Glucan, Water Dikinase Exerts Little Control over Starch Degradation in Arabidopsis Leaves at Night[1][W][OPEN]

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The first step on the pathway of starch degradation in Arabidopsis (*Arabidopsis thaliana*) leaves at night is the phosphorylation of starch polymers, catalyzed by glucan, water dikinase (GWD). It has been suggested that GWD is important for the control of starch degradation, because its transcript levels undergo strong diel fluctuations, its activity is subject to redox regulation in vitro, and starch degradation is strongly decreased in *gwd* mutant plants. To test this suggestion, we analyzed changes in GWD protein abundance in relation to starch levels in wild-type plants, in transgenic plants in which *GWD* transcripts were strongly reduced by induction of RNA interference, and in transgenic plants overexpressing GWD. We found that GWD protein levels do not vary over the diel cycle and that the protein has a half-life of 2 d. Overexpression of GWD does not accelerate starch degradation in leaves, and starch degradation is not inhibited until GWD levels are reduced by 70%. Surprisingly, this degree of reduction also inhibits starch synthesis in the light. To discover the importance of redox regulation, we generated transgenic plants expressing constitutively active GWD. These plants retained normal control of degradation. We conclude that GWD exerts only a low level of control over starch degradation in Arabidopsis leaves.

Many plants accumulate starch as a product of photosynthesis during the day and then degrade it at night, thereby ensuring a continued supply of sugars when primary photoassimilate is unavailable. In Arabidopsis (*Arabidopsis thaliana*) plants grown in standard controlled conditions, starch accumulation typically accounts for 40% of the carbon assimilated during the day. Degradation is initiated at the beginning of the night and proceeds at a constant rate such that around 95% is used by dawn. Remarkably, if plants are subject to an unexpectedly early night (e.g. a shift from a 12-h day to an 8-h day), the rate of starch degradation is immediately decreased relative to that on the previous night, such that reserves last until dawn (Lu et al., 2005; Graf et al., 2010). Likewise, if plants are subjected to a single day of decreased light, leading to a low starch content at the end of the day, then the rate of starch degradation is adjusted in the subsequent night such that reserves last until dawn (Scialdone et al., 2013).

This exquisite regulation of starch degradation is crucial for normal growth. Root extension growth falls by more than 50% in 10 h in Arabidopsis plants subject to an unexpected extension of the night, after starch reserves are exhausted (Yazdanbakhsh et al., 2011), and application of exogenous Suc slows this decline in growth rate. An extended night is accompanied by the activation of a large set of starvation genes, many involved in catabolic processes (Thimm et al., 2004; Usadel et al., 2008), that are expressed only at very low levels during the normal day/night cycle. Plants that exhaust their starch reserves before dawn due to growth in day/night cycles longer than their circadian clock period also show the activation of starvation genes at the end of each night and decreased productivity relative to plants in which the length of the day/night cycle matches the clock period (Graf et al., 2010).

Despite its importance for productivity, we do not yet know how the rate of starch degradation is controlled. Although the major enzymes that hydrolyze the 1,4- and 1,6-linkages of the Glc polymers that constitute the starch granule, BETA-AMYLASE3 (BAM3) and ISOAMYLASE3 (ISA3), are inhibited by oxidation in vitro (Glaring et al., 2012), there is no evidence that modulation of their activities is important for flux control in vivo. Instead, attention has focused on a cycle of phosphorylation and dephosphorylation of the starch granule surface that is necessary for normal rates of starch degradation (Weise et al., 2012). Glc residues within amylopectin polymers at the starch surface are phosphorylated by glucan, water dikinase (GWD) and phosphoglucan, water dikinase (PWD) at the 6- and 3-positions, respectively (Baunsgaard et al., 2005; Kötting et al., 2005; Ritte et al., 2006; Hejazi

[1] This work was supported by European Union Framework Program 7 Collaborative Project TiMet (grant no. 245143), an Institute Strategic Programme Grant to the John Innes Centre from the Biotechnology and Biological Sciences Research Council (grant no. BB/J004561/1), and Ph.D. studentships from the Biotechnology and Biological Sciences Research Council and the John Innes Foundation (to A.W.S., A.G., and Z.D.).

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[W] The online version of this article contains Web-only data.

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www.plantphysiol.org/cgi/doi/10.1104/pp.114.237016

et al., 2009). These phosphate groups are subsequently removed by two glucan phosphate phosphatases. STARCH EXCESS4 (SEX4) removes phosphate groups from both the 6- and 3-positions (Niittylä et al., 2006; Kötting et al., 2009); LIKE SEX FOUR2 (LSF2) is specific for the 3-position (Santelia et al., 2011). Although phosphorylation and dephosphorylation occur during both the synthesis and degradation of starch (Ritte et al., 2004; Santelia et al., 2011), the importance of the cycle is thought to be in facilitating degradation by BAM3 and ISA3 (Edner et al., 2007; Hejazi et al., 2008, 2009; Kötting et al., 2009). Plants lacking GWD, PWD, or SEX4 accumulate abnormally high levels of starch, consistent with limited hydrolysis during the night (Yu et al., 2001; Baunsgaard et al., 2005; Kötting et al., 2005; Niittylä et al., 2006). Studies of the actions of recombinant forms of these enzymes on starch granules and crystallized maltodextrins in vitro reveal that phosphorylation disrupts the crystalline packing of helices formed by adjacent Glc chains and may unwind helices (Hejazi et al., 2008, 2009, 2010; Blennow and Engelsen, 2010). This amorphization (Blennow and Engelsen, 2010) facilitates the actions of starch-hydrolyzing enzymes on the granule surface in vitro (Edner et al., 2007). The removal of phosphate groups by SEX4 and LSF2 then permits the complete degradation of Glc chains by the hydrolyzing enzymes (Kötting et al., 2009; Santelia et al., 2011).

The starch-phosphorylating enzymes are attractive candidates for the control of flux through starch degradation. They are effectively the first step on the pathway, and modulation of their activities could potentially determine the rate of degradation of the granule. Both enzymes possess properties consistent with modulation of their activities over the day/night cycle. Transcript levels undergo large daily changes from high at the end of the day to very low at the end of the night (Smith et al., 2004; Baunsgaard et al., 2005). Immunoblots suggest that protein levels do not undergo such large daily changes as transcript levels (Yu et al., 2001; Kötting et al., 2005), and activity in crude extracts of leaves is reported to be similar regardless of the time of harvest (Ritte et al., 2003), but no quantitative data are available. GWD is also subject to redox regulation. A disulfide bond can be reduced in vitro by micromolar concentrations of reduced thioredoxins, resulting in activation of the enzyme. GWD in the soluble fraction of plant extracts is in the reduced form, but a fraction of the enzyme bound to starch granules was reported to be in an oxidized, inactive form (Mikkelsen et al., 2005). The reactions catalyzed by both dikinases involve ATP hydrolysis and glucan hydration and so are likely to be associated with large, negative free energy changes. Some modulation of activity must occur in order to prevent the excessive consumption of ATP by these enzymes.

Recent work indicates that PWD is a target for mechanisms that integrate information about starch content and time until dawn to set an appropriate rate of starch degradation. We showed that a mutant lacking PWD failed to adjust the rate of starch degradation in response to an unexpectedly early night, whereas adjustment occurred in several mutants lacking other components of the starch degradation pathway, including SEX4, BAM3, and ISA3 (Scialdone et al., 2013). It is not possible to examine whether GWD is also required for the adjustment of starch degradation according to the length of the night, because the rate of degradation is too low to measure accurately in this mutant. However, GWD would seem to be an excellent candidate for a control point in the pathway. It is effectively upstream of PWD in the pathway, because the action of PWD requires prior disruption of the ordered structure of the starch granule surface (Hejazi et al., 2009). Its loss brings about the strongest starch degradation phenotype reported for any single mutant: starch contents are up to 5 times greater than in wild-type plants at the end of the day, and there is very little change in starch content over the day/night cycle (Yu et al., 2001).

