Diurnal Changes in the Transcriptome Encoding Enzymes of Starch Metabolism Provide Evidence for Both Transcriptional and Posttranscriptional Regulation of Starch Metabolism in Arabidopsis Leaves[1]

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To gain insight into the synthesis and functions of enzymes of starch metabolism in leaves of Arabidopsis L. Heynth, Affymetrix microarrays were used to analyze the transcriptome throughout the diurnal cycle. Under the conditions employed, transitory leaf starch is degraded progressively during a 12-h dark period, and then accumulates during the following 12-h light period. Transcripts encoding enzymes of starch synthesis changed relatively little in amount over 24 h except for two starch synthases, granule bound starch synthase and starch synthase II, which increased appreciably during the transition from dark to light. The increase in RNA encoding granule-bound starch synthase may reflect the extensive destruction of starch granules in the dark. Transcripts encoding several enzymes putatively involved in starch breakdown showed a coordinated decline in the dark followed by rapid accumulation in the light. Despite marked changes in their transcript levels, the amounts of some enzymes of starch metabolism do not change appreciably through the diurnal cycle. Posttranscriptional regulation is essential in the maintenance of amounts of enzymes and the control of their activities in vivo. Even though the relationships between transcript levels, enzyme activity, and diurnal metabolism of starch metabolism are complex, the presence of some distinctive diurnal patterns of transcripts for enzymes known to be involved in starch metabolism facilitates the identification of other proteins that may participate in this process.

Starch is the major form in which carbon is stored in plants, the major source of calories in the human diet, and an important industrial commodity (Jobling, 2004). However, our understanding of the nature and regulation of the pathways of starch synthesis and degradation is incomplete. The Arabidopsis genome sequence, together with extensive functional genomics resources, is facilitating investigations to better understand starch metabolism in plants (Zeeman et al., 2002; Smith et al., 2003). Starch is synthesized in many organs of Arabidopsis, including leaves, flowers, developing seeds, and root caps, and the structure and composition of starch isolated from leaves are similar to that from crop plants (Zeeman et al., 2002). Use of leaves enables both synthesis and degradation to be studied within a 24-h period independently of changes in plant development. The two processes are integrated with each other, and their rates are related to the duration of day and night. Characteristic changes in the content of sugars and maltooligosaccharides are also observed throughout the diurnal cycle, showing a complex integration of starch and sugar metabolism. The Arabidopsis leaf thus provides an excellent model system with which to elucidate the pathways and regulatory mechanisms of starch synthesis and breakdown in the plastids of living cells (Zeeman et al., 2002; Smith et al., 2003).

The Arabidopsis genome sequence reveals many genes encoding enzymes that may be involved in starch synthesis and degradation, and in the synthesis of Suc from starch. Some of these enzymes have been extensively studied in other species, and their functions are well established (such as starch synthases and branching enzymes), while the functions of others are uncertain. In many cases, several genes in Arabidopsis encode different putative isoforms of the same enzyme, in some instances with different subcellular targeting information. With the increasing availability

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of functional genomic resources in Arabidopsis, the comprehensive analysis of the function of all putative enzymes of starch metabolism is now possible.

Isolation of mutants using both forward and reverse genetics approaches has provided important information about some of these enzymes. The results have led to some surprising discoveries: enzymes previously assumed to be important have no obvious role and proteins of previously unknown function have been shown to be important. A mutant (*dbe1*) identified as a result of unusual iodine staining of leaf starch was found to contain both starch and phytoglycogen (Zeeman et al., 1998a). The mutation responsible for this phenotype occurs in a gene encoding an isoamylase-type starch debranching enzyme (ISA2 or DBE1). This mutant provides evidence that debranching enzyme is required for normal starch synthesis in Arabidopsis, as it is in other plants (Myers et al., 2000). However, the precise function of debranching enzymes in starch synthesis is still uncertain. A mutant of Arabidopsis (*sex1*) with reduced rates of starch degradation (Yu et al., 2001) has been shown to carry a mutation in a gene encoding an R1 protein, a glucan, water dikinase (GWD) necessary for normal starch degradation in potato (*Solanum tuberosum*; Lorberth et al., 1998; Ritte et al., 2002). This enzyme phosphorylates the amylopectin component of starch, but the reason that it is required for normal starch degradation has not yet been established. The enzyme(s) responsible for primary attack on the starch granule to initiate breakdown is still unknown. Research from our laboratories shows that neither α-amylase (AMY; H. Dunstan, D. Fulton, and S. Smith, unpublished data) nor starch phosphorylase (Zeeman et al., 2004a) is required for starch breakdown in Arabidopsis leaves, but β-amylase (BAM) does appear to be required (D. Fulton, H. Dunstan, S. Zeeman, and S. Smith, unpublished data). However, significant progress has been made in establishing the pathway of metabolism of starch breakdown products. Disproportionating enzyme (DPE1) has been shown to be required for conversion of maltotriose into larger maltooligosaccharides, which can be further attacked by amylolytic enzymes, and Glc, which is exported from the plastid (Critchley et al., 2001). Studies of a high-starch mutant that accumulates maltose (*mex1*) led to the discovery that maltose is the major product of starch breakdown (consistent with a key role for BAM). The mutated gene was found to encode a novel transporter that is required for maltose export from the chloroplast (Niittylä et al., 2004). The immediate fate of maltose exported from the chloroplast has been revealed by knockout mutants that lack a cytosolic disproportionating enzyme-like protein (DPE2). These plants accumulate very high levels of maltose (Lu and Sharkey, 2004; Chia et al., 2004). DPE2 has been shown to transfer a glucosyl unit from maltose to glycogen in vitro, suggesting a novel pathway of carbohydrate metabolism in the cytosol of Arabidopsis leaves at night (Chia et al., 2004).

Microarray analyses have revealed that transcripts for at least some of these enzymes show strong diurnal changes (Harmer et al., 2000). It has been speculated that these may be important in the regulation of the processes of synthesis and degradation. However, there has been no systematic attempt to relate the transcriptome to the amounts and activities of enzymes and to the fluxes through the pathways of starch synthesis and degradation over 24 h. Here, we present results for transcript levels over a day-night cycle. We use our previous data on amounts and activities of enzymes and of amounts of starch and its metabolites to discover the extent to which changes in the starch transcriptome reflect and may be related to changes in metabolic fluxes into and out of starch.

RESULTS

Physiological Context of Transcriptome Analysis

Conditions chosen for investigation of the starch transcriptome throughout the diurnal cycle were those used routinely in studies of starch metabolism in our laboratories (e.g. Zeeman et al., 1998a, 2002; Critchley et al., 2001). These studies provide data on starch synthesis, structure, and degradation, the amounts and activities of many enzymes of starch metabolism, and amounts of Suc, hexoses, maltose, and maltooligosaccharides in leaves. They therefore provide an excellent basis for the interpretation of information about changes in the transcriptome. Under the conditions we normally use in our experiments—a 12-h-light/12-h-dark cycle at growth stage 3.90 (Boyes et al., 2001)—starch is degraded progressively throughout the dark period and accumulates progressively throughout the light period. Pulse-chase experiments with $^{14}CO_2$ indicate that there is no significant starch degradation during the light period (Zeeman et al., 2002). Changes in the content of starch, maltooligosaccharides, Suc, and hexoses in such plants are shown in Figure 1. All of the major diurnal trends and relative amounts of these metabolites are highly robust and reproducible between batches of plants.

