Regulation of Transcript Levels of a Potato Gibberellin 20-Oxidase Gene by Light and Phytochrome B[1]

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Up to three gibberellin (GA) 20-oxidase genes have now been cloned from several species including Arabidopsis, bean (*Phaseolus vulgaris*), and potato (*Solanum tuberosum*). In each case the GA 20-oxidase genes exhibit different patterns of tissue expression. We have performed extensive northern analysis on one of the potato GA 20-oxidase genes (*StGA20ox1*), which is the only one that shows significant transcript levels in leaves. We show that levels of *StGA20ox1* transcript are elevated in transgenic antisense plants that have reduced levels of phytochrome B (PHYB) compared with wild-type plants, implicating PHYB in the control of GA biosynthesis. We show that *StGA20ox1* transcript levels vary in leaves of different age throughout the plant and cycle throughout the day, furthermore they are up-regulated by light and down-regulated in the dark. The degree of the response to the light-on signal is similar in potato plants deficient in phytochrome A or PHYB and wild-type plants. The induction of *StGA20ox1* by blue light raises the possibility that a blue light receptor may be involved in the control of this gene by light.

Tuberization of certain lines of potato (*Solanum tuberosum* subsp. *andigena*) is a strict short-day (SD) photoperiodic response. Several lines of evidence implicate gibberellins (GAs) in the inhibition of the tuberization in this potato species in long days (LD); levels of GA-like activity decrease in leaves of potato upon transfer from LD to SD conditions (Railton and Wareing, 1973), treating plants with ancymidol, an inhibitor of GA biosynthesis, enables them to tuberize in LD (Jackson and Prat, 1996), and a dwarf mutant of potato that is partially blocked in the 13-hydroxylation of GA_{12}-aldehyde to GA_{53} (Van den Berg et al., 1995b) is also able to tuberize in LD. The early 13-hydroxylation pathway has been shown to be the main pathway for GA biosynthesis in potato (Van den Berg et al., 1995a), thus the reduction in the levels of GAs subsequent to this step in the pathway must be the reason that the dwarf mutant is able to tuberize in LD. Phytochrome B (PHYB) deficient potato transgenic antisense plants are also able to tuberize in LD (Jackson et al., 1996), although these plants have elongated internodes and reduced chlorophyll levels, which is the converse phenotype to that of the dwarf mutant or of wild-type (WT) plants treated with GA inhibitors. *PhyB* mutants of sorghum and *Brassica rapa* are reported to have increased GA levels (Rood et al., 1990; Foster et al., 1994), whereas other studies of *phyB* mutants of pea, cucumber, and Arabidopsis suggest that GA sensitivity is affected (Weller et al., 1994; Lopez-Juez et al., 1995; Reed et al., 1996).

Genes for enzymes involved in several steps of the GA biosynthetic pathway have now been cloned from various different species and the expression of many of these genes is regulated by light (Hedden and Kamiya, 1997; Kamiya and Garcia-Martinez 1999). It has been shown that genes encoding 3β-hydroxylases from lettuce and Arabidopsis are under phytochrome control (Toyomasu et al., 1998; Yamaguchi et al., 1998) and in the case of *GA4H* from Arabidopsis it was shown to be under the control of PHYB. At least three GA 20-oxidase genes have been cloned from Arabidopsis, bean (*Phaseolus vulgaris*), and potato (Phillips et al., 1995; Garcia-Martinez et al., 1997; Carrera et al., 1999). In these species the individual GA 20-oxidase genes exhibit different levels and patterns of expression, indicating that they probably have separate roles to play in specific aspects of the growth and development of the plant such as stem elongation or fruit development. Red light did not induce the expression of either of two GA 20-oxidases from lettuce, and with one of the genes (*Ls20ox2*) it was found that red light reduced its expression suggesting that Pfr may inhibit expression of this GA 20-oxidase (Toyomasu et al., 1998). However, red light was found to induce the expression of a GA 20-oxidase in pea (Ait-Ali et al., 1999).