To discover the importance of GWD for modulation of the flux through starch degradation, we generated transgenic Arabidopsis lines in which GWD protein levels could be manipulated through inducible RNA interference (RNAi) and lines in which native GWD was replaced by a form that is constitutively reduced, and therefore active, under all conditions. This material was used to assess the importance of daily transcriptional regulation for GWD protein abundance, to measure the flux control coefficient of the enzyme with respect to starch degradation, and to discover whether redox inactivation of the enzyme is important for its role in vivo.

RESULTS

GWD Shows Strong Oscillations in Transcript Abundance But Not in Protein Abundance

To obtain quantitative information about GWD protein levels over a day/night cycle with sufficient sensitivity to detect small changes, we used a label-free, peak area-based mass spectrometric method. Soluble protein was extracted from leaves harvested at four time points over 24 h. Following SDS-PAGE, gel slices containing proteins of greater than 100 kD, including the 156-kD GWD protein, were subjected to tryptic digestion. Eluted peptides were analyzed using an LTQ Orbitrap XL mass spectrometer. Normalization and scaling were applied to outputs to provide quantitative data on relative protein abundance between samples. We found no statistically significant variation in GWD protein abundance over a day/night cycle (Fig. 1A). This result contrasts with the previously reported substantial fluctuation in transcript levels of GWD over a day/night cycle, with highest values at the end of the light period and lowest values at the end of the night (Smith et al., 2004; Bläsing et al., 2005). Publicly available *GWD* transcript values from an experiment using the same growth conditions as ours (Bläsing et al., 2005) are shown for comparison in Figure 1B.

We used the same protein and transcript data sets to investigate whether the striking difference between patterns of abundance of transcript and protein for

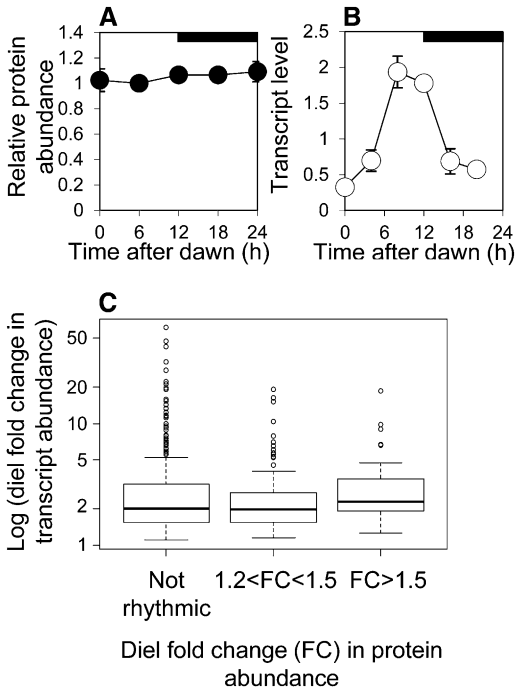

Figure 1. Changes in protein and transcript levels over 24 h. A, GWD protein levels over 24 h measured using quantitative proteomics. The black bar shows the dark period. Values are relative to the geometric mean of all GWD abundance values for the time course. Values are means of measurements on eight, four, seven, seven, and eight individual rosettes for time points 0, 6, 12, 18, and 24 h, respectively, ± SE. B, GWD transcript levels over 24 h taken from a published experiment using the same growth conditions as in A (Bläsing et al., 2005; sourced from the National Center for Biotechnology Information Gene Expression Omnibus data browser [www.ncbi.nlm.nih.gov/gds], record no. GDS1757). Values are means ± SE of measurements using the GeneChip Arabidopsis ATH1 genome array on three biological replicates. C, Box plots showing the diel fold change in transcript abundance for nonrhythmic proteins, rhythmic proteins with a fold change (FC) between 1.2 and 1.5, and rhythmic proteins with a fold change greater than 1.5. The boxes show the positions of the 25th, 50th, and 75th percentiles, and whiskers denote 1.5× the interquartile range.

GWD is exceptional. The strong pattern of change in transcript abundance seen for GWD is also seen for at least 10 other enzymes of starch metabolism (Smith et al., 2004; Santelia et al., 2011), three of which were represented in our protein data set. These were the chloroplastic α-amylase AMY3, the starch-branching enzyme SBE3, and the cytosolic glucanotransferase DISPROPORTIONATING ENZYME2 (DPE2; Smith, 2012). For all three, there was no significant diurnal change in protein abundance (using cutoff criteria of a 1.2-fold change and $P < 0.05$).

This experiment provided good-quality data for a further 387 proteins (Supplemental Table S1). To compare the patterns of change in the abundance of proteins with those of their transcripts, we used data for transcript abundance over 24 h obtained by Bläsing et al. (2005) from plants grown in the same conditions (sourced from the National Center for Biotechnology Information Gene Expression Omnibus data browser; record no. GDS1757). We defined proteins with diel changes in abundance as those with a maximum fold change greater than a given cutoff (1.2 or 1.5) and a statistically significant variation over the time course (ANOVA, $P = 0.05$; multiple testing correction as in Benjamini and Hochberg, 1995). Using these criteria, the abundance of 35% of proteins changed more than 1.2-fold during 24 h but only 8% (31 proteins) changed in abundance by more than 1.5-fold. For most proteins, diel changes in abundance did not directly reflect diel changes in transcript levels (Fig. 1C). In general, proteins varied less in abundance over the diel cycle than their transcripts (Supplemental Fig. S1). An exception to this trend was the major small subunit of ADPglucose pyrophosphorylase (ADG1; At5g48300), which showed a greater fold change in protein abundance than in transcript abundance over the day/night cycle (Supplemental Fig. S2). This result is consistent with a previous report that the change in AGPase activity over the day/night cycle is greater than the change in *ADG1* transcript abundance (Gibon et al., 2004a). In addition, the times of peak abundance for proteins and transcripts were often different. The phase of the protein oscillation in some cases matched and in other cases was different from that of the transcript. Further information about and discussion of the relationship between protein and transcript levels over a day/night cycle can be found in Supplemental Figure S3.

GWD Has a Long Half-Life and a Low Flux Control Coefficient for Starch Degradation

The results above indicate that transcriptional regulation of GWD protein levels is unlikely to be important for the control of starch degradation. However, they do not rule out this possibility. This is because the transcriptional oscillations could be driving large daily changes in GWD protein turnover, perhaps with regulatory consequences. For a better description of the system, the half-life of the GWD protein should be measured and its flux control coefficient calculated. We achieved both of these objectives by generating Arabidopsis plants expressing a dexamethasone (dex)-inducible silencing (RNAi) construct targeted at *GWD* (referred to as the RNAi lines) and, as controls, plants transformed with the same construct without *GWD* sequences (referred to as the control line). In a preliminary experiment, plants of two RNAi lines and a control line were sprayed with dex 2 h after dawn and at the same time point 2 and 8 d later. Plants were harvested for starch measurements at the end of the night at intervals up to 9 d after the first dex treatment. Starch content remained low in the control line but started to rise progressively between 2 and 6 d after the first dex treatment in both RNAi lines. By 14 d, starch content at the end of the night in the two RNAi lines was 6 and 12 times greater than that in the control line, respectively. Subsequent experiments were carried out on the stronger RNAi line (Fig. 2A).