Experimental Design of Transcriptome Analysis

We harvested leaves at the 11 time points we routinely use for metabolite analyses (Fig. 1). The harvesting is particularly focused on the periods immediately after the transitions from dark to light and from light to dark, since changes in carbohydrate metabolism are most pronounced at these times. The first sample was taken at the end of the light period, and subsequent samples were taken after 1, 2, 4, 8, and 12 h of darkness, then after 1, 2, 4, 8, and 12 h of light. Thus, the last sample is a replicate of the first.

For each sample, we harvested three leaves from each of eight plants to reduce biological variability as

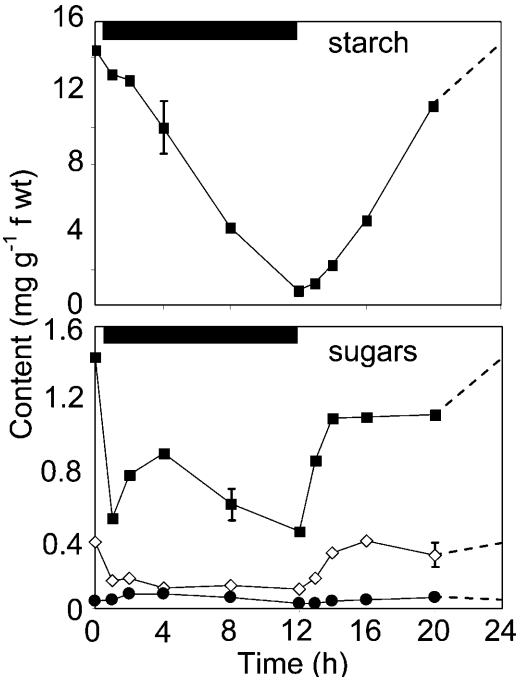

Figure 1. Starch and sugar content of leaves over 24-h diurnal cycle. Plants were grown under conditions identical to those for plants harvested for transcript analysis. Measurements were made according to Critchley et al. (2001). Values are means of five measurements, each made on a separate plant. Suc, Black squares; hexoses, white diamonds; maltooligosaccharides, black circles. SES greater than 10% of means are shown as bars, where these are larger than symbols. For each metabolite, the same major trends in values over 24 h were displayed by at least two further, separately grown batches of plants.

far as realistically possible. We used single microarrays at each time point because the Affymetrix genome arrays are highly reproducible (less than 0.6% of genes show a 2-fold difference in expression due to technical variability; http://nasc.nott.ac.uk/). Evidence for the reliability of the data was obtained by observing progressive changes in the levels of individual transcripts at successive time points in the diurnal cycle and by observing very similar expression patterns for coordinately regulated genes (see below). Finally, by repeating the whole experiment on plants grown in the same growth chamber, we were able to demonstrate the reproducibility of the results (see below).

Choice of Genes for Analysis

We focused our analysis on genes encoding enzymes involved in starch synthesis from the Calvin cycle intermediate Fru-6-P, and in the conversion of starch to maltose and Glc in the dark (Table I). For starch synthesis, we included genes encoding chloroplastic phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM), subunits of ADP-Glc pyrophosphorylase (AGPase), starch synthase (STS) and starch branching enzyme (SBE) isoforms, and the two isoamylases (ISA1 and ISA2) homologous to the isoamylase proteins that constitute the major isoamylase activity of the potato tuber (Hussain et al., 2003). These enzymes are believed to be located in the chloroplast, and their genes encode predicted plastidial transit peptides. One exception is a predicted glucan synthase-like protein (GLS; At5g65685), showing about 30% amino acid sequence identity to starch synthases, which has no predicted targeting information.

The pathway by which starch is degraded in leaves at night is far from understood (Smith et al., 2003; Zeeman et al., 2004b), so we have examined transcript levels of all of the enzymes either shown to be involved in this pathway, or predicted from their sequences or known activities to be capable of catalyzing relevant reactions. Thus, we include genes encoding GWD and GWD-like enzymes, AMYs, BAMs, disproportionating enzyme (DPE1) and the related DPE2 protein, a third isoamylase (ISA3), the pullulanase or limit dextrinase (LDA), glucan (starch) phosphorylases (PHS), and putative α-glucosidases (AGL). For some of these enzymes there is strong evidence that they occur in the plastid, and some are encoded by genes including predicted transit peptides for plastid targeting (Table I). AMY2 and some putative AGLs are apparently synthesized with putative signal sequences for endomembrane targeting. Other proteins contain no apparent targeting information and are therefore assumed to be cytosolic. The functions of these nonplastidial enzymes is unknown, but they could potentially be involved in metabolism of products of starch breakdown exported from the plastid—or in the degradation of starch in lysosome-like vacuoles, or in the metabolism of extracellular glucans.

We also included genes encoding transporters on the chloroplast envelope that are capable of transferring metabolites related to starch metabolism between the chloroplast and the cytosol, including a recently discovered maltose transporter (Niittylä et al., 2004). Current evidence indicates that maltose and Glc are the main forms in which products of starch breakdown at night are exported to the cytosol from the chloroplast (Critchley et al., 2001; Niittylä et al., 2004; Weise et al., 2004). Recent studies show that a glucosyltransferase-like enzyme (DPE2) is required for maltose metabolism outside the chloroplast (Lu and Sharkey, 2004; Chia et al., 2004), so we have included this in our analysis. In total, we present data for the expression of 48 genes, although we recognize that there may be others with potential roles in starch synthesis or breakdown, such as the 14-3-3 protein proposed to interact with a starch synthase (Sehnke et al., 2001) and protein kinases and phosphatases involved in the phosphorylation of enzymes of starch synthesis (Tetlow et al., 2004). The microarray data for all other genes can be mined as appropriate in the future.