GA 20-oxidase is thought to be a key regulatory enzyme in the GA biosynthetic pathway, its expres-

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sion is subject to feedback inhibition by GAs further down the pathway, suggesting that GA biosynthesis has an auto-regulatory component (Hedden and Croker, 1992; Phillips et al., 1995). It is regulated in the pericarp of peas by the presence of seeds or the shoot apex (Garcia-Martinez et al., 1997; Van Huizen et al., 1997), and transcript levels are reported to be higher in LD than SD in the LD plants spinach and Arabidopsis (Wu et al., 1996; Xu et al., 1997), although no evidence has yet been found for this in SD potato (Carrera et al., 1999). Evidence for photoperiodic regulation of the steps catalyzed by the GA 20-oxidase originally came from gas chromatography-mass spectrometry measurements of cell-free extracts and endogenous GA levels of spinach plants grown in either LD or SD conditions (Gilmour et al., 1986; Talon et al., 1991). These results showed that the enzyme activities catalyzing the conversion of GA_{53} to GA_{44}, and GA_{19} to GA_{20}, increased upon transfer from non-inducing SD conditions to inducing LD conditions, whereas the activity of an enzyme catalyzing the intermediate conversion of GA_{44} to GA_{19} remains high in both photoperiods. These activities could be separated by HPLC (Gilmour et al., 1987), and thus there are at least two GA 20-oxidases in leaves of spinach plants, one whose activity is photoperiodically regulated and one with high constitutive activity.

In addition to the existence of multiple GA 20-oxidases, which have different activities and/or are subject to different tissue and light regulation, several time course studies show that GA levels fluctuate throughout the day (Talon et al., 1991; Foster and Morgan, 1995; Lee et al., 1998), indicating that the activity or expression of genes involved in the biosynthetic pathway might also fluctuate throughout the day. This has been observed to some extent for a GA 20-oxidase and a 3β-hydroxylase from pea (Ait-Ali et al., 1999). It is thus difficult to draw general conclusions from measurements of GA levels or GA 20-oxidase expression levels from samples harvested at just a single time point.

In an attempt to understand some of the factors affecting GA biosynthesis and the photoperiodic control of tuber induction in potato, we have performed some detailed northern analysis on one of the three GA 20-oxidases (*StGA20ox1*) that were recently cloned from potato (Carrera et al., 1999). As the principle site of photoperiodic perception is young mature leaves, rather than the apex or other parts of the plant, we chose to analyze *StGA20ox1* because it is the only one of the three clones that shows significant expression in the leaves. Through comparisons between WT plants and transgenic plants antisensed for the potato *PHYB1* gene (that have reduced levels of PHYB1 and possibly also PHYB2), we show that PHYB and possibly a blue-light photoreceptor are involved in regulating the transcript levels of this gene.

RESULTS

StGA20ox1 Transcript Levels Are Higher in *PHYB* Antisense Plants Than WT

In a preliminary experiment the levels of *StGA20ox1* transcript were examined on a northern blot of leaf tissue harvested around mid-day from WT control, antisense *PHYB* 4 (α-4), antisense *PHYB* 10 (α-10), and antisense *PHYB* 2 (α-2) plants. α-4 and α-10 are transgenic plants that have greatly reduced levels of PHYB and which are able to tuberize in LD (Jackson et al., 1996). α-2 is a transgenic plant that has a slight reduction in PHYB levels yet is unable to tuberize in LD and thus behaves as WT control plants. Figure 1a shows the levels of *StGA20ox1* transcript in those plants at that particular time point, the