In experiments to discover the relationship between *GWD* transcript, GWD protein, and starch content, plants

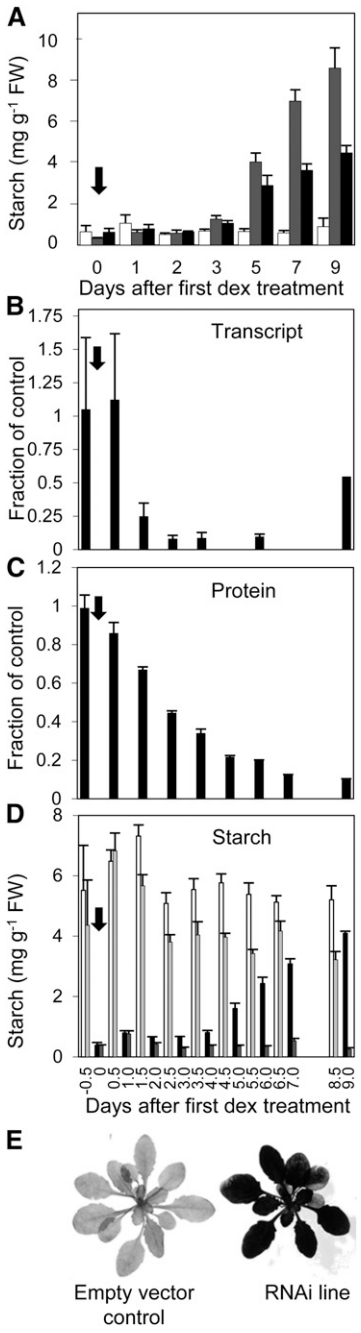

Figure 2. Time course of changes following the induction of *GWD* RNAi. Eighteen-day-old plants were sprayed with dex to induce RNAi at day 0 (arrows) and again at intervals specified in the text. A, Starch contents at the end of the night in a control line (white bars) and two RNAi lines (dark gray and black bars). Values are means ± SE of measurements made on six rosettes. The RNAi line represented by the dark gray bars was used in the experiments shown in B to E. FW, Fresh weight. B, *GWD* transcript abundance in the RNAi line at the end of the day, expressed as a fraction of that in the control line at the same time point. Values are ratios of means ± SE. For each line and time point, measurements were made by quantitative PCR on four biological replicates, each of four plants. C, GWD protein abundance in the RNAi line, expressed as a fraction of the geometric mean of all values for the control line. Values are ratios of means ± SE. Measurements were made by quantitative proteomics on four biological replicates, each of four plants. D, Starch contents at the end of the day (white and light gray bars) and the end of the night (black and dark gray bars) in the RNAi line (white and black bars) and the control line (light and dark gray bars). Values are means ± SE of measurements made on four or five individual rosettes. E, Iodine-stained rosettes at the end of the night, 10 d after the first dex treatment. Left, control line; right, RNAi line.

were treated with dex at 2, 4, and 6 d after the first treatment and harvested at intervals over 9 d after the first treatment. Treated plants appeared identical to untreated plants grown at the same time under the same conditions. *GWD* transcript and protein levels and starch content were essentially the same in the RNAi line and the empty vector control prior to dex treatment. *GWD* transcript abundance at the end of the day declined in the RNAi line to 25% of the control value within 1.5 d after the first dex treatment (Fig. 2B) and plateaued at 8% of the control value by 2.5 d after the first dex treatment. There was partial recovery of transcript levels 8.5 d after the first dex treatment (2.5 d after the last dex treatment). GWD protein levels decreased at a much slower rate, falling below 50% of the control value by 3 d and reaching 20% at 5 d and 11% at 9 d after the first treatment (Fig. 2C). Starch levels at the end of the night began to increase in the RNAi line 5 d after the first dex treatment. Between 4 and 7 d after treatment, the amount of starch degraded during the night fell by 57% (Fig. 2D). By 9 d, the RNAi line contained more than 16 times as much starch as the control line at the end of the night. Iodine staining showed that the starch content in the RNAi line at the end of the night was high throughout the rosette at this point (Fig. 2E). In the control line, none of the parameters measured showed any obvious or consistent variation over the time course.

The half-life of the GWD protein was calculated from its abundance between 2 and 7 d after the first dex treatment. We assumed that GWD protein synthesis was negligible in this period due to the very low transcript levels. Nonlinear regression was used to fit an exponential decay curve to the data, according to $G = A + B \times C^t$, where G is GWD protein level, t is time, and A, B, and C are constants. Given the data, it was reasonable to set A to 0 and constrain C to values less than 1 during the fitting process. The fit to the data was excellent ($r^2 = 0.98$; Fig. 3A). The constant B was estimated at 1.17 ± 0.04 (SE) and C at 0.69 ± 0.008 (SE). The half-life of the protein ($t_{1/2}$) was then calculated as $t_{1/2} = \log_c(0.5)$, yielding a value of 1.93 d (46 h) with a 95% confidence interval of 1.81 to 2.06 d.

Values for protein abundance and starch content following dex treatment were used to calculate an approximate flux control coefficient for GWD with respect to starch degradation. The flux through starch degradation was estimated as the proportion of starch present at the end of the day that was degraded by the end of the night. The estimation assumes that the degradation rate is essentially linear: this has consistently been

found to be the case in many experiments (Kötting et al., 2005, 2009; Comparot-Moss et al., 2010; Scialdone et al., 2013). Flux control calculations (Kacser and Burns, 1973) require that the metabolic system is at steady state. This is not strictly the case in our experiment, because protein levels changed throughout the time course. However, levels changed by a maximum of 10% in any one night, so analysis of these data should provide a good approximation of the flux control coefficient.

The flux control coefficient was determined by fitting the hyperbolic function

$$J = a + \frac{b}{1 + d \times G}$$

to a plot of GWD protein abundance at the end of the day against estimated flux through starch degradation at night (Fig. 3; Small and Kacser, 1993), where J is the flux, G is the amount of GWD protein, and a, b, and d are constants. The function provided a good fit to the data ($r^2 = 0.966$). The hyperbolic function was differentiated with respect to G to yield the following:

$$\frac{dJ}{dG} = \frac{-b \times d}{(1 + d \times G)^2}$$

Estimated values for the constants were substituted into the equation, yielding a flux control coefficient of 0.058 at wild-type protein levels ($G = 1$).

It should be noted that a good fit could not be achieved if the origin was constrained (i.e. $a = -b$), as would normally be the case when dealing with enzymes acting on soluble substrates (Small and Kacser, 1993). It is reasonable not to constrain the origin to 0 in this case because the precise effects of GWD activity on starch structure and its susceptibility to degradation by hydrolytic enzymes are not known. For example, it may be that a minimum density of phosphate groups must be added to amylopectin before crystalline packing is sufficiently disrupted to permit degradation.

Starch Synthesis as Well as Degradation Are Affected following Induction of RNAi

A dex treatment of the RNAi line resulted in decreased rates of starch synthesis during the day as well as decreased rates of starch degradation during the night. Although the amount of starch remaining at the end of the night increased from about 5 d after treatment, there was no consistent change in the amount of starch present at the end of the day (Fig. 2D). Thus, in this experiment, rates of starch synthesis in the light period decreased from the point at which GWD protein levels were sufficiently low to inhibit starch degradation. This was confirmed in an independent experiment in which starch content over the 10th light period after the first dex treatment increased more than 7-fold in the control line (end of night, 0.64 ± 0.05 mg g^{-1} fresh weight; end of day, 4.53 ± 0.51 mg g^{-1} fresh weight [mean ± SE from five rosettes]) but showed no statistically significant increase in the RNAi line (end of night, 5.96 ± 0.22 mg g^{-1} fresh weight; end of day, 6.68 ± 1.21 mg g^{-1} fresh weight [mean ± SE from five rosettes]). Although these results must be regarded as preliminary, they imply that GWD may have a previously unknown role in starch synthesis.

We first considered whether the reduction in starch synthesis in the RNAi line might be due to an unanticipated off-target effect rather than to decreased GWD abundance. To assess the likelihood of this problem, we looked for changes in the abundance of other proteins included in the proteomics data set over the time course of dex treatment. Of the 663 proteins identified in this experiment, only one protein other than GWD changed significantly ($P = 0.05$) in abundance in the RNAi line relative to the control, the stromal cyclophilin ROTAMASE CYP4 (ROC4; also called CYCLOPHILIN20-3; At3g62030; Supplemental Table S2). ROC4 was represented by a single peptide, and its appearance in the data set is surprising since its molecular mass is 19.9 kD and the data set comprised proteins of 100 kD and greater. Further work is required to understand the significance of this finding. Overall, the lack of change in the abundance of proteins other than GWD provides confidence that the reduction in starch synthesis is due to the reduction in GWD protein rather than an off-target effect.