Table I. *Genes included in transcriptome analysis*

Enzyme	EC No.[a]	CAZy Family[b]	Genome Locus	Gene Name[c]	Target[d]	Affy Signal[e]
Phosphoglucoisomerase	5.3.1.9		At4g24620	*PGI1*	P	331
Phosphoglucomutase	5.4.2.2		At5g51820	*PGM1*	P	376
ADP-Glc pyrophosphorylase large subunit 1	2.7.7.27		At5g19220	*APL1*	P	587
ADP-Glc pyrophosphorylase large subunit 2	2.7.7.27		At1g27680	*APL2*	P	67
ADP-Glc pyrophosphorylase large subunit 3	2.7.7.27		At4g39210	*APL3*	P	99
ADP-Glc pyrophosphorylase large subunit 4	2.7.7.27		At2g21590	*APL4*	P	11*
ADP-Glc pyrophosphorylase small subunit	2.7.7.27		At5g48300	*APS1*	P	701
ADP-Glc pyrophosphorylase small subunit-like			At1g05610	*APS2*	P	8*
Starch synthase I	2.4.1.21	GT5	At5g24300	*STS1*	P	320
Starch synthase II	2.4.1.21	GT5	At3g01180	*STS2*	P	122
Starch synthase III	2.4.1.21	GT5	At1g11720	*STS3*	P	144
Starch synthase IV	2.4.1.21	GT5	At4g18240	*STS4*	P	271
Granule-bound starch synthase	2.4.1.21	GT5	At1g32900	*GBS1*	P	603
Glucan synthase-like		GT5	At5g65685	*GLS1*	(C)	142
Starch branching enzyme I	2.4.1.18	GH13	At3g20440	*SBE1*	P	35*
Starch branching enzyme II	2.4.1.18	GH13	At5g03650	*SBE2*	P	282
Starch branching enzyme III	2.4.1.18	GH13	At2g36390	*SBE3*	P	379
Starch debranching enzyme: Isoamylase I	3.2.1.68	GH13	At2g39930	*ISA1*	P	164
Starch debranching enzyme: Isoamylase II	3.2.1.68	GH13	At1g03310	*ISA2, DBE1*	P	227
Starch debranching enzyme: Isoamylase III	3.2.1.68	GH13	At4g09020	*ISA3*	P	234
Starch debranching enzyme: Limit dextrinase	3.2.1.142	GH13	At5g04360	*LDA1*	P	124
Glucan water dikinase 1	2.7.9.4		At1g10760	*GWD1, SEX1*	P	711
Glucan water dikinase-like 2			At4g24450	*GWD2*	(C)	52*
Glucan water dikinase-like 3			At5g26570	*GWD3*	(P)	494
Glucanotransferase (DPE1)	2.4.1.25	GH77	At5g64860	*DPE1*	P	121
Transglucosidase (DPE2)		GH77	At2g40840	*DPE2*	C	622
Glucan phosphorylase (plastidial)	2.4.1.1	GT35	At3g29320	*PHS1*	P	566
Glucan phosphorylase (cytosolic)	2.4.1.1	GT35	At3g46970	*PHS2*	C	1,141
α-Amylase 1	3.2.1.1	GH13	At4g25000	*AMY1*	(S)	39*
α-Amylase 2	3.2.1.1	GH13	At1g76130	*AMY2*	(C)	146
α-Amylase 3	3.2.1.1	GH13	At1g69830	*AMY3*	P	414
β-Amylase 1	3.2.1.2	GH14	At3g23920	*BAM1, BMY7*	(P)	275
β-Amylase 2	3.2.1.2	GH14	At4g00490	*BAM2, BMY9*	(P)	430
β-Amylase 3	3.2.1.2	GH14	At4g17090	*BAM3, BMY8, ctBMY*	P	2,012
β-Amylase 4	3.2.1.2	GH14	At5g55700	*BAM4*	(P)	49*
β-Amylase 5	3.2.1.2	GH14	At4g15210	*BAM5, BMY1, RAM1*	S	139
β-Amylase 6	3.2.1.2	GH14	At2g32290	*BAM6*	(C)	79
β-Amylase 7	3.2.1.2	GH14	At2g45880	*BAM7*	(C)	15*
β-Amylase 8	3.2.1.2	GH14	At5g45300	*BAM8*	(C)	67
β-Amylase 9	3.2.1.2	GH14	At5g18670	*BAM9, BMY3*	(C)	246
α-Glucosidase-like 1		GH31	At3g23640	*AGL1*	(C)	103
α-Glucosidase-like 2		GH31	At5g63840	*AGL2*	(S)	220
α-Glucosidase-like 3		GH31	At3g45940	*AGL3*	(S)	3
α-Glucosidase-like 4	3.2.1.20	GH31	At5g11720	*AGL4*	(S)	143
α-Glucosidase-like 5		GH31	At1g68560	*AGL5*	(S)	260
Triose phosphate/Pi translocator			At5g46110	*TPT1*	P	3,454
Glc transporter			At5g16150	*GLT1*	P	711
Maltose exporter			At5g17520	*MEX1*	P	306

[a]EC numbers are omitted where there is appreciable doubt. [b]CAZy families are available from http://afmb.cnrs-mrs.fr/CAZY/index.html. [c]Gene names have been adopted, or in some cases modified, to conform to a three-letter-one-number convention. [d]Target indicates subcellular locations as: P, plastid; C, cytosol; S, secreted. Those in parentheses indicate uncertainty or are predicted based only on the gene sequence. Those assignments not in parentheses are supported by experimental evidence in Arabidopsis or for equivalent proteins in other species. [e]Affy signal is the mean Affymetrix signal for all 11 time points for each gene in experiment 1. Those with asterisks have 6 or more time points with values below 50 and so are excluded from Figure 2A.

Overview and Validation of Results

Microarray analysis using the Affymetrix ATH1 genome array was carried out by the Nottingham Arabidopsis Stock Centre (NASC) using RNA delivered to them. The microarray data are available for public access on the NASC Web site (http://nasc.nott.ac.uk/). To obtain an overview of the trends in expression of the transcriptome in the first experiment, genes with a low expression signal (arbitrarily chosen as 6 or more time points with values below 50) were excluded from the total set of 22,810 genes, leaving a subset of 9,437 genes. The expression values for each gene in the subset were normalized to the value at time zero (0 h), and expressed as fold change in signal at each subsequent time point. The results (Fig. 2A) reveal several striking features. First, changes in gene expression are most pronounced immediately after the transitions from light to dark and from dark to light. Some genes in this set show changes in expression of up to 50-fold. Others not included in this set show even greater changes in gene expression throughout the diurnal cycle (data not shown). Second, some transcripts increase in amount prior to the light period and thereafter continue to increase, suggesting that they could be under circadian control (Harmer et al., 2000; Schaffer et al., 2001). Third, the signal for the great majority of transcripts at the 24-h time point is very similar to that at the 0-h time point, as expected of replicate samples. It was found that of 9,852 genes with signals of 50 or more at both 0 h and at 24 h, only 39 showed more than a 2-fold difference in expression between these samples. By contrast, between 12 and 13 h, 444 such differences were seen, and between 16 and 20 h, 603 such differences were seen (data not shown). The data for the second experiment showed these same general features (data not shown).

For a very small number of genes, the signal at 24 h is severalfold greater than that at 0 h, in both experiments. Some of these genes, such as those encoding *VSP1* (Guerineau et al., 2003) and a superoxide dismutase (At4g25100), may be highly responsive to biotic and abiotic factors, implying that during the leaf-harvesting process, the plants were stimulated in some way, even though great care was taken to minimize disturbances to the plants. The *RAM1* (*BAM5*) gene encoding a nonplastidial BAM is another gene for which expression is appreciably higher at the 24-h time point than at 0 h (see below). However, overall, the degree of correspondence between values at 0 h and at 24 h is excellent.

Next, we sought some indicators of the reliability of the signals produced by each genome array and of the comparability of the two experiments. First, we examined expression patterns of genes that have previously been extensively studied in the context of diurnal or circadian regulation. For these purposes and for all subsequent evaluation of expression profiles, we normalized the data for each gene as a percentage relative to the mean of its expression level throughout the diurnal cycle (Harmer et al., 2000). This avoided normalization to single values for each gene. The genes *CCA1* and *LHY* encode MYB transcription factors, which are believed to be components of the central oscillator of the circadian clock (Hayama and Coupland, 2003). The expression patterns of these two genes are remarkably similar to each other, both within and between experiments (Fig. 2, B and C), and consistent with published data (Wang and Tobin, 1998). The genes *TOC1* and *ELF3* are regulated by the clock and show similar patterns of expression, which are highly reproducible between experiments (Fig. 2, B and C), and consistent with published data (Strayer et al., 2000; Liu et al., 2001). *TOC1* regulates timing of expression of genes encoding chlorophyll *a*/*b*-binding proteins (CAB). Of the 21 *CAB*-related genes in Arabidopsis, 16 are represented on the ATH1 genome array, but not specifically *CAB1* and *CAB2*, which have been most extensively studied (Millar and Kay, 1991). Most of the *CAB* genes have similar expression profiles, and two are shown as examples (Fig. 2, B and C). These genes have similar expression patterns within each experiment, consistent with published data for the diurnal regulation of *CAB* genes as reported previously (Millar and Kay, 1991).

