A red and far-red light receptor mutation confers resistance to the herbicide glyphosate

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SUMMARY

Glyphosate is a widely applied broad-spectrum systemic herbicide that inhibits competitively the penultimate enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) from the shikimate pathway, thereby causing deleterious effects. A glyphosate-resistant Arabidopsis mutant (gre1) was isolated and genetic analyses indicated that a dysfunctional red (R) and far-red (FR) light receptor, phytochrome B (phyB), caused this phenotype. This finding is consistent with increased glyphosate sensitivity and glyphosate-induced shikimate accumulation in low R:FR light, and the induction of genes encoding enzymes of the shikimate pathway in high R:FR light. Expression of the shikimate pathway genes exhibited diurnal oscillation and this oscillation was altered in the phyB mutant. Furthermore, transcript analysis suggested that this diurnal oscillation was not only dependent on phyB but was also due to circadian regulatory mechanisms. Our data offer an explanation of the well documented observation that glyphosate treatment at various times throughout the day, with their specific composition of light quality and intensity, results in different efficiencies of the herbicide.

Keywords: Arabidopsis thaliana, glyphosate, herbicide resistance, shikimate pathway, phyB, circadian rhythm.

INTRODUCTION

Glyphosate (N-phosphonomethyl glycine) is a highly effective, post-emergence, broad-spectrum, systemic herbicide that mobilizes to the chloroplasts of actively growing sink tissues and inhibits irreversibly a shikimate pathway component EPSPS (EC 2.5.1.19) (Figure S1), causing subsequent plant death due to the disturbance of the carbon metabolism required to produce aromatic amino acids and their derivatives in plants (Amrhein et al., 1980; Herrmann, 1995b; Bromilow and Chamberlain, 2000). Since 1996, glyphosate-resistant (GR) crops have been in wide commercial use partly due to the apparently low toxicity (Franz et al., 1997; Williams et al., 2000) and fast immobilization and degradation characteristics of glyphosate in soils (Rüppel et al., 1977; Zhou et al., 2004). The glyphosate application range has increased dramatically mainly because of their cost-effective, superior weed control technology and no-till monoculture systems (Gianessi, 2008; Duke and Powles, 2009). Widespread glyphosate use causes high selection pressure on weeds that has resulted in weed population shifts and the evolution of over 20 GR weed species (Benbrook, 2012; Heap, 2013). The mechanism(s) of resistance of most of these species, while not well characterized, are believed to be due mainly to alterations of the target enzyme. In the cases of goosegrass (Eleusine indica) and ryegrass species (Lolium rigidum, and Lolium multiflorum) a mutation at Pro106 in EPSPS confers glyphosate resistance (Baerson et al., 2002; Wakelin and Preston, 2006; Jasieniuk et al., 2008; Kaundun et al., 2011), whereas in populations of Palmer’s amaranth (Amaranthus palmeri) and in Italian ryegrass (Lolium multiflorum), an amplification of EPSPS underlies the increased glyphosate resistance (Gaines et al., 2010; Salas et al., 2012). In addition, high pressure liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) analyses have revealed tissue-specific translocation rates and vacuolar sequestration in ryegrass (Lolium rigidum) and horseweed (Conyza canadensis) populations (Lorraine-Colwill et al., 2002; Ge et al., 2010). Furthermore, several reports
have suggested that glyphosate efficiency is affected by the time of day at which it is sprayed (Martinson et al., 2005) and have attributed this effect to factors such as leaf angle changes (Mohr et al., 2007).

In order to gain a better understanding of glyphosate-resistance mechanisms, we performed a forward genetic screen using a T-DNA tagged (pGPTV-Bar) Arabidopsis thaliana mutant population, ecotype Col0, and obtained a number of mutants that are either hypersensitive to or hypo-sensitive to glyphosate. One mutant, the glyphosate response 1 (gre1), showed an elevated resistant phenotype in shoot and root growth caused by a loss of function of the red and far-red (FR) light receptor, phytochrome B (phyB). Here we demonstrate the role of phyB on glyphosate sensitivity and present a model that links phyB signaling and circadian clock associated control mechanism to the complex regulation of the shikimate pathway.

RESULTS

Characterization of the GR mutant gre1

A glyphosate response mutation screen was performed on agar plates under 16 h white light at a fluence rate of 22 ± 2 μmol m⁻² s⁻¹ (Figure S2) and this screen led to the isolation of the gre1 mutant. The gre1 plant showed GR traits such as vigorous shoot growth and new leaf formation, development of lateral root branches and nodules, and high chlorophyll content and reduced shikimate levels in the shoot in response to 15–50 μM glyphosate treatments over 12 days (Figure 1). Glyphosate treatments ≤5 μM did not affect shoot growth, however they did cause induction of lateral root development in both genotypes (Figure 1a). Glyphosate concentrations ≥15 and ≥30 μM inhibited lateral root and primary leaf formation in the wild-type (wt) only, whereas, in gre1 lateral roots, root nodules and primary rosette leaves developed on glyphosate concentrations up to 50 μM (Figure 1a). We determined that 15 and 30 μM glyphosate caused marginal and complete chlorosis respectively in newly formed primary leaves in the wt but not in gre1 (Figure 1a). The glyphosate-response phenotype was confirmed by fresh weight comparisons, chlorophyll content analyses and shikimate accumulation assays, and it was shown that gre1 plants gain more fresh weight, contain more chlorophyll and less shikimate in response to glyphosate treatment with the largest differences noticed at an exposure to 15 μM glyphosate (Figure 1b–d).