Two main reasons for the decrease in starch synthesis can be proposed. First, GWD may be required for normal rates of starch synthesis during the day. In this case, reductions in its activity would directly inhibit starch accumulation. It is well established that phosphate groups are added to amylopectin during its synthesis (Nielsen et al., 1994; Ritte et al., 2004; Santelia et al., 2011; Scialdone et al., 2013), but the significance of this phenomenon is not known. By analogy with starch degradation, the presence of phosphate groups during synthesis might retard the semicrystalline organization of the granule matrix and thus facilitate the actions of starch synthases and branching enzymes at the granule surface. Second, decreased GWD activity at night may indirectly affect starch synthesis during the following day. For example, the reduction in starch degradation might bring about metabolic changes that affect starch synthesis during the following day. Alternatively, the surfaces of starch granules remaining at the end of the night in plants with low GWD activity may be poor substrates for starch synthesis during the following day.

To look for a direct requirement for GWD in starch synthesis, we compared starch synthesis in plants with low GWD (due to induction of RNAi) with that in control plants following a prolonged period of darkness sufficient to destarch the leaves. We reasoned that destarching would place the RNAi and control plants in the same metabolic state at the start of starch synthesis. Any differences in starch synthesis would then be due to a direct requirement for GWD rather than to knockon effects of differences in the extent of starch degradation during the previous dark period.

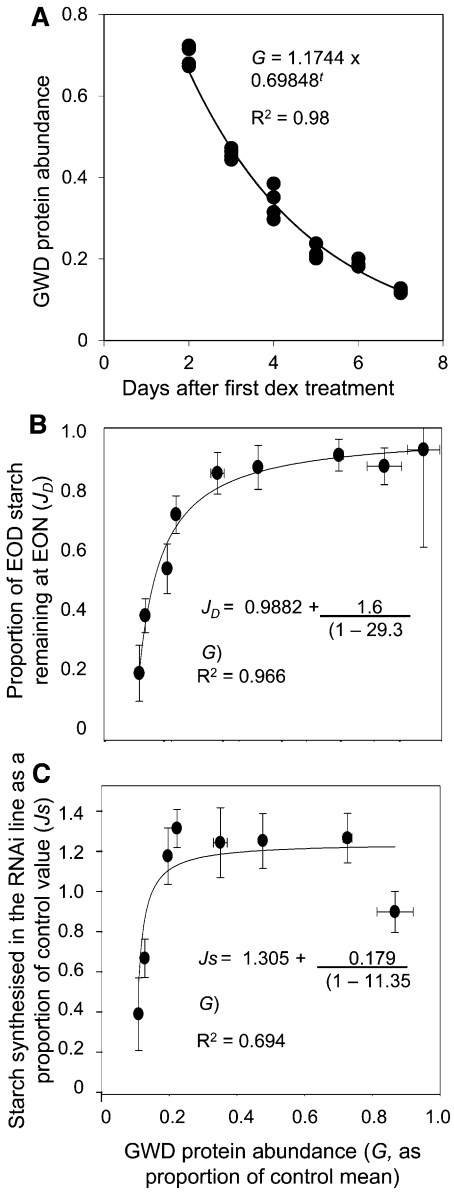

Figure 3. Plots for the estimation of GWD half-life and flux control coefficients. A, GWD protein abundance in the RNAi line during the portion of the time course in which *GWD* transcript was at low levels. Values are fractions of the geometric mean of all values for the control line and are the same as those used in Figure 2B. The equation for the fitted exponential decay curve is shown on the plot. B, Flux through starch degradation (J_D) plotted against GWD protein abundance (G). Values are proportions of end-of-day (EOD) starch remaining at the end of the night (EON) and are the same as those used in Figure 2C. The equation for the fitted hyperbola is shown on the plot. C, Flux through starch synthesis (J_S) plotted against GWD protein abundance (G). Values are amounts of starch made during the day in the RNAi line as a proportion of that made in the control line and are the same as those used in Figure 2C. The equation for the fitted hyperbola is shown on the plot.

At the end of day 3 after the first dex treatment, when starch turnover was not yet altered in the RNAi line (Figs. 2D and 4A), RNAi and control plants were placed in darkness. They were returned to the light after 60 h, at a point at which GWD protein levels in the RNAi line were expected to be about 20% of those in the control line (6 d after the initial dex treatment; Fig. 2B). Both sets of plants had extremely low starch levels at this point. The two sets of plants synthesized starch at the same rate over 12 h and made slightly more starch over this period than in the light period prior to destarching (Fig. 4A). To check whether prolonged darkness had unanticipated effects on GWD levels, plants were stained with iodine at the end of the night following the return to the light (i.e. the night from 6.5 to 7 d after the first dex treatment). The control line had no detectable starch at this point, and the RNAi line had a high starch content (Fig. 4B). This result confirms that GWD levels were decreased as expected in the RNAi line and sufficient for normal starch breakdown in the control line. Taken as a whole, these data are consistent with an indirect effect of the loss of GWD on starch synthesis rather than a direct requirement for GWD during starch synthesis.

To assess the potential importance of GWD for starch synthesis, we calculated an approximate flux control coefficient. Data from Figure 2 were used to estimate the flux through starch synthesis by expressing starch

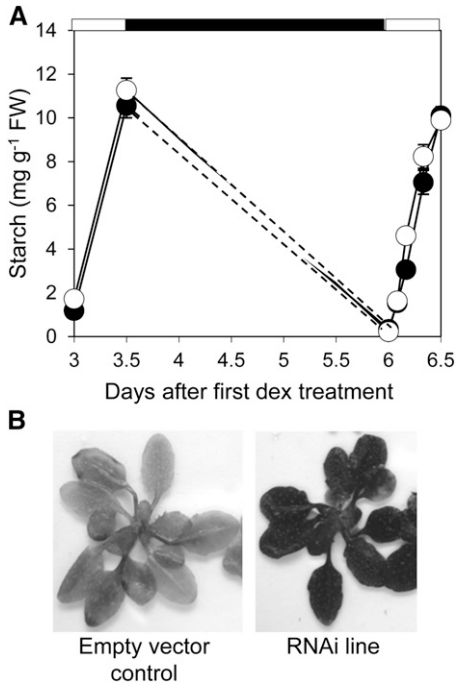

Figure 4. Effect of destarching on starch synthesis in the RNAi line and the control line. A, Starch contents of the RNAi line (black circles) and the control line (white circles) subjected to 60 h of darkness (indicated by the back bar) at 3.5 d after the first dex treatment followed by a 12-h light period. Values are means ± SE of measurements on five rosettes. FW, Fresh weight. B, Iodine-stained rosettes from the experiment in A. Plants were harvested at the end of the night following the light period from 6 to 6.5 d after the first dex treatment. Left, control line; right, RNAi line.

accumulated during the day in the RNAi line as a proportion of that accumulated in the empty vector control. The estimation assumes that the synthesis rate is essentially linear: this has consistently been found to be the case in many experiments (Gibon et al., 2004b; Smith et al., 2004; Comparot-Moss et al., 2010). As discussed above, it is probable that the system approximates to the steady state required for flux control analysis. The function

$$J = a + \frac{b}{1 + d \times G}$$

was fitted to a plot of the estimated flux through starch synthesis, J_S, against GWD protein abundance (Fig. 3C). The hyperbola was a reasonable fit to the data, accounting for 69% of the variation. Estimated values for the constants were substituted into the differentiated function, yielding a flux control coefficient for GWD with respect to starch synthesis of 0.019 at wild-type protein levels ($G = 1$).

Overexpression of GWD Does Not Affect Starch Turnover

The low flux control coefficients of GWD with respect to starch degradation and synthesis suggest that GWD overexpression should have little effect on these processes. To test whether this is the case, a *gwd* mutant (SALK_077211) was transformed with constructs encoding wild-type GWD, with or without a C-terminally fused GFP, and on either a 35S promoter or the native GWD promoter. As a control, the *gwd* mutant was also transformed with the empty vector (referred to as the empty vector control). The construct contained the *PHOSPHINOTHRICIN ACETYL TRANSFERASE* gene, enabling the selection of homozygous transgenic lines based on resistance to the herbicide BASTA (with active ingredient phosphinothricin). Transgenic lines were checked for the presence of the transfer DNA (T-DNA) insertion in the native GWD gene.