To test the relevance of relatively small changes in apparent levels of gene expression between time points, we focused initially on the gene encoding the triose phosphate transporter (TPT1). The expression level of this gene changes little in magnitude but exhibits a complex pattern of change. GeneSpring software (http://www.silicongenetics.com) was used to search for genes with similar expression profiles in the first experiment. The seven genes with profiles most similar to *TPT1* (Fig. 2D) all encode chloroplast proteins, all with functions in photosynthesis. One of these genes encodes glyceraldehyde 3-phosphate dehydrogenase, which produces the substrate for TPT1. The same seven genes show coexpression in the second experiment (Fig. 2E), although the pattern differed in detail from that of the first. The small differences between experiments indicate that an aspect of the physiology of the leaves may have been different in each experiment. However, the expression patterns of genes not directly related to photosynthesis was remarkably consistent between experiments (Fig. 2, B and C; see below). The coincidence of successive values for these related genes throughout the diurnal cycle provides confidence that individual values for each gene are reliable and that even relatively small changes in transcript level at successive time points can be meaningful. A similar conclusion was reached by Menges et al. (2003) in a study using ATH1 microarrays to analyze progressive changes in gene expression in cell cultures.

Transcript signal strengths for the 48 genes under investigation varied widely (Table I). Individual values for some genes were appreciably below the commonly accepted cutoff value of 100. Caution should be exercised when interpreting results with low signal

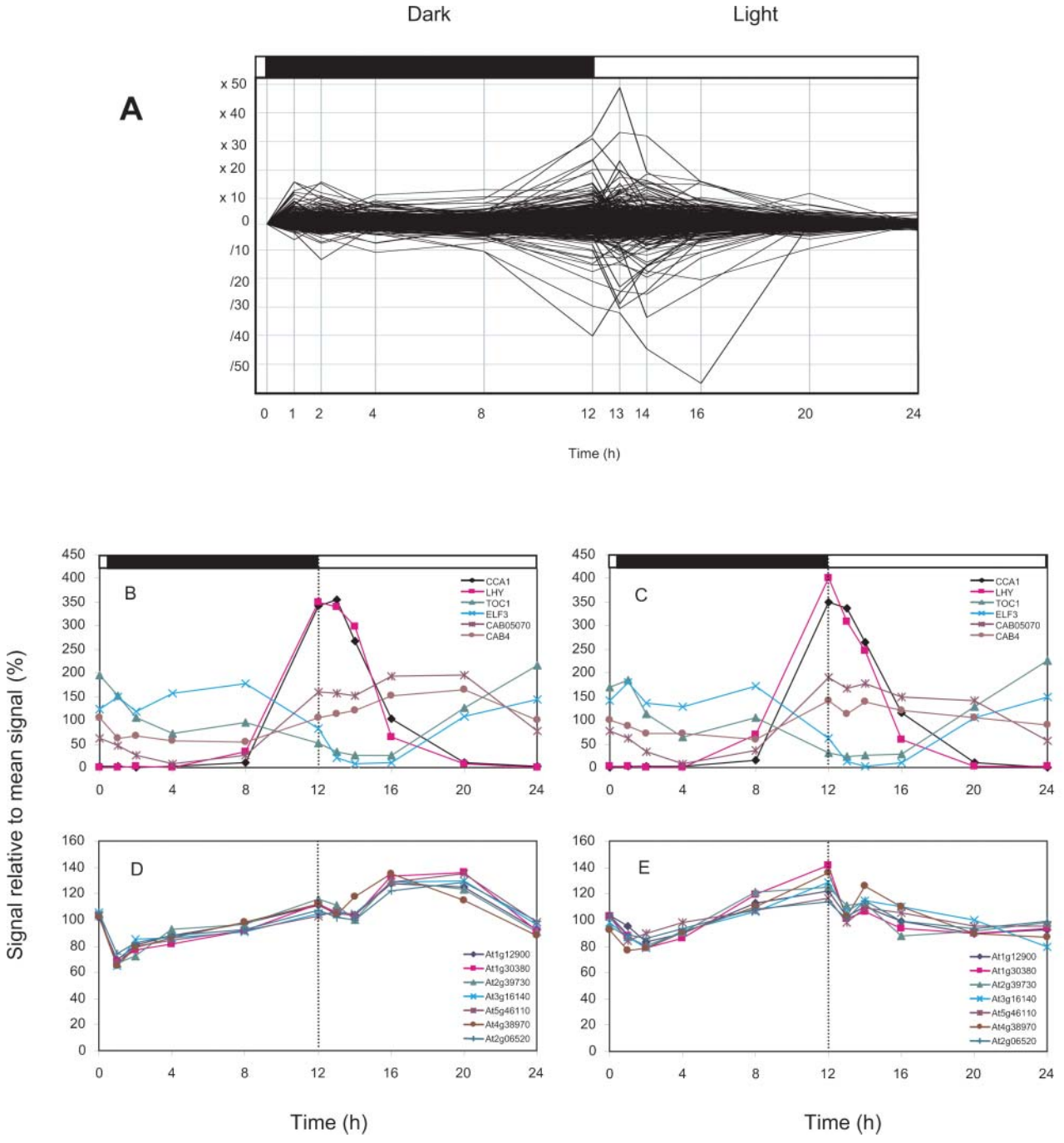

Figure 2. Comparison of expression profiles of gene subsets and genes with known diurnal regulation. A, Expression profile for a subset of 9,437 genes, which excludes those expressed at a very low level (6 or more signal values of less than 50). Expression values are expressed as fold change relative to values at time zero. The two genes showing greatest fold increase both encode proteins of unknown function (At5g54130 and At3g54500), while that showing greatest fold decrease encodes a putative Gly-rich RNA-binding protein (At2g21660). Four genes that show an approximately 4-fold increase in expression at 24 h relative to 0 h are predicted to encode a lipid transfer protein (At5g59320), a ubiquitin-related protease (At3g28220), vacuolar storage protein Vsp1 (At5g24780), and myrosinase-binding protein (At1g52040). B, Expression of *CCA1* (At2g46830), *LHY* (At1g01060), *TOC1* (At5g61380), *ELF3* (At2g25930), *CAB05070* (At2g05070), and *CAB4* (At3g47470) in experiment 1. C, Expression of the same genes shown in B, in experiment 2. D, Expression of TPT gene (At5g46110) and the six genes showing most similar expression profiles in experiment 1. Those genes encode glyceraldehyde 3-phosphate dehydrogenase (At12900), PSI subunit X (At1g30380), Rubisco activase (At2g39730), PSI subunit VI (At3g16140), Fru-1,6-bisphosphate aldolase (At4g38970), and a PSII protein (At2g06520). E, Expression of the same genes shown in D, in experiment 2.

strengths. The relationship between signal strength and mRNA levels was investigated by comparing Affymetrix signal strengths to mRNA abundance estimated by Massively Parallel Sequence Signatures (MPSS; Brenner et al., 2000), in rosette leaves 2 h after the transition from light to dark (http://dbixs001.dbi.udel.edu/MPSS4/java.html). The correspondence between the two sets of values was generally very good (data not shown), indicating that Affymetrix signals for most genes provide a good measure of mRNA abundance relative to the total amount of RNA in the sample. In a few cases in which there was not good correspondence, we compared both values with the frequencies of expressed sequence tags reported for each gene. Such considerations led us to the conclusion that for some genes (e.g. *PHS2* and *ISA3*), the Affymetrix signal strength probably overestimates mRNA abundance, while for others (e.g. *BAM9*) it underestimates (data not shown). However, for the purposes of the analysis presented here, the absolute amounts of each mRNA are generally less important than the temporal pattern of expression.