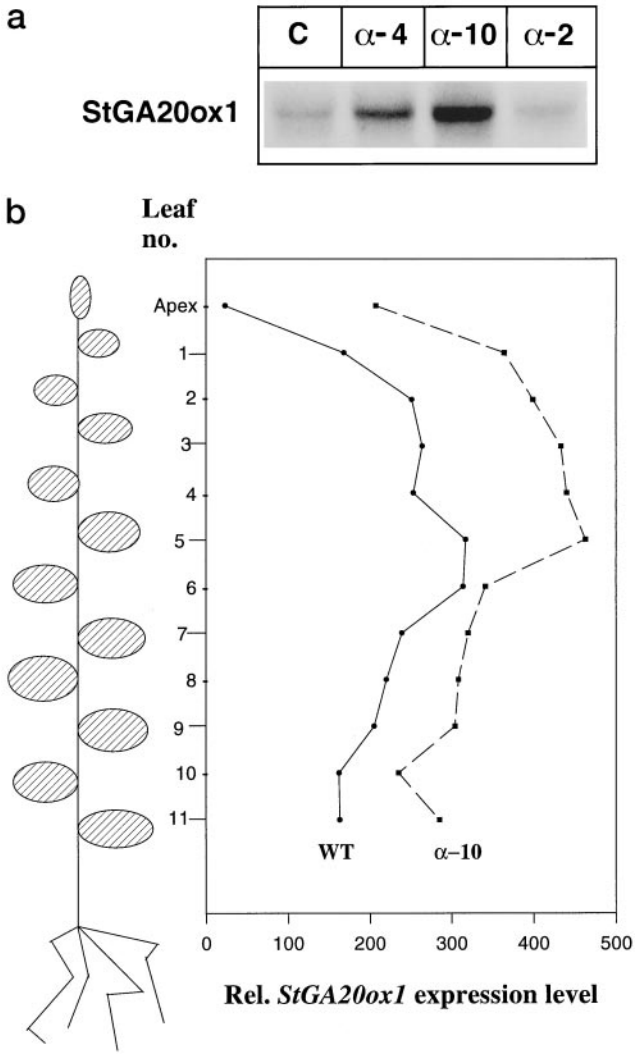

Figure 1. a, Expression of *StGA20ox1* in WT control (C), antisense *PHYB* 4 (α-4), antisense *PHYB* 10 (α-10), and antisense *PHYB* 2 (α-2) plants. b, Expression levels of *StGA20ox1* relative to the *S4* ribosomal protein gene in the apex and in leaves of increasing age down the plant.

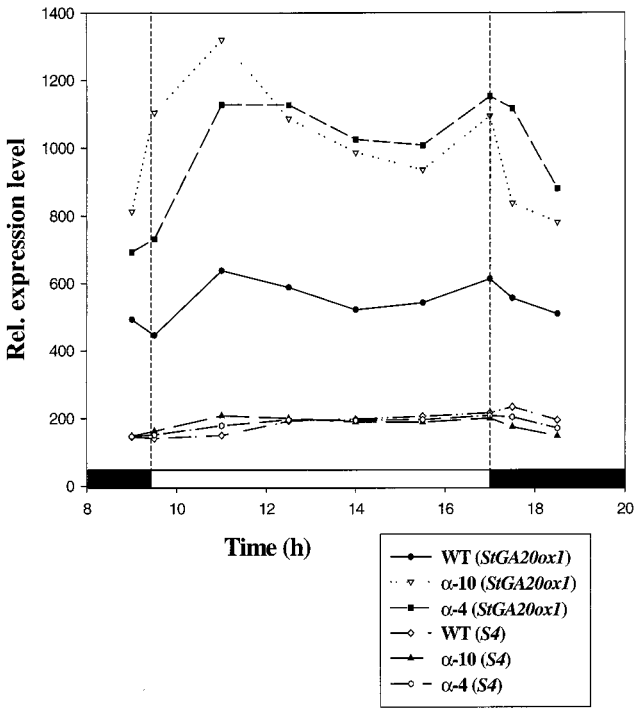

Figure 2. Time course of the relative expression level of *StGA20ox1* in WT, α-4, and α-10 plants over a short (8-h) photoperiod. The white bar denotes the light period. Also shown is the variation in *S4* transcript levels in these tissues in the different plants over the time course.

levels in α-4 and α-10 being much higher than in WT and α-2 plants.

Looking in more detail at transcript levels throughout the plant we harvested samples from the apex and then from individual leaves all the way down the plant from WT and α-10 plants. The relative levels of *StGA20ox1* transcript compared with a constitutively expressed ribosomal protein gene (*S4*) were calculated. As shown in Figure 1b, higher *StGA20ox1* transcript levels were observed in the antisense *PHYB* plants compared with WT plants all the way down the plant from the apex to leaves that had just started to senesce. Furthermore, transcript levels were found to vary with the age of the leaf in both types of plant. These results demonstrate that the variation in transcript levels in leaves of different ages is an important factor to be considered in the analysis of *StGA20ox1* expression, therefore in all subsequent experiments we only harvested the first three fully mature leaves (usually leaf nos. 3, 4, and 5 counting down from the apex).