To assess GWD protein content in transgenic lines, blots of leaf extracts were probed with an antiserum raised to potato (*Solanum tuberosum*) GWD (Ritte et al., 2000). Immunoblots of extracts from wild-type plants had a band of the expected mass of GWD (156 kD), but no band was present for extracts from the empty vector control (Fig. 5A). Leaves of numerous transgenic lines expressing GWD gave much more intense bands than leaves of wild-type plants, indicating substantially elevated levels of GWD protein (Fig. 5, B and C). For lines expressing native GWD, the band was of the same apparent molecular mass as for wild-type plants, whereas for lines expressing GWD-GFP fusion proteins, the band was of a higher molecular mass, consistent with the additional mass of the GFP (expected mass of the fusion protein is 183 kD).

Expression of either native GWD or the GWD-GFP fusion protein successfully complemented the high-starch phenotype of the *gwd* mutant. Both *gwd* and the empty vector control had very high starch contents at the end of the night (Fig. 5E). Lines expressing GWD from its native promoter had end-of-night starch contents very similar to those of wild-type plants. Lines expressing GWD-GFP fusion proteins from a 35S promoter had higher end-of-night starch contents than wild-type plants, but contents were at least 6-fold lower than in the empty vector control (data from two independently derived lines of these genotypes are shown in Fig. 5E). Despite the very high GWD protein levels of the transgenic lines, the pattern of starch turnover during the day/night cycle was very similar to that of wild-type plants. The rates of starch accumulation during the day and mobilization during the night were essentially linear (Fig. 5F). When transgenic plants were subjected to an unexpectedly early night (after 8 h of light rather than the normal 12 h), they responded in the same way as wild-type plants. The rate of starch degradation remained linear but was decreased so that end-of-night starch content was the same as on previous nights (Fig. 5F).

Restoration of normal patterns of starch turnover was accompanied by accelerated growth rates. Whereas fresh weights of *gwd* and empty vector control rosettes were 10% or less of wild-type fresh weights after 21 d of growth, *gwd* plants expressing either wild-type GWD or the GWD-GFP fusion protein had fresh weights comparable with wild-type plants (Fig. 5E).

Plants Expressing Redox-Insensitive GWD Have Normal Starch Turnover

To examine the importance of the redox regulation of GWD for the control of starch degradation, the *gwd* mutant was transformed with a construct encoding a mutant form of GWD in which Cys residue 1,019 was replaced with a Ser residue. This mutation prevents the formation of the disulfide bond responsible for the oxidative inactivation of the enzyme in vitro, giving rise to a redox-insensitive enzyme that is active over a large range of redox potentials (Mikkelsen et al., 2005).

Two independent, homozygous transgenic lines expressing high levels of the redox-insensitive GWD as a GFP fusion protein (Fig. 5D) displayed good complementation of the *gwd* phenotype. End-of-night starch was restored to near wild-type levels. A normal pattern of starch turnover was restored, and so was plant growth rate (Fig. 5, E and F, bottom graph). Importantly, the response of starch degradation to an unexpectedly early night was the same as in wild-type plants.

DISCUSSION

Transcriptional Regulation of GWD Is Not Important for the Daily Regulation of Starch Degradation

We found that the large, daily changes in *GWD* transcript abundance are not reflected at the protein level. GWD protein abundance, measured using label-free quantitative proteomics, showed no statistically significant variation over the diel cycle. This result is

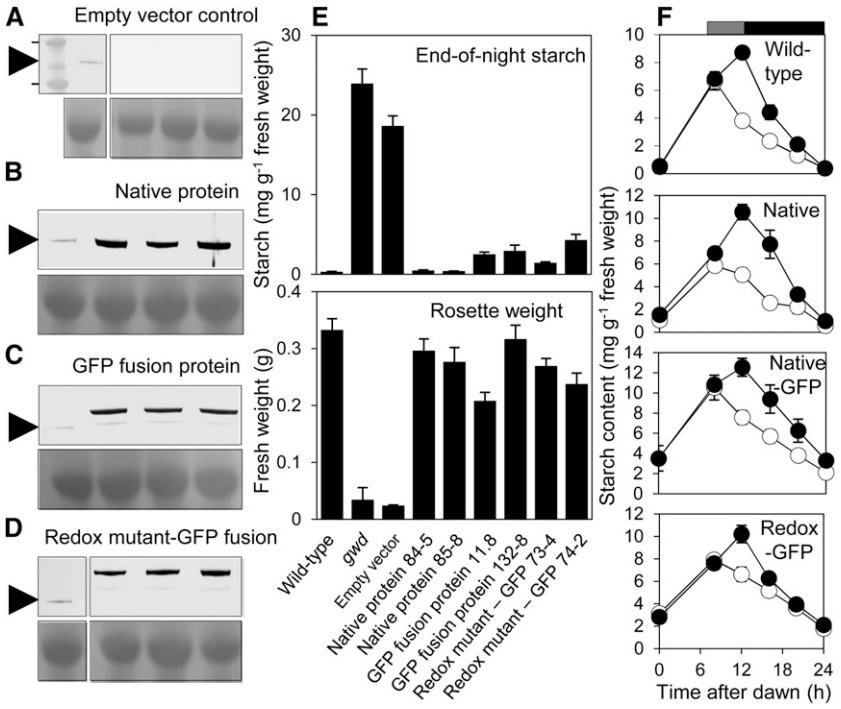

Figure 5. A to D, Immunoblots of rosette extracts probed with an antibody against potato GWD (top). Ponceau stains of each blot (bottom) show the band attributable to the Rubisco large subunit (55 kD) as a loading control. Each lane was loaded with extract from a different, individual plant. For each line, a lane from the same blot loaded with Col-0 extract is included (second lane in A, first lane in B–D). The band attributable to native GWD is indicated with the black arrowheads in B to D. The positions of molecular mass markers (230 and 150 kD) in the first lane in A are indicated as well. A, *gwd* transformed with an empty vector (empty vector control line). B, *gwd* expressing native Arabidopsis GWD from its native promoter (line 85-8). C, *gwd* expressing native Arabidopsis GWD C-terminally fused to GFP from a 35S promoter (line 11-8). D, *gwd* expressing Arabidopsis GWD mutant C1019S C-terminally fused to GFP from a 35S promoter (line 74-2). E, End-of-night starch contents and fresh weights of 21-d-old rosettes of wild-type plants (Col-0), the *gwd* mutant, the empty vector control, two lines of *gwd* expressing native Arabidopsis GWD, two lines of *gwd* expressing the GWD-GFP fusion protein, and two lines of *gwd* expressing the C1019S mutant form of GWD. For starch, values are means ± SE of measurements on five rosettes; for fresh weight, values are means ± SE of measurements on 15 rosettes. F, Changes in starch content over 24 h in conditions of 12 h of light and 12 h of dark (black circles) and an early imposition of the night (8 h of light and 16 h of dark; white circles). Lines of plants were as in A to D. Plants expressing native GWD, the GWD-GFP fusion, and the mutant GWD C1019S GFP fusion were from transgenic lines 84-5, 11-8, and 73-4, respectively. Values are means ± SE of measurements on four to eight rosettes at 21 d old.

consistent with previous qualitative studies of GWD protein abundance using immunoblotting (Yu et al., 2001; Lu et al., 2005). However, the lack of change in GWD protein abundance does not rule out the possibility of large diel changes in GWD protein turnover, masked by reciprocal changes in synthesis. Such changes might be important for the control of starch degradation. To assess the rate of GWD protein turnover, we made use of inducible RNAi to prevent production of the GWD protein in otherwise wild-type plants. Induction of RNAi rapidly decreased *GWD* transcript to very low levels; thus, the subsequent decay of GWD protein abundance was due to protein turnover. The decay curve revealed that GWD protein has a half-life of about 2 d. This finding demonstrates that the protein is relatively stable and that the diel oscillations in GWD transcript do not drive significant diel variation in the rate of GWD protein synthesis.