Expression of Genes Encoding Enzymes of Starch Synthesis

Results for experiments 1 and 2 were very similar for all genes of starch metabolism, so only those of experiment 1 are shown in subsequent sections, except where indicated. There are single genes encoding chloroplastic PGI and PGM, the enzymes responsible for conversion of Fru-6-P to Glc-6-P and Glc-1-P for starch synthesis. The transcripts for these two enzymes show different diurnal patterns: *PGI1* transcript changes relatively little, while *PGM1* transcript decreases during the dark and increases during the light (Fig. 3A). A total of six genes encode the large and small subunits of AGPase: four large subunit (*APL*) genes and two small subunit (*APS*) genes. Mutational analysis shows that most of the activity in the leaf comes from the *APS1* and *APL1* genes. Mutations in *APS1* result in plants (*adg1* mutants) with no measurable AGPase activity in the leaves and essentially no leaf starch (Lin et al., 1988a; Wang et al., 1998). Mutations in *APL1* result in plants (*adg2* mutants) lacking 95% of the AGPase activity and 60% of leaf starch (Lin et al., 1988b; Wang et al., 1997). Consistent with the major role for these two genes, their transcript levels are much higher than those of other AGPase genes (Table I). Transcripts for these two subunits show relatively little change in the diurnal cycle (Fig. 3B), although that of *APL1* does increase in the light phase, similarly to that of *PGI1*. It has been shown that *APS2* does not encode a functional AGPase, probably due to the absence of key amino acid residues (Crevillén et al., 2003; Hendriks et al., 2003), and its transcript level is very low.

There are five genes for STS and three for SBE, representing classes that are conserved across higher plants (Ball and Morell, 2003). Mutational analysis in species other than Arabidopsis suggests that each isoform plays a specific role in determining the struc-

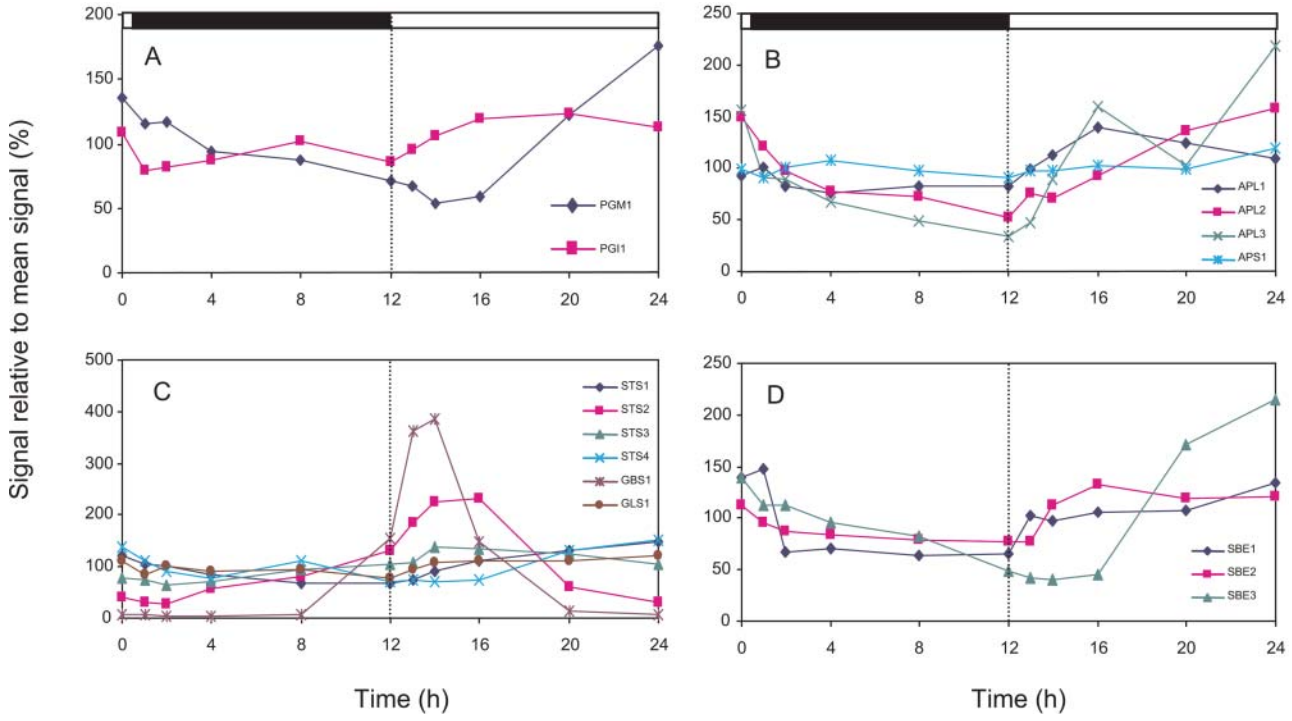

Figure 3. Expression patterns of genes encoding enzymes of starch synthesis. All data are from experiment 1. The key to gene nomenclature is given in Table I. Data for APL3 and APS2 are excluded due to very low expression (Table I).

ture and composition of the starch granule during its synthesis (e.g. Edwards et al., 1999; Schwall et al., 2000; Fulton et al., 2002). Transcript levels for these eight genes do not change in a coordinated way over 24 h (Fig. 3, C and D). In fact, no two genes show exactly the same pattern. Transcripts for granule-bound starch synthase (GBS) show a dramatic diurnal change, far greater than for the other STS and SBE enzymes. This change has been observed in other species and has been shown to be under circadian control (Merida et al., 1999; Wang et al., 1999). GBS differs from the other starch synthases in being located solely within the matrix of the starch granule and active in the synthesis of the amylose component of starch in that location. We speculate that the profound diurnal change in transcript levels might reflect a need for resynthesis of the GBS protein following granule degradation at night. Immunoblot analysis of GBS levels in the insoluble (starch-containing) and soluble fractions of the leaf during a 12-h light period support this idea. GBS protein is not detectable at any point in the soluble fraction of the leaf (data not shown). On a fresh-weight basis, the amount in the insoluble fraction of the leaf is very low at the end of the night. It increases dramatically during the first 4 h of the day (Fig. 4A, compare time 0 and 4 h). On a starch-weight basis, the protein is most abundant at the end of the night and falls in amount throughout the day (Fig. 4). Taken together with the diurnal pattern of synthesis and degradation of starch (Fig. 1), these data suggest that GBS is destroyed as it is released from granules during degradation at night. At the end of the night, the amount in the leaf is very low. GBS synthesis occurs during the first part of the light period, then slows or stops while starch synthesis continues. Thus, GBS protein is apparently more abundant toward the center of the granule than at the periphery, and, hence, the residual starch at the end of the night has a very high GBS content. Therefore, the rise in GBS transcript level in the latter part of the night and the first 2 h of the day would be associated with the daily period of synthesis of this protein.

A second starch synthase gene *STS2*, representing the SSII class, also shows a marked diurnal change in transcript level. Although this isoform is probably active in amylopectin synthesis at the outer edge of the granule, in several plant organs it is known to become buried within starch granules as starch synthesis proceeds (e.g. Denyer et al., 1993). It is possible that the diurnal behavior of transcript and protein of this isoform is the same as that proposed for GBS above.

Levels of transcripts for *SBE1* and *SBE2* show small changes at the start and end of the night, but *SBE3* shows a distinctive pattern of decline through the night and a rapid increase after 4 h in the light period (Fig. 3D). We observed a pattern very similar to that of *SBE3* for several enzymes with putative roles in starch degradation (see below). Levels of transcript for both of the isoamylases involved in starch synthesis (ISA1 and ISA2) are generally higher during the day than at night, and their patterns of diurnal change have some similar features (Fig. 5A). The pattern for the third isoamylase gene, *ISA3*, matches closely the pattern shown by several genes encoding enzymes putatively involved in starch degradation (see below).