The Levels of *StGA20ox1* Transcript Fluctuate throughout the Day

As previous time course analysis of the *StGA20ox1* gene (Carrera et al., 1999), and also of a GA 20-oxidase from pea (Ait-Ali et al., 1999), had found differences in transcript levels over a 24-h time period we decided to look at fluctuations in *StGA20ox1* transcript levels over the course of a day.

In the first experiment we looked at *StGA20ox1* transcript levels in WT and antisense *PHYB* plants (both α-4 and α-10) over a short (8 h) photoperiod. Harvesting every 1.5 h, we obtained the time course shown in Figure 2 starting before lights on and continuing through into the following dark period. Samples for time points during the dark period were harvested using a dim green safe light (for durations of less than 5 min), which does not elicit phytochrome responses. Consistent with our previous observations the transcript levels of *StGA20ox1* were much higher in the antisense *PHYB* plants compared with WT plants throughout the time-course experiment. Transcript levels showed a strong induction by the light-on signal, and this occurred in the antisense *PHYB* plants as well as the WT plants suggesting that PHYB may not be necessary for this induction to occur. After this initial induction the transcript levels fall and then start to rise again until lights off when they fall again. This cycling in the levels of *StGA20ox1* transcript and the fairly rapid down-regulation in the dark implies that the transcript is turned over rapidly and is amenable to fine control.

The cycling of *StGA20ox1* transcript levels that we observed in the SD time course is even more apparent in the second experiment where the time course consisted of a LD followed by continuous darkness. Samples from WT, α-4, and α-10 plants were harvested as before, and the *StGA20ox1* transcript levels relative to the *S4* gene were calculated and are shown in Figure 3. The levels in α-4 and α-10 are very similar, and consistent with previous observations, the transcript levels of *StGA20ox1* are much higher in

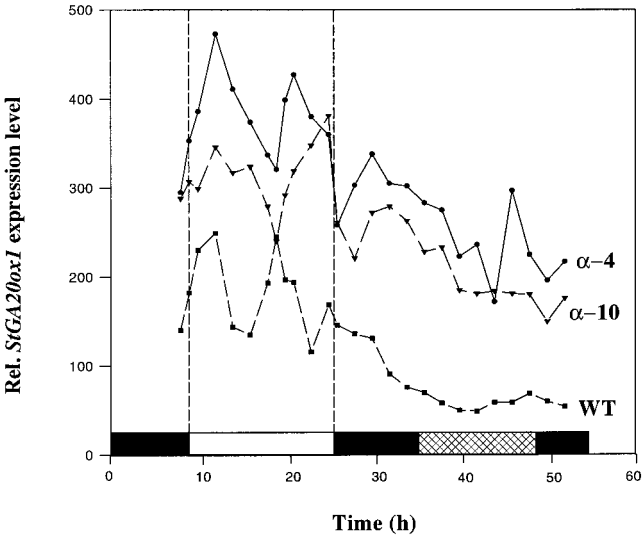

Figure 3. Time course of the relative expression level of *StGA20ox1* (in WT, α-4, and α-10 plants) over a long (16-h) photoperiod followed by continuous darkness. The white bar denotes the light period, the hatched bar represents the next subjective day during the subsequent dark period.