Given that transcriptional regulation of GWD is not important for the daily control of starch degradation, it is interesting that the *GWD* transcript shows such pronounced changes in abundance over the day/night cycle. Based on extensive comparisons of protein and transcript levels, Stitt and colleagues (Gibon et al., 2004a, 2006; Bläsing et al., 2005; Piques et al., 2009) hypothesize that transcript levels for enzymes of central metabolism represent an immediate readout of the metabolic status of the plant. Rapid, transient changes in transcripts are integrated over a longer period of time to set appropriate levels of enzyme proteins. Thus, enzymatic capacities are buffered against the metabolic changes that occur over the day/night cycle and against short-lived metabolic perturbations. They are adjusted only in response to sustained metabolic changes over longer periods of time. Intriguingly, *GWD* has a complex promoter region containing motifs with the potential to confer responsiveness

to diverse endogenous and exogenous signals (Supplemental Fig. S4). For example, there are three evening elements, known to confer evening-specific transcription by recruiting the MYB repressors LATE ELONGATED HYPOCOTYL and CIRCADIAN CLOCK ASSOCIATED1 in the morning (Harmer et al., 2000; Alabadí et al., 2001) and the activator REVEILLE8 in the evening (Hsu et al., 2013). There are also G-boxes, which mediate light responses (Gangappa et al., 2013), abscisic acid, ethylene, and gibberellin response elements, and a low-temperature response element. Thus, consistent with the general proposals of Stitt and colleagues (Gibon et al., 2004a, 2006; Bläsing et al., 2005; Piques et al., 2009), the GWD transcriptional system could sensitively integrate multiple input signals while the long protein half-life effectively acts as a noise filter, ensuring that protein levels change only when environmental or metabolic perturbations are sustained.

Daily Control of Starch Degradation Is Likely To Be at a Posttranslational Level

In general, it seems likely that the diel regulation of starch degradation does not require changes in the abundance of starch-degrading enzymes. In addition to GWD, two other proteins involved in starch degradation and present in our data set (AMY3 and DPE2) were essentially unchanged in abundance over the day despite large daily oscillations in the abundance of their transcripts (Smith et al., 2004). Our results provide quantitative confirmation of the apparently constant diel abundances of these proteins observed by immunoblotting (Smith et al., 2004; Lu et al., 2005; Yu et al., 2005). Although at least 10 enzymes of starch metabolism, including GWD, show similar daily patterns of change in transcript abundance, with a strong peak at dusk (Smith et al., 2004; Kötting et al., 2005; Niittylä et al., 2006; Santelia et al., 2011), there is no evidence thus far that any of the encoded proteins undergo marked changes in abundance.

A lack of correspondence between patterns of transcript and protein abundance is observed for many proteins of moderate to high abundance in Arabidopsis leaves. Most of the 387 proteins we detected showed no daily change or fractional daily changes in abundance that were smaller than those of their transcripts. In addition, the pattern of change in protein abundance over 24 h was not well predicted by the pattern of change in the corresponding transcripts. Baerenfaller et al. (2012) also found that only a very small fraction of proteins in the Arabidopsis leaf showed diel changes in abundance despite large changes in the abundance of their transcripts. They reported that only two proteins out of more than 2,000 measured showed significant ($P < 0.05$) variation of at least 1.5-fold between the end of the day and the end of the night. The fact that we found rather more rhythmic proteins than Baerenfaller et al. (2012; 8% of those measured) probably reflects the greater number of time points and hence the greater power to detect changes in our study. Other, independent approaches confirm that adjustments in the abundance of enzymes of central metabolism tend to be dampened and slow compared with changes in the abundance of their transcripts. For example, Gibon et al. (2004a) showed that transcript abundances for about 20 enzymes of central metabolism varied more than their maximum catalytic activities over 24 h and that changes in activity following environmental perturbation were generally less than and slower than the changes in the transcripts encoding them. Using measurements of protein and transcript abundance and ribosome number and occupancy, Piques et al. (2009) estimated that for 23 out of 35 enzymes studied, the time required to synthesize all of the protein in the rosette would be at least 3 d, and over 10 d in eight cases. Thus, daily modulation of fluxes through central metabolism, including starch metabolism, is unlikely to be brought about at the level of transcription.

GWD May Be Required for Normal Starch Synthesis as Well as Degradation

The precise role of GWD in starch metabolism cannot be deduced from the study of mutants lacking the enzyme. *gwd* mutants have six times more starch than wild-type plants at the end of the day and show little or no net starch synthesis or degradation. Despite elevated levels of sugars during the day, mutant plants are slow growing and exhibit symptoms of carbohydrate starvation (Yu et al., 2001; Yazdanbakhsh et al., 2011; Andriotis et al., 2012; A.W. Skeffington, A. Graf, Z. Duxbury, W. Gruissem, and A.M. Smith, unpublished data). These phenotypes are, or are likely to be, long-term consequences of the loss of GWD. Our use of inducible RNAi largely circumvented the problem of longer-term effects, allowing putative primary role(s) of the enzyme to be identified. As expected, progressive loss of the enzyme protein following the induction of RNAi was accompanied by elevated levels of starch at the end of the night, consistent with a primary role in starch degradation. Surprisingly, starch contents at the end of the day were not similarly elevated. This result implied that starch synthesis as well as degradation were inhibited, suggesting that GWD has a role in starch synthesis as well as degradation. Further research is required to confirm and define this role. However, our results and recent discoveries by Mahlow et al. (2014) allow the following conclusions and proposals.

The reduction in starch synthesis in plants with low GWD does not appear to reflect a direct requirement for GWD in this process. Following complete destarching in a prolonged period of darkness, plants with low and normal amounts of GWD had the same rate of starch synthesis. This result suggests that the effects of decreased GWD on starch synthesis in normal day/night conditions are an indirect consequence of decreased starch degradation at night. We propose three ways in which decreased starch degradation caused by lower levels of GWD might lead to decreased starch synthesis on the following day.

First, it might result in metabolic conditions in the leaf unfavorable for starch synthesis. This possibility seems relatively unlikely. Numerous mutants lacking components of the starch degradation apparatus have rates of starch degradation at night that are within the range seen in our RNAi lines, yet these mutants retain substantial rates of starch synthesis (Kötting et al., 2005; Delatte et al., 2006; Fulton et al., 2008; Comparot-Moss et al., 2010). Thus, the inhibition of starch synthesis seen in our RNAi lines would seem to be specific for plants with decreased GWD rather than a general effect of decreased starch degradation.

Second, there may be a mechanism that adjusts the rate of starch synthesis so that a particular target starch content is achieved at the end of the day. If considerable amounts of starch remain at the end of the night, then starch synthesis during the day will be proportionately slower. This possibility seems relatively unlikely, because several mutants lacking components of the starch degradation apparatus have high starch contents at the end of the night, higher than those seen in our RNAi lines, yet have substantial rates of starch synthesis during the day (Kötting et al., 2005; Delatte et al., 2006; Fulton et al., 2008; Comparot-Moss et al., 2010).

Third, the surfaces of starch granules remaining at the end of the night in plants with decreased GWD may be poor substrates for starch synthesis during the following day. In mature leaves, starch synthesis is thought to occur largely on granules present at the end of the night rather than on granules initiated de novo at the start of the day (Crumpton-Taylor et al., 2012). Thus, if reductions in GWD produce granule surfaces on which only limited starch synthesis can occur at the start of the day (e.g. due to lower phosphate content and a consequent high level of organization of starch polymers), starch synthesis as a whole may be inhibited. This possibility is consistent with recent experiments on starch granules isolated from wild-type and GWD-deficient leaves (Mahlow et al., 2014). Wild-type granules were found to have longer outer glucan chains than *gwd* mutant granules. Recombinant starch synthase I, the dominant form of starch synthase in Arabidopsis leaves (Delvallé et al., 2005), had higher activity on wild-type granules than on *gwd* mutant granules. Prephosphorylation of granules by incubation with recombinant GWD and ATP further increased the activity of starch synthase I. Mahlow et al. (2014) concluded that wild-type granules are potentially better substrates for starch synthesis than *gwd* granules because they have longer outer glucan chains and because phosphorylation results in a less crystalline surface that is more accessible to starch synthases.