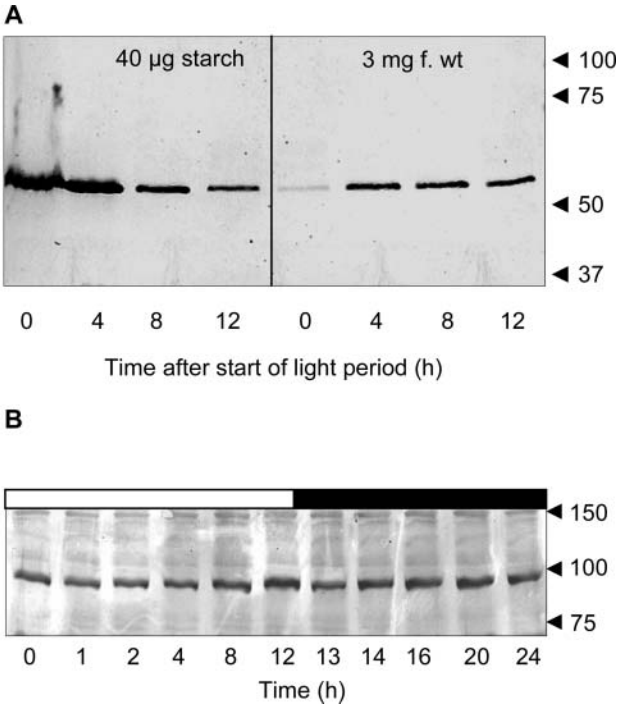

Figure 4. Diurnal changes in amounts of GBS and DPE2 proteins. A and B show immunoblots of 7.5% SDS-polyacrylamide gels. Positions of molecular mass markers are indicated (masses in kD). A, Immunoblot developed with an antiserum to GBS (raised against the pea embryo protein, used at a dilution of 1:500). A, left, Lanes are loaded with 40 μg of starch purified from leaves harvested at the times indicated, from plants grown in 12-h-light/12-h-dark regime. A, right, Lanes are loaded with buffer-insoluble material from the equivalent of 3 mg fresh weight of leaf, from leaves harvested at the times indicated. B, Immunoblot developed with a peptide-specific antiserum to DPE2 (used at a dilution of 1:3,000). Lanes are loaded with soluble extract from the equivalent of 15 mg fresh weight of leaf, from leaves harvested at the times indicated after the start of the light period, from plants grown in 12-h-light/12-h-dark regime.

A Coexpressed Gene Set Encoding Putative Enzymes of Starch Degradation

Transcripts encoding enzymes potentially involved in starch breakdown exhibit a wide range of expression profiles (Fig. 5). Emerging from this range is a distinctive pattern shown by nine genes, of slow decline during the night to a low level during the first few hours of the light, followed by a rapid increase between 4 and 8 h of the light (Fig. 6). This distinctive expression pattern is highly consistent between the two experiments (Fig. 6, A and B) and is suggestive of coordinate regulation. This set includes genes encoding three enzymes shown to be necessary for normal

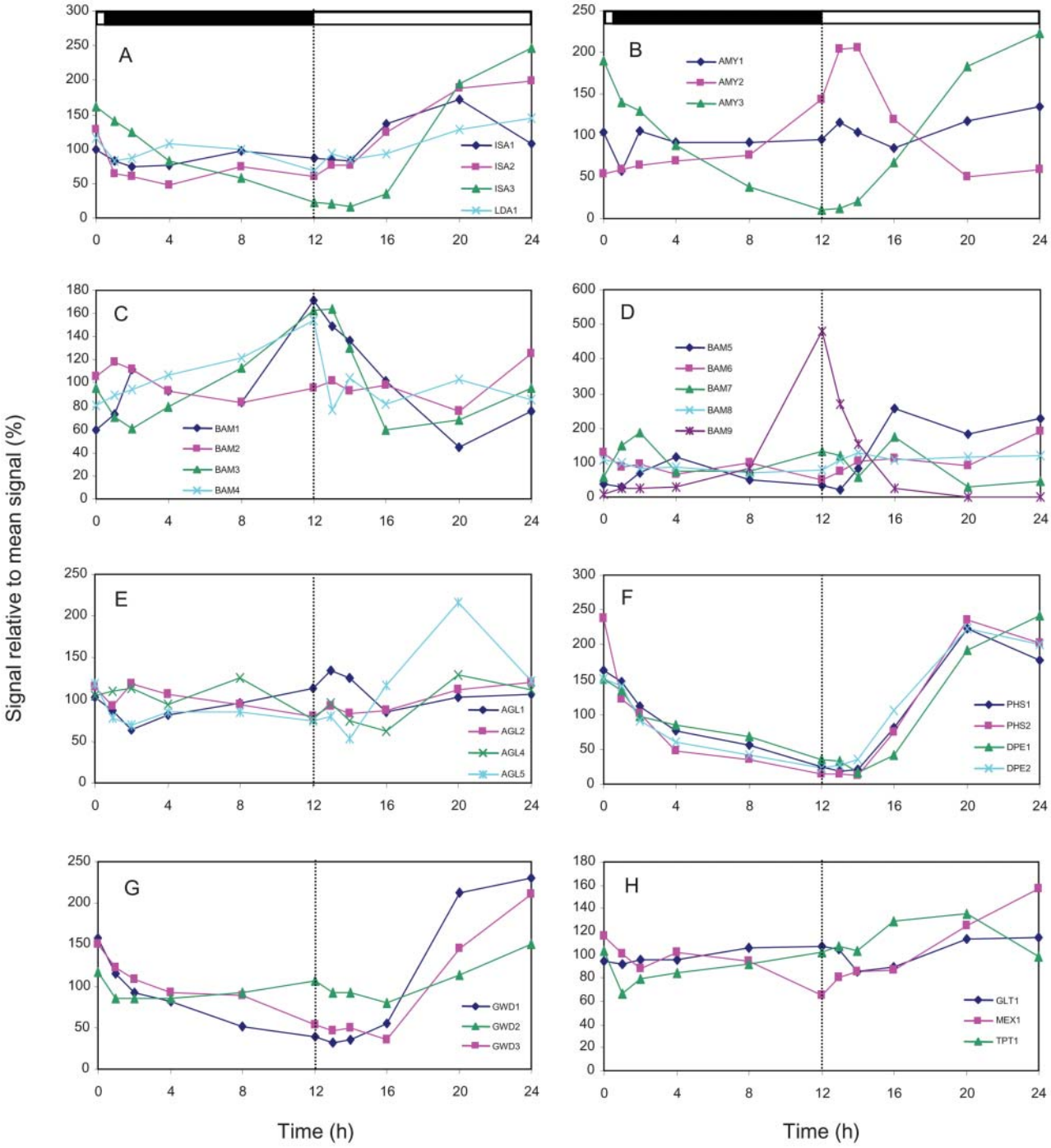

Figure 5. Expression patterns of genes encoding enzymes potentially involved in starch breakdown. All data are from experiment 1. The key to gene nomenclature is given in Table I. Data for AGL3 are excluded due to very low expression (Table I).

rates of starch degradation, namely GWD1 (Yu et al., 2001), DPE1 (Critchley et al., 2001), and DPE2 (Lu and Sharkey, 2004; Chia et al., 2004). It also includes other enzymes that might reasonably be expected to participate in starch degradation. These include AMY3, plastidial starch phosphorylase (PHS1), a debranching enzyme (ISA3), and GWD2, which is closely related in sequence to GWD1. However, PHS1 and AMY3 appear thus far to be dispensable for starch degradation (Zeeman et al., 2004a; H. Dunstan, D. Fulton, and S. Smith, unpublished data), and the functions of ISA3 and GWD2 have not yet been reported. Another enzyme in this group is cytosolic glucan phosphorylase (PHS2). A possible role for