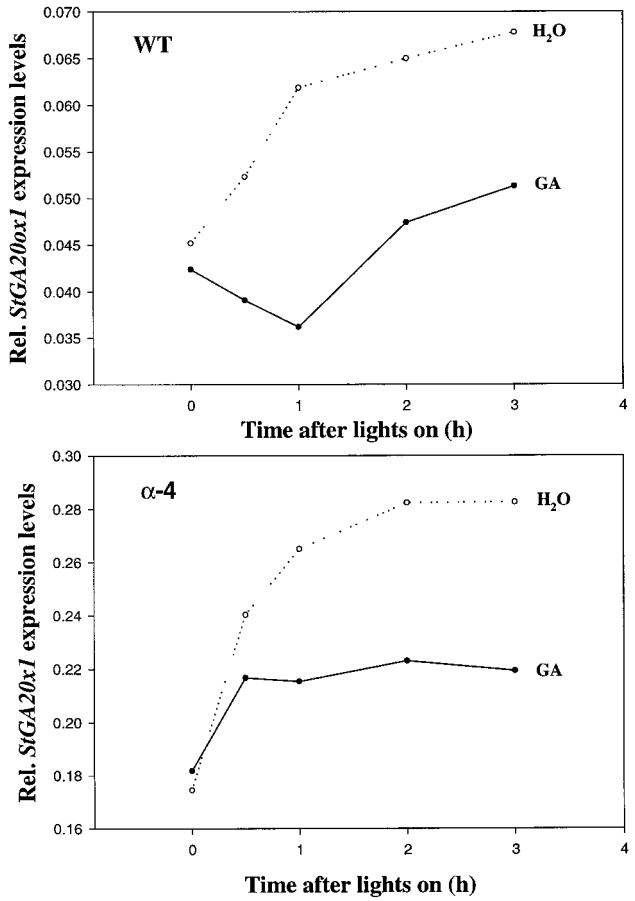

Figure 4. Relative expression levels of *StGA20ox1* in WT and α-4 plants treated with water or 50 μM GA_3.

the antisense *PHYB* plants compared with WT. Again we observe the induction by the light-on signal in both types of plant and also the down-regulation in the dark. During the extra 8 h of light in this LD time course, compared with the previous SD time course, we observe a second peak in levels and possibly the start of a third peak in WT plants. Being able to distinguish two complete peaks in the longer time course enables a second difference between WT and antisense *PHYB* plants to be seen. The period of the cycles of *StGA20ox1* transcript levels appears to be longer in the antisense *PHYB* plants, the second peak in levels in WT plants occurring at the same time as the first trough in the antisense *PHYB* plants. To verify this, however, a more sensitive assay would probably be needed such as promoter-reporter gene analysis.

What Is the Cause of the Cycling of *StGA20ox1* Transcript Levels?

The levels of *StGA20ox1* transcript are down-regulated in the dark although some cycling does appear to persist (Fig. 3), especially in the antisense *PHYB* plants. As *StGA20ox1* transcript levels cycle in both antisense *PHYB* and WT plants, the cause of the rhythm is present in both types of plant even though the period of the rhythm appears to be different. The cycling of *StGA20ox1* transcript levels may be explained by the feedback inhibition of GA 20-oxidases by GA_1. The fact that cycling is observed in the antisense *PHYB* plants as well as WT plants implies that if the cycling is due to negative feedback by GA_1 then this feedback control is still present in the antisense *PHYB* plants. This was shown in an experiment where WT and α-4 transgenic plants were sprayed to run off with 50 μM gibberellic acid (GA_3), or water, on d 1 and then again 30 mins before the start of the light period on the following day. The levels of *StGA20ox1* transcript were monitored after spraying on d 2 just before lights on and for the first 3 h of the light period. The reduced levels of *StGA20ox1* transcript in WT and α-4 plants sprayed with GA_3 is shown in Figure 4, confirming that the negative feedback mechanism is still present in the PHYB-deficient plants.

It may be possible that the cycling of *StGA20ox1* is due to some input from the circadian clock, although the period of the rhythm is very short for that. When *StGA20ox1* transcript levels were analyzed in plants after growing them in constant light for 3 d, no cycling was observed, and they were maintained at a constant high level (data not shown). Therefore, if the cycling is the result of a circadian rhythm, the rhythm has a very short period and damps out fairly quickly.

Reduced Levels of Phytochrome A (PHYA) Does Not Affect the Light Induction of *StGA20ox1*

Although PHYB is involved in controlling overall levels of *StGA20ox1* transcript, the reduced levels of PHYB in the antisense plants does not appear to affect the diurnal regulation of the levels of this transcript by light, the induction by the light-on signal, and the repression in the dark occurring in a similar manner to WT plants. To assess the potential involvement of PHYA, we used potato plants (cv Désirée) that are antisensed for the *PHYA* gene

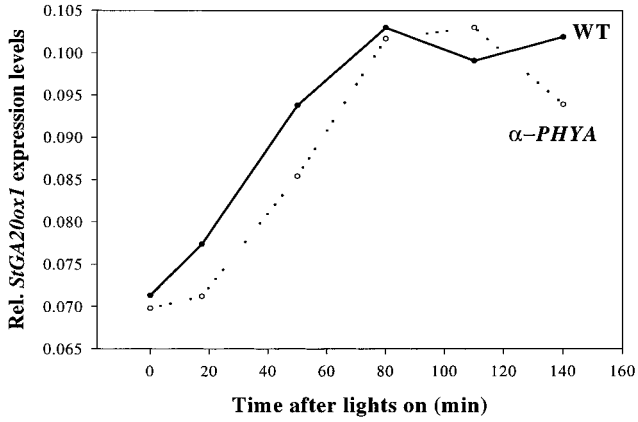

Figure 5. Relative levels of *StGA20ox1* induction by light in WT and antisense *PHYA* (α-*PHYA*) plants.

