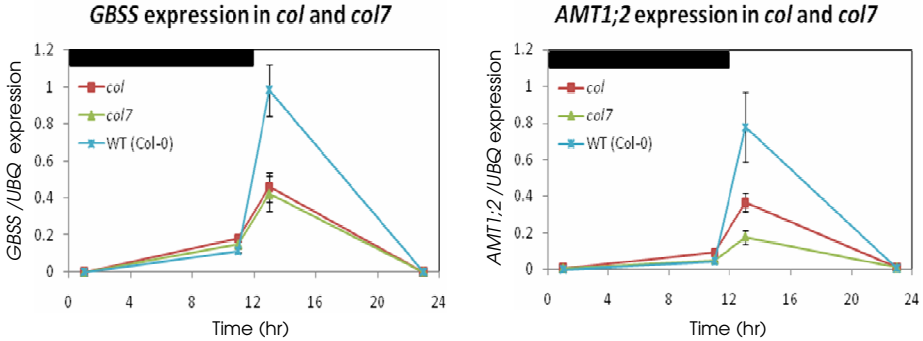

Figure 4. Expression patterns of *GBSS* and *AMT1;2* in *col* and *col7* mutants comparing to the wild type under a diurnal cycle. Error bars represent SEs (n=3).

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