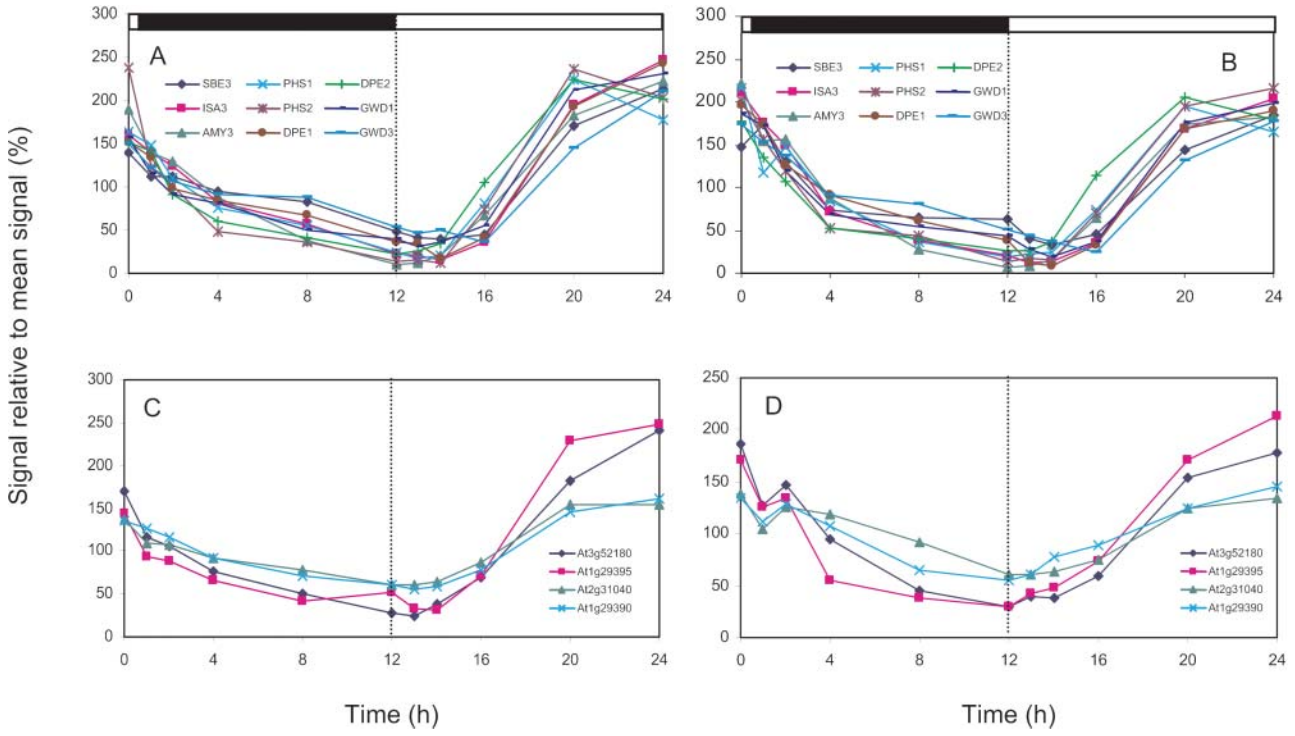

Figure 6. Coexpression of genes of starch metabolism plus four genes encoding proteins of unknown function. Data are from experiment 1 (A and C) and experiment 2 (B and D). The key to gene nomenclature is given in Table I except for the four genes of unknown function.

cytosolic glucan phosphorylase in the conversion of starch to Suc is suggested by the discovery that DPE2 transfers a glucosyl moiety from maltose to an unknown cytosolic acceptor likely to be a glucan (Chia et al., 2004). The PHS2 enzyme could convert glucosyl moieties from the glucan acceptor into Glc-1-P, which would then be available for Suc synthesis. The fact that the expression profile of cytosolic starch phosphorylase is the same as that of DPE2 and other enzymes known to be involved in starch degradation supports this hypothesis. The ninth member of this group, SBE3, is more unexpected since branching enzymes are considered to be involved in starch synthesis. For at least some of the transcripts showing this distinctive diurnal pattern, the amounts of the proteins that they encode do not change consistently or appreciably through the diurnal cycle. Under the same growth conditions as those used for transcript analysis, immunoblots revealed no appreciable differences in amount of GWD1 (Yu et al., 2001) or AMY3 (J.-C. Chen, unpublished data). Similarly, DPE2 does not change in amount (Fig. 4B). Thus, the amounts of these proteins are controlled posttranscriptionally.

Genes Encoding Other Enzymes Putatively Involved in Starch Degradation

Transcript levels of AMY1 and 2, GWD3, the debranching enzyme LDA1, and the AGLs do not conform to the coordinated pattern described above (Figs. 5 and 6). Each shows a distinctive pattern of change over 24 h. Transcript levels of AMY2 show a particularly strong diurnal change. There is a marked increase at the end of the night and a fall in the first few hours of the day. The functions of these enzymes are not yet known. LDA1 and GWD3 are predicted to be plastidial, but the others have no obvious plastid transit peptides. Knockout mutants of AMY2 and 3 and LDA1 are not impaired in leaf starch breakdown when grown under the conditions used here (H. Dunstan, D. Fulton, S. Zeeman, and S. Smith, unpublished data). There is no obvious requirement for α-glucosidases (maltases) in the conversion of starch to Suc. The massive accumulation of maltose in *mex1* and *dpe2* mutants (Chia et al., 2004; Niittylä et al., 2004) suggests that maltose produced via BAM in the chloroplast is exported and then metabolized in the cytosol via a glucosyltransferase reaction. None of the nine BAM genes has the coordinated diurnal pattern of expression discussed above (Fig. 5). Each of the four BAMs predicted to be plastidial shows a different pattern of diurnal changes in transcript levels (Fig. 5C). Our preliminary analysis of knockout mutants indicates that BAM4 is necessary for normal rates of starch degradation (D. Fulton, H. Dunstan, S. Zeeman, and S. Smith, unpublished data), but the roles of the other BAMs has yet to be investigated. Surprisingly, the transcript signal for BAM4 is very low (Table I), and MPSS data confirm that this gene is only weakly expressed in leaves. Among the isoforms predicted to

be extraplastidial, one isoform (BAM9) shows a massive diurnal change in transcript level, as reported previously (Chandler et al., 2001). This pattern is similar to that of AMY2, with a dramatic increase at the end of the night and a fall in the first few hours of the day. BAM5 is responsible for most of the BAM activity of the Arabidopsis leaf and is believed to be located in the phloem (Wang et al., 1995). Mutations eliminating this protein (at the *RAM1* locus) have no obvious effect on plant growth and metabolism, and the function of this enzyme remains to be discovered (Laby et al., 2001). In experiments 1 and 2, respectively, the *BAM5* transcript level is 5 and 2 times higher at 24 h than at 0 h. This might reflect disturbances to the plants during leaf harvesting and the responsive nature of this gene to environmental or metabolic changes (Caspar et al., 1989; Laby et al., 2001).

The transcript levels for the TPT1, GLT1, and MEX1 transporters show intricate and different patterns of diurnal change, of relatively low amplitude (Fig. 5H). Our recent work shows that most of the carbon derived from starch is exported by the maltose transporter MEX1, with a smaller contribution from GLT1 (Niittylä et al., 2004). The pattern of expression of these genes does not resemble the coordinated pattern for nine starch-degrading enzymes described above or the patterns for any of the BAMs that generate maltose in the chloroplast.