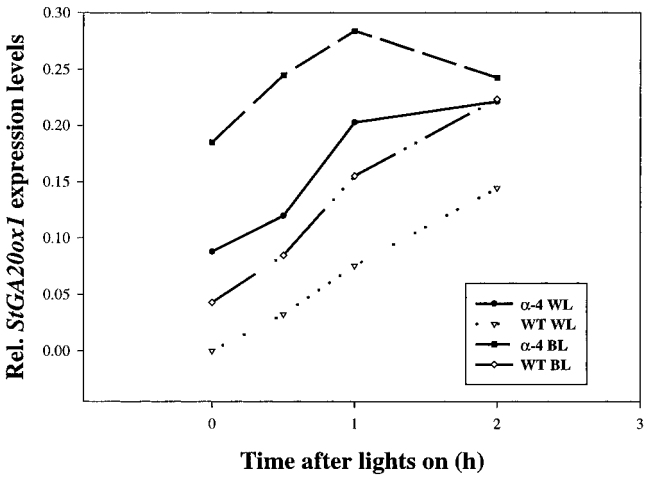

Figure 6. Relative levels of *StGA20ox1* in WT and α-4 plants upon induction by white (WL) and blue (BL) light.

(Heyer et al., 1995). We grew these antisense *PHYA* plants (line Ap9) and WT cv Désirée plants in the same conditions as before and looked at the *StGA20ox1* transcript levels in these plants during the first couple of hours after lights on. As is shown in Figure 5, the rate of induction of *StGA20ox1* transcript levels is similar in both antisense *PHYA* and WT plants, suggesting that PHYA is probably also not involved in the induction of this gene by light.

StGA20ox1 Transcript Levels Are Induced by Blue Light

To test whether the levels of *StGA20ox1* transcript are affected by blue light we grew potato plants in a cabinet fitted with a blue filter that only transmitted light of wavelengths between 400 and 500 nm. The filters reduced the light levels to 10 μmol m^{-2} s^{-1} and so control plants were grown under similar levels of white light. The induction of *StGA20ox1* was analyzed for the first couple of hours after lights on in WT and antisense *PHYB* plants grown under white and blue light. Figure 6 shows that blue light can induce the levels of *StGA20ox1* transcript in both WT and antisense *PHYB* plants. Whereas the levels of *StGA20ox1* transcript are higher in the antisense *PHYB* plants compared with WT plants as has been observed in all previous experiments, it appears that blue light can also result in an increased level of *StGA20ox1* transcript in both antisense *PHYB* and WT plants compared with white light.

In similar experiments using red filters we were not able to obtain a consistent induction of *StGA20ox1* by red light, and in some cases a decrease in the level of expression was observed (data not shown).

DISCUSSION

GA 20-oxidase genes have now been isolated from several species, and in both spinach and Arabidopsis the transcript levels of at least one of the GA 20-oxidase genes was shown to be up-regulated in LD (Wu et al., 1996; Xu et al., 1997). These analyses, however, were based on single time point measurements that may be difficult to interpret in the light of these and previous results, showing that the transcript levels of a GA 20-oxidase from potato (*StGA20ox1*) cycles throughout the day and furthermore that the degree of cycling is variable (Carrera et al., 1999; Figs. 2 and 3). Analysis of this gene in different photoperiods found no difference in transcript levels between LD and SD, a result supported by gas chromatography-mass spectrometry analysis of [^{14}C]GA_{12} feeding studies of potato plants grown in LD and SD (Van den Berg et al., 1995b). However, the gene was expressed for a longer period in LD than SD and was also up-regulated by a light treatment in the middle of the night (Carrera et al., 1999), indicating that the gene is regulated by light rather than photoperiod in potato plants. The difference between potato and spinach and Arabidopsis may lie in the fact that the latter two are LD plants whereas potato is a SD plant or in the fact that both spinach and Arabidopsis "bolt" in response to photoperiod whereas potato does not.