GWD Exerts Little Control over Starch Turnover

Analysis of the relationship between GWD levels and the rates of starch degradation and synthesis following the induction of RNAi revealed that the enzyme has a very low flux control coefficient with respect to both processes. Accumulation of starch at the end of the night, indicating reduced starch degradation, did not occur until GWD protein levels fell to 20% to 30% of wild-type values. Consistent with a low flux control coefficient, substantial overexpression of GWD did not affect the pattern of starch synthesis and degradation. The long half-life and low flux control coefficients of GWD mean that changes in the amount of the protein are unlikely to be regulatory on a daily time scale.

Although GWD is essential for starch degradation in a wide range of organs and species (e.g. Arabidopsis embryos [Andriotis et al., 2010], *Lotus japonicus* roots and leaves [Vriet et al., 2014], potato leaves [Lorberth et al., 1998], maize [*Zea mays*] leaves [Weise et al., 2012], and tomato [*Solanum lycopersicum*] pollen [Nashilevitz et al., 2009]), insufficient data are available to allow speculation about its importance for the control of starch turnover in these contexts. Overexpression of potato GWD in developing barley (*Hordeum vulgare*) grains increased the level of starch phosphate and altered granule morphology (Carciofi et al., 2011), but the effect on starch content was not reported. Reduction of GWD in developing wheat (*Triticum aestivum*) grains did not alter the starch content of mature grains (Ral et al., 2012). However, despite the grain-specific expression of the transgene, plant biomass and grain size were both increased in transgenic plants. The mechanism underlying these pleiotropic effects is not known.

Redox Regulation of GWD Is Not Required for the Adjustment of Starch Degradation Rates

Redox regulation has emerged as a strong theme in the search for mechanisms controlling starch degradation (Mikkelsen et al., 2005; Sokolov et al., 2006; Sparla et al., 2006; Seung et al., 2013; Silver et al., 2013). A recent, comprehensive survey established or confirmed that β-amylase, isoamylase, limit dextrinase, and α-amylase (AMY3) activities from Arabidopsis all display reductive activation in vitro (Glaring et al., 2012). For potato GWD, mutation of a Cys near the active site generates an enzyme active over a much larger range of redox potentials than the wild-type enzyme (effectively a constitutively activated enzyme; Mikkelsen et al., 2005). However, an in vivo role for redox regulation in starch degradation has yet to be established.

We found no evidence that redox regulation of GWD is important for the control of starch degradation in Arabidopsis leaves at night. Transgenic plants expressing a form of Arabidopsis GWD containing the Cys mutation that gives constitutive activation of the potato enzyme initiated starch degradation normally at the start of the night and showed decreased starch degradation rates in response to an unexpected early night, in a manner similar to wild-type plants. Thus, redox regulation of the enzyme is not required for either the initiation or the adjustment of the rate of starch degradation.

The lack of requirement for oxidative inactivation of GWD under the plant growth conditions we studied is consistent with the fact that the midpoint potential of

potato GWD (−255 mV at pH 7, the pH of the chloroplast stroma at night) is one of the most positive of any enzyme characterized (Mikkelsen et al., 2005). Thus, it is unlikely that the enzyme undergoes full redox-mediated inactivation under normal physiological conditions (Seung et al., 2013). It is also not clear how the enzyme would become reduced and thus activated at night. Although Mikkelsen et al. (2005) showed that specific thioredoxins could reduce and thus activate the enzyme in vitro, the chloroplast stroma as a whole and thioredoxins in particular are in a more reduced state during the day than at night (Lemaire et al., 2007). However, the chloroplast is not a uniform redox environment: electrons are channeled to particular targets in a highly specific manner dependent on protein-protein interactions. Thus, it remains possible that electrons from NADPH generated through the oxidative pentose phosphate pathway at night are used to specifically reduce and activate enzymes of starch degradation. NADPH-dependent thioredoxin reductase C (NTRC) is an unusual thioredoxin that uses such a metabolically generated reductant (Michalska et al., 2009). It can reduce and thus activate the chloroplastic β-amylase isoform BAM1 in vitro (Valerio et al., 2011). NTRC could thus potentially channel reductant at night for the regulation of the enzymes of starch degradation. Our experiments do not exclude the possibility that GWD is activated by reduction at night and that its redox regulation plays a role in the adjustment of starch degradation under some specific environmental conditions. However, this form of regulation appears to be unimportant for the control of starch turnover and for its adjustment to an unexpectedly early night under controlled environment conditions.

Further questions about the role of redox regulation of GWD are raised by a wide survey of predicted GWD amino acid sequences (Supplemental Table S3). The two Cys residues shown to form a disulfide bridge in the oxidized form of GWD from potato, Cys-1004 and Cys-1008 (Mikkelsen et al., 2005), are present in GWD from 13 dicotyledonous species and the moss *Physcomitrella patens*; in fact, all of these species have the same CFATC motif. However, the first of the two Cys residues is replaced by a Lys in GWD from grasses, in a region of the protein that is otherwise highly conserved (Supplemental Table S3). Some grasses have two Cys residues within eight residues of each other in this region (a CKVLFATC motif), but in others, the region contains only a single Cys. Species with a single Cys residue include two in which GWD is known to be necessary for normal leaf starch turnover, maize (Weise et al., 2012) and rice (*Oryza sativa*; Hirose et al., 2013).

Starch Phosphorylation, Dephosphorylation, and the Control of Starch Degradation

The cycle of phosphorylation and dephosphorylation at the starch granule surface remains a key step at which flux through starch degradation could be controlled. It was recently shown that a mutant lacking the second dikinase, PWD, does not adjust its starch degradation rate in response to an early night (Scialdone et al., 2013), strongly implicating some aspect of starch granule phosphorylation in implementing this adjustment. Several pieces of evidence point to strong regulation of the activities of enzymes of the phosphorylation-dephosphorylation cycle. For example, starch surface phosphate levels increase on the light-to-dark transition in potato leaves, the turnover of starch phosphate is much higher in the dark than in the light in *Chlamydomonas reinhardtii* (Ritte et al., 2004), and the level of Glc-6-P residues per Glc residue in Arabidopsis leaf starch increases during the day and decreases during the night (Scialdone et al., 2013). Precisely how GWD, PWD, SEX4, and LSF2 might be regulated to limit the ATP consumption of the system while permitting an appropriate flux through the starch degradation pathway remains to be discovered. The work presented here has begun to tackle this question by establishing that translational control and redox activation, which were attractive and previously postulated mechanisms for the regulation of GWD, are of limited relevance for the control of starch turnover in Arabidopsis leaves.

MATERIALS AND METHODS

Plant Material

Wild-type Arabidopsis (*Arabidopsis thaliana*) plants (ecotype Columbia-0 [Col-0]; NI1093) and the *gwd* T-DNA insertion line (SALK_077211; characterized by Ritte et al., 2006) were from the Nottingham Arabidopsis Stock Centre (University of Nottingham). Seeds were germinated on soil in the conditions of the experiment. Plants for the RNAi experiment were grown in a controlled environment room at 20°C and 75% humidity in a 12-h photoperiod with 180 μmol photons m^{-2} s^{-1}. For dex treatment, leaf surfaces were wetted by spraying with 30 μM dex and 0.2% (v/v) Triton X-100. Plants for other experiments were grown in cabinets at 20°C, 80% humidity, 160 μmol photons m^{-2} s^{-1}, and photoperiods as shown in the figures. Leaves or whole rosettes were harvested into liquid nitrogen. For SDS-PAGE and starch analyses, leaf material was ground to a fine powder in a ball mill in the presence of dry ice.

Generating the dex-Inducible RNAi Line

The *GWD*-targeted, dex-inducible RNAi construct was generated using the pOpOff2(hyg) vector system (Wielopolska et al., 2005). A unique section of *GWD* complementary DNA, from 984 to 1,476 bp after the transcription start site, was amplified (for primer sequences, see Supplemental Table S4) and cloned into the Gateway entry vector pCR8/GW/TOPO TA (Invitrogen). Site-directed mutagenesis was employed to modify the codon for Cys-1019 (TGT) to a Ser codon (TCT) to generate the redox-insensitive GWD. The mutation was confirmed by sequencing. Wild-type and mutated clones were transferred into the destination vector by recombination. Transformed plants were selected on hygromycin, and single-copy homozygous lines were isolated.