The pathway by which maltose exported from the chloroplast is converted to Suc in the cytosol is not fully understood (Chia et al., 2004). However, it seems highly likely that Suc synthesis at night will at least in part involve the same enzymes as those active in this process during the day. Accordingly, we inspected transcripts for cytosolic enzymes required to convert Glc to Suc (hexokinase, cytosolic PGI and PGM, UDP-Glc pyrophosphorylase, Suc phosphate synthase, and Suc phosphate phosphatase). These show complex patterns of changes through the diurnal cycle. While some of these transcripts show similar changes to each other, none shows a pattern similar to those of transcripts constituting the starch transcriptome (data not shown).

DISCUSSION

Analysis of diurnal changes in the starch transcriptome reveals that it is very complex. For the most part, transcripts encoding enzymes thought to function in the same pathway do not show similar patterns of change over 24 h. For example, mutational and transgenic analysis in several species reveals that starch structure is determined by the coordinated actions of the multiple isoforms of starch synthase and starch branching enzyme (Schwall et al., 2000; Fulton et al., 2002; Jobling et al., 2002). In spite of this, no two isoforms of these enzymes show the same diurnal pattern of transcript change. Changes in transcript abundance are also not coordinated for the major two subunits of AGPase expressed in the leaf, or for the chloroplastic BAMs, disproportionating enzyme (DPE1), and the maltose and Glc transporters (MEX1 and GLT1, respectively), which together constitute a pathway of glucan degradation and export to the cytosol at night (Chia et al., 2004; Niittylä et al., 2004).

We identified a set of nine genes that appear to be coordinately expressed. Transcripts decline during the dark and characteristically increase rapidly between 4 and 8 h in the light. Transcripts for eight of these genes encode enzymes that are either known to be required for starch breakdown or could reasonably be expected to be. However, they do not constitute a complete pathway (for example, no BAMs are included), and they include proteins apparently not necessary for starch degradation (PHS1 and AMY3; Zeeman et al., 2004a; H. Dunstan, D. Fulton, and S. Smith, unpublished data). Occurrence of this set may nonetheless reflect interactions between all of these enzymes at some level, and its discovery prompts a more detailed examination of the in vivo functions of all enzymes in this group.

This lack of correspondence between RNA and protein for three members of the coordinately expressed group (AMY3, GWD1, and DPE2) indicates the importance of posttranscriptional control in regulating amounts of enzymes of starch metabolism and the fluxes through the key pathways. Control over amounts of protein may be exerted at the levels of both translation and protein stability. It is possible, for example, that the changes in transcript level for the coordinated set of enzymes reflect a substantial turnover of these proteins on a diurnal basis. The simultaneous occurrence of translation and protein degradation during a specific, limited period of the day would allow protein levels to be maintained at constant levels throughout the day. Control of flux through the pathways of starch synthesis and degradation is likely to be exerted primarily through modulation of enzyme activity. Little is known about control of starch degradation, but synthesis is thought to be controlled primarily via modulation of AGPase activity via metabolites and via redox-mediated changes in enzyme structure (Neuhaus and Stitt, 1990; Hendriks et al., 2003).

The exception to this picture is GBS1 (and possibly STS2). Because of the location of this protein inside the starch granule and the requirement for the granule matrix for its activity and stability (Denyer et al., 1999; Edwards et al., 1999; Tatge et al., 1999), the protein is lost during the dark period as starch is degraded. It is resynthesized during the first part of the light period, when transcript levels are at their maximum.

Although there is no obvious correspondence between transcript levels, levels of the encoded proteins, and fluxes in starch metabolism, the information obtained about individual transcripts may nonetheless help to direct future experiments to reveal the functions of the encoded proteins. For example, the isoamylase ISA3 is expressed coordinately with genes encoding degradative enzymes, while *ISA1* and *ISA2*,

shown to be required for starch synthesis, show a different pattern. Thus, ISA3 merits investigation as a debranching enzyme involved in starch degradation in the chloroplast. Changes in levels of transcript of BAM9 are strikingly similar to those of AMY2. Both enzymes are extraplastidial; perhaps both are involved in the degradation of same, as yet unknown glucan.

A potential use of the coordinately expressed gene set is to search for other genes showing a similar pattern of expression. This may help to identify new proteins involved in starch metabolism. We searched the entire transcriptome for transcripts showing this same pattern of expression, expressed to an appreciable level, and encoding proteins with putative plastid transit peptides. This search identified a small number of genes encoding proteins of unknown function. The expression patterns for four of these genes are shown for experiments 1 and 2 (Fig. 6, C and D). Transcripts for these genes show a small increase in level between 1 and 2 h in experiment 2 but not in experiment 1 (Fig. 6, C and D). Transcripts for AMY 3 and PHS1 show a similar increase in level in experiment 2 but not in experiment 1 (Fig. 6, A and B). Such subtle differences in expression level observed between different experiments can be particularly helpful in identifying coexpressed genes. The possibility that these proteins of unknown function are involved in starch degradation is under investigation.

The coexpression of nine genes of starch metabolism suggests that they could be coregulated. The promoter sequences of these genes range from 550 to 4,680 bp (defined as the intergenic regions upstream of the ATG translation start codon). Searches for previously identified putative cis-acting regulatory elements, using programs such as PLACE (Higo et al., 1999), revealed several such elements of 4 or 5 bp in all nine promoters, including the GATA and I box core sequences found in light-regulated genes (Terzaghi and Cashmore, 1995). However such elements were also found in promoters of other genes with quite different transcript profiles (data not shown). Searches for novel motifs in all nine promoters using a range of programs (Rombauts et al., 2003) failed to find any conserved motifs greater than 5 bp. Similarly, comparisons of the 3′ untranslated regions of all nine genes failed to find conserved motifs. Future analysis of the sequences that might regulate expression of this gene set will require experimental manipulation of the DNA sequences.

In summary, it is clear that the relationships between transcript levels, protein levels, enzyme activities, and fluxes of carbon into and out of starch are extremely complex. Nevertheless, analysis of the starch transcriptome can provide clues about the possible roles of individual proteins and can help to identify new proteins involved in starch synthesis and breakdown. Analyses of changes in the transcriptome in other situations, such as in response to sugar feeding of heterotrophic tissues or during the development of seeds, may provide yet further insight into the starch transcriptome and the regulation of starch synthesis and breakdown. Our results have already identified new targets for study and new hypotheses to test.

MATERIALS AND METHODS

Plant Material

Arabidopsis Col-0 was obtained from the NASC (accession no. N1093). It was sown in a peat-based compost and incubated at 4°C for 3 d, then grown at 20°C, 75% relative humidity, in a 12-h-light/12-h-dark photoperiod with an irradiance of 180 μmol m^{-2} s^{-1}. Plants were grown to growth stage 3.90 (Boyes et al., 2001) and labeled for leaf harvesting using a random number generator. At each of 11 time points, three fully expanded (source) leaves were harvested from each of eight plants and frozen in liquid nitrogen. RNA was isolated and purified using Qiagen RNeasy kits (Crawley, UK).

Microarray Analysis

RNA was freeze-dried and sent to NASC, where quality control analysis was carried out before labeling and hybridization to 22,800 Affymetrix ATH1 genome arrays (Santa Clara, CA). Procedures for hybridization and data handling are given on the NASC Web site (http://nasc.nott.ac.uk/). Clustering of transcript patterns was carried out using Silicon Genetics GeneSpring 6 (http://www.silicongenetics.com; Redwood City, CA).

GBS and DPE2 Immunoblots

Immunoblotting for DPE2 was according to Chia et al. (2004) and for GBS was according to Zeeman et al. (2002).

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