PHYB is involved in controlling the level of *StGA20ox1* gene transcript in potato. Increased levels of expression are observed in PHYB-deficient antisense transgenic plants in all leaves of the plant and at all times of the day and night. The increased levels of *StGA20ox1* mRNA could possibly lead to higher levels of GA_{20} and GA_1, and this may explain some of the observed phenotypes of the *PHYB* antisense plants such as increased internode elongation and reduced chlorophyll levels (Jackson et al., 1996), both of which can be caused by increased GA levels. Whether the increased levels of *StGA20ox1* transcript are responsible for the reduced photoperiodic sensitivity of the *PHYB* antisense plants enabling them to tuberize in LD is still to be determined. It is known, however, that a PHYB mutant of sorghum, the *S. bicolor* ma_3^R mutant, which also exhibits a reduced sensitivity to photoperiod (Pao and Morgan, 1986), has higher levels of GA_{20} and reduced levels of GA_{53} than WT plants (Foster and Morgan, 1995; Lee et al., 1998). These authors also showed that the levels of all GAs, from GA_{12} to GA_1, cycle throughout the day and night in sorghum and furthermore that a shift in the rhythm of GA_{20} levels is reported in the PHYB-deficient ma_3^R mutant compared with WT sorghum plants. This reflects our observations of the longer period of *StGA20ox1* cycling in the *PHYB* antisense potato plants compared with WT. Thus there are striking similarities in the effects of PHYB deficiency on *StGA20ox1* transcript levels in potato and on GA levels in *S. bicolor*, both potato and *S. bicolor* being SD plants.

It is known that GA 20-oxidase genes are subject to feedback inhibition by active GAs further down the

pathway such as GA_1 and are accordingly expressed at higher levels in GA-deficient mutants (Phillips et al., 1995; Xu et al., 1997; Carrera et al., 1999). It is possible that the cycling of *StGA20ox1* transcript levels are caused by a build-up of GA_1 that inhibits expression of *StGA20ox1*. This in turn would lead to reduced levels of GA_1 and a resulting increase of *StGA20ox1* transcript. It should be noted that whereas the cycling of *StGA20ox1* transcript levels has been a consistent observation, the degree of cycling does vary between experiments (compare Figs. 2 and 3). This may reflect different GA statuses of the plants in the different experiments, perhaps caused by different ages of the plants at the time of the experiments.

Whereas the feedback inhibition of GA 20-oxidase expression is still present in the antisense *PHYB* plants, the increased transcript levels in these plants may be explained by a reduced level of feedback inhibition by GA_1, resulting in overall higher levels of *StGA20ox1* mRNA. Such a reduced feedback mechanism in the *PHYB* antisense plants and *phyB* mutants may explain why they appear to have a greater response to GAs (Weller et al., 1994; Lopez-Juez et al., 1995; Reed et al., 1996). If the oscillations in *StGA20ox1* transcript levels are due to feedback inhibition, a weaker feedback mechanism may also explain why the cycling of *StGA20ox1* is different in the antisense *PHYB* plants compared with WT. Reduced levels of PHYB do not affect other aspects of the control of *StGA20ox1* mRNA levels such as the induction by light or the down-regulation in the dark. Likewise, reduced levels of PHYA also do not affect the induction of *StGA20ox1* transcript by light, which is consistent with the fact that *PHYA* antisense potato plants do not exhibit elongated internodes and reduced chlorophyll levels in white light as are observed in *PHYB* antisense plants. A photoreceptor other than PHYA or PHYB is probably responsible for the light induction of this GA 20-oxidase.