Generating Complemented Lines and Lines Expressing Modified GWD

The *GWD* sequence was amplified from Col-0 genomic DNA with or without the promoter (the 1,487-bp intergenic region upstream of the start codon) and with or without the stop codon (for primer sequences, see Supplemental Table S4), then cloned into pCR8/GW/TOPO TA (Invitrogen) and transferred to pB7FWG2 (making 35S-driven, *GFP*-tagged constructs) or pB7GW (no promoter or tag) by recombination (Karimi et al., 2005). The resulting constructs were stably transformed into a *gwd* mutant (SALK_077211) by floral dipping mediated by *Agrobacterium tumefaciens* (GV3101). BASTA-resistant transformants were selected, and single-copy homozygous lines were generated. A set of lines checked for the presence of the T-DNA insertion at the native *GWD* locus (for primer sequences, see Supplemental Table S4) was used for subsequent analysis.

Immunoblotting

Powdered frozen leaves were suspended in 100 mM HEPES, pH 7, 33 μL mL^{-1} plant protease inhibitor (Sigma), and 20 mg mL^{-1} polyvinylpyrrolidone at 4°C and centrifuged at 20,000*g* for 10 min at 4°C. The supernatant was diluted 1:1 (v/v) in 2× Laemmli sample buffer (120 mM Tris-HCl, pH 6.8, 3.4% [w/v] SDS, 12% [v/v] glycerol, 200 mM dithiothreitol, and 0.04% [w/v] Bromphenol Blue) and then incubated at 80°C for 10 min. Samples were run on NuPAGE 4% to 12% Bis-Tris gradient gels (Invitrogen) and then blotted onto nitrocellulose. Blots were developed with an antiserum raised in rabbit to potato (*Solanum tuberosum*) GWD (Ritte et al., 2000) at a 1:1,000 dilution according to Barratt et al. (2001) using a secondary antibody conjugated to alkaline phosphatase and the SIGMAFAST 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium reagent.

Quantitative Protein Measurements

Protein Extraction and Tryptic Digestion

Powdered frozen plant material (5–100 mg) was suspended in 200 μL of SDS extraction medium (4% [w/v] SDS, 40 mM Tris, and 60 μL mL^{-1} protease inhibitor cocktail [Roche]), mixed vigorously, and centrifuged for 10 min at 16,000*g*. The supernatant was further centrifuged at 100,000*g* for 45 min. The resulting supernatant was diluted 1:1 (v/v) in Laemmli sample buffer and incubated at 65°C for 20 min. Approximately 105 μg of protein per sample was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel at 50 V overnight. Gels were stained for 45 min in Coomassie Blue solution (20% [v/v] methanol, 10% [v/v] acetic acid, and 0.1% [w/v] Coomassie Brilliant Blue R) and then incubated in 50% (v/v) methanol and 10% (v/v) acetic acid for 2 h at room temperature. Gel slices that included all material above the 100-kD marker were cut from the top of each lane, transferred to a 96-well plate, and washed three times with 50% (v/v) methanol and 100 mM ammonium bicarbonate, incubating each time for 1 h at 37°C. After in-gel tryptic digestion (Shevchenko et al., 1996), peptides were purified by reverse-phase chromatography on Finisterre C18 SPE columns (Teknokroma) and dried at 45°C in a vacuum centrifuge.

Mass Spectrometric Analysis

Peptides were resuspended in 40 μL of 3% (v/v) acetonitrile and 0.1% (v/v) formic acid. Measurements were performed on an LTQ-Orbitrap XL ETD (Thermo Scientific) coupled with a NanoLC 1D (Eksigent). Samples were loaded onto a laboratory-made capillary column (75 μm i.d., 8 cm long), packed with Magic C18 AQ beads (5 μm, 100 Å; Microm) at a flow rate of 0.5 μL min^{-1} in 3% (v/v) acetonitrile and 0.2% (v/v) formic acid, and eluted with a 5% to 40% (v/v) acetonitrile concentration gradient over 70 min, followed by 80% (v/v) acetonitrile for 10 min, at 0.25 μL min^{-1}. Peptide ions were detected in a full scan from mass-to-charge ratio 300 to 2,000. Tandem mass spectrometry (MS/MS) scans were performed for the five peptides with the highest mass spectrometry signal (minimal signal strength, 500 hits; isolation width mass-to-charge ratio, 3; relative collision energy, 35%). Peptides for which MS/MS spectra had been recorded were excluded from further MS/MS scans for 2 min.

Peak Area-Based Protein Quantification

Quantitative analysis of MS/MS measurements was performed with Progenesis LCMS software (Nonlinear Dynamics). The full mass-to-charge ratio range was imported; the peak picking time was limited to between 20 and 80 min of the LCMS run. One run was selected as a standard, and for each run 15 vectors were placed manually on prominent peaks before applying the automatic alignment function of Progenesis, followed by the peak picking function. Progenesis-calculated normalization factors, all between 0.8 and 1.2, were applied across the runs. Following this, the best six spectra for each peak were exported to Mascot.

Mascot Search Parameters

Mascot search parameters were set as follows: The Arabidopsis Information Resource 10 genome annotation, requirement for tryptic ends, one missed cleavage allowed; fixed modification, carbamidomethylation (Cys); variable modification, oxidation (Met); peptide mass tolerance = ±1.2 D, MS/MS tolerance = ±0.6 D, allowed peptide charges of +2 and +3. Spectra were also searched against a decoy database of the Arabidopsis proteome in reverse amino acid sequences. This allowed the estimation of false positive rates as 0.16% for the 24-h experiment and less than 0.14% for the RNAi experiment. Identifications below rank 1 or with a Mascot ion score below 25 were excluded. Peak identities were imported into Progenesis, quantitative peak area information was extracted, and the results were exported for data plotting and statistical analysis. Peptides from which proteins were identified are given in Supplemental Table S5.

Starch Quantification and Staining

For quantification, powdered frozen leaf material was extracted in 0.7 M perchloric acid, washed three times in 80% (v/v) ethanol, heated in water at 90°C for 15 min, and incubated with α-amylase and amyloglucosidase prior to determination of Glc (Critchley et al., 2001). For staining, rosettes were harvested into 80% (v/v) ethanol, heated until colorless, drained, stained for several minutes in 50% (v/v) aqueous Lugol's iodine solution, and then washed in water.

Transcript Quantification

mRNA was isolated from leaves using the RNeasy kit (Qiagen), and complementary DNA was synthesized using the SuperScript III kit (Invitrogen) and oligo(dT) primers. Real-time quantitative PCR was carried out using the SYBR Green system (Invitrogen) on an Opticon 4 thermal cycler (Bio-Rad). Three reference genes were used for normalization, chosen for their stability over diurnal and developmental time courses (Czechowski et al., 2005). Primer sequences are given in Supplemental Table S4.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Frequency distribution of the amplitude of diel changes in transcript and protein abundance.

Supplemental Figure S2. Diel changes in transcript and protein abundance for ADG1.

Supplemental Figure S3. Analysis of the relationship between daily changes in the abundance of transcripts and proteins.

Supplemental Figure S4. Structure of the *GWD* promoter.

Supplemental Table S1. Diel changes in protein abundance.

Supplemental Table S2. Changes in protein abundance following dex induction of the RNAi line and the control line.

Supplemental Table S3. Alignments of predicted amino acid sequences of GWD from different organisms.

Supplemental Table S4. Primer sequences used in this work.

Supplemental Table S5. Peptides detected in proteomic experiments.

ACKNOWLEDGMENTS

We thank Katja Baerenfaller, Sam Zeeman, and Oliver Kötting (Eidgenössisch Technische Hochschule) and Richard Morris (John Innes Centre) for advice and assistance.

Received January 30, 2014; accepted April 28, 2014; published April 29, 2014.

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