As we observed no induction by red light, an observation also reported for the two GA 20-oxidase genes from lettuce *Ls20ox1* and *Ls20ox2* (Toyomasu et al., 1998), it suggests that none of the phytochromes are involved in the induction of GA 20-oxidase by light. This is in contrast to the findings of Ait-Ali et al. (1999) who observed induction of a GA 20-oxidase in pea under their red light conditions. This induction is also observed in PHYA-deficient and PHYB-deficient mutants of pea, indicating that as in potato these phytochromes are unlikely to be involved in the induction of the GA 20-oxidase by light and leading the authors to propose that other phytochromes may be involved. That blue light alone can induce the levels of *StGA20ox1* transcript to the same, if not greater, extent supports this conclusion and implies that a blue light receptor may be involved. If a blue light receptor does play a role in the control of *StGA20ox1* transcript levels this may explain some of the differences in expression pattern that have been observed. In previous experiments where incandescent lights were used, a different time course with a much slower induction by light was observed (Carrera et al., 1999). This weaker induction may be explained by the lower levels of blue light present in the incandescent lights compared with the fluorescent lights we have used in our experiments, which have greater amounts of blue light and induce *StGA20ox1* much more rapidly. The higher levels of expression in blue light compared with white light can either be explained by a reduced level of feedback inhibition in blue light or alternatively by some degree of inhibition by a component of white light other than the 400- to 500- nm wavelengths transmitted through the blue filter.

We have demonstrated that PHYB levels can affect *StGA20ox1* transcript levels, however, it is clear that other photoreceptors are also likely to be involved in the control of this gene and of other genes involved in GA biosynthesis, which would at least in part explain how light quality can have such diverse and dramatic effects on plant development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

WT potato (*Solanum tuberosum* cv Désirée), and the photoperiodic *S. tuberosum* L. subsp. *andigena* WT line 7540 were obtained from the Institute für Pflanzenbau und Pflanzenzüchtung Bundesforschungsanstalt für Landwirtschaft Braunschweig-Volkenrode (Braunschweig, Germany). The antisense *PHYB* transgenic lines were produced as described by Jackson et al. (1996). These antisense lines are deficient in PHYB1, however they may also have reduced levels of PHYB2 and therefore are referred to as antisense *PHYB* lines. The antisense *PHYA* transgenic lines were produced as described in Heyer et al. (1995). Plants were derived from in vitro grown plants that had been planted out into soil and then subsequently propagated through stem cuttings. The plants were grown in soil in growth cabinets (Sanyo, Gallenkamp PLC, Loughborough, UK) under cool-white Pluslux 3,500 fluorescent tubes (Thorn, Borehamwood, Herts, UK) at light levels of 100 μmol m^{-2} s^{-1} and at constant 70% humidity and 22°C. The blue filter (no. 119, Dark Blue) was obtained from LEE Filters Ltd. (Andover, UK).

For each time point or leaf position, two samples were taken from duplicate plants (i.e. four leaves, two per plant) and combined before RNA extraction in an attempt to average out differences between leaves and plants and to obtain a more representative picture of the effects of the light environment on *StGA20ox1* transcript levels. Each data point therefore represents the average transcript level from four separate leaves and two different plants.

Northern-Blot Analysis

RNA was extracted from leaves as described (Logemann et al., 1987). Thirty micrograms of total RNA was loaded

per track and run out on agarose/formaldehyde gels. The RNA was blotted onto a nylon membrane and hybridized with a radioactively labeled (Rediprime kit, Amersham Life Science, Buckinghamshire, UK) 180-bp *StGA20ox1* probe fragment that specifically recognizes *StGA20ox1* and no other potato GA 20 oxidase (fragment 7 in Carrera et al., 1999). Hybridization conditions were as described by Amasino (1986), filters were washed twice in 3× SSC, 0.5% (w/v) SDS, at 60°C. The strength of the signal on the filters were analyzed with a phosphor imager using ImageQuant software (version 5.0, Molecular Dynamics, Sunnyvale, CA).

To correct for any differences in the amounts of RNA loaded in each sample, the northern blot was subsequently hybridized with a probe to the constitutively expressed *S4* ribosomal protein gene of potato (Braun et al., 1994), and the relative levels of *StGA20ox1* transcript were calculated. Due to the uniformity of the tissue analyzed (only leaf tissue and in most experiments just the first three fully expanded leaves were sampled), and the spectrophotometric quantitation of the amount of RNA loaded, we observed little variation in *S4* gene transcript levels in our experiments (Fig. 2). The *S4* signal levels obtained from the northern blots correlated well with the levels of RNA in the ethidium bromide-stained gels.

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