The gre1 phenotype at 30 μM glyphosate treatment is strictly fluence rate-dependent, with the most pronounced characteristics in light intensities between 15 and 25 μmol m⁻² s⁻¹ (Figure S3). Glyphosate resistance in gre1 is lost when plants are exposed to light intensities outside this range. Gre1 also exhibits morphological differences from the wt, such as longer hypocotyls and shorter roots, earlier flowering, paler green leaves and bright yellow colored seeds (Figure S4).

![Figure 1. Gre1 plants show glyphosate resistant phenotypes.](image)

(a) Phenotypes of wt and gre1 plants screened on 15 or 30 μM glyphosate medium for 12 days under L1 = 22 ± 2 μmol m⁻² s⁻¹, 16 h light regime. Close-up pictures show shoot and root phenotypes of wt and gre1 plants. White scale bars represent 5 mm, and empty arrowheads point to the primary leaves.

(b–d) Fresh weight, relative chlorophyll content and shikimate level of wt and gre1 plants in glyphosate dose-response studies. Fresh weight data were taken at day 12 and chlorophyll content and shikimate analyses were done at day 9. Each data point represents mean ± standard errors (SEs), with 15 plants analysed for fresh weight, and five plants for chlorophyll and shikimate content analyses. Double and single asterisks indicate P-values < 0.05 and < 0.1 respectively.

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In addition to the phenotypic characterization of gre1 plants grown on agar plates, we also determined glyphosate resistance of soil grown plants (Figure S5) under a higher fluence rate (75 ± 5 μmol m<sup>-2</sup> s<sup>-1</sup>). The higher fluence rate in soil was required to avoid elongated hypocotyl and petiole due to the Shade Avoidance Syndrome (SAS). In contrast with wt plants, gre1 plants continued to grow after treatment with 200 μM glyphosate (0.2535 mg m<sup>-2</sup>) and produced seeds even after the death of older leaves (Figure S5). These phenotypic and physiological characteristics of gre1 clearly point to the presence of a glyphosate-resistance mechanism that prevents the irreversible inhibitory effects on the target enzyme and/or a compensating activity on the shikimate pathway.

The glyphosate resistant phenotype of gre1 is caused by dysfunctional phyB

A back-cross analysis (wt × gre1) was performed on gre1 plants to confirm the inheritance of glyphosate resistance (Table S1). In the analysis, all tested 134 F<sub>1</sub> plants inherited both glyphosate- and bialaphos-resistant phenotypes from the gre1 parental line and these phenotypes segregated 1:3 (sensitive:resistant) in the F<sub>2</sub> progenies, indicating that resistance is a dominant trait. We performed a genetic co-segregation analysis in the F<sub>3</sub> GR homozygote progeny using both glyphosate and bialaphos selection procedures and all the 380 lines tested were resistant to both herbicides and confirmed the tight linkage between T-DNA insertion and the glyphosate-response locus.

A thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) analysis of the mutant identified a T-DNA insertion (triangle) at the first exon of PHYB (At2g18790) that encodes an apoprotein of the red (R) and far-red (FR) light receptor phyB (Figure 2a). We then performed a PHYB expression analysis (Figure 2b) and showed the absence of the PHYB amplicon in gre1. The GR phenotypes of gre1 lines were confirmed by testing another allelic phyB loss-of-function mutant line (phyB-9) that also confers resistance to glyphosate (Figure 2c). This result further supported the conclusion that a dysfunctional phyB can cause glyphosate resistance, and suggested that over-expression of PHYB may increase sensitivity to glyphosate. When PHYB was over-expressed on wt and gre1 genetic backgrounds (pbc1 and gpbcl), hypersensitive phenotypes were observed in response to glyphosate with traits that included arrested shoot and root development, twisted cotyledons and shoot anthocyanin accumulation (Figure 2c).

We also tested the response of phyA (the light labile phytochrome) deficient (phyA-201) and phytochromobilin (the light absorbing unit of phytochromes) deficient (hy1-100) plants on agar containing 30 μM glyphosate (Figure 2c). The analysis revealed that phyA deficiency does not lead to glyphosate resistance, whereas phytochromobilin deficiency conferred glyphosate resistance and that the glyphosate response is similar to that of gre1 and phyB-9 plants (Figure 2c).

Arabidopsis glyphosate hypersensitive phenotypes are far-red-light specific

Phytochrome exists in two distinct photo-reversible isoforms, Pr and Pfr in FR (near 730 nm) and in R (near 660 nm) respectively (Andel et al., 1996; Rockwell et al., 2006). It is generally accepted that the physiologically active form of phytochrome is Pfr (Rudiger et al., 1983), as it localizes in the nucleus where it interacts with transcription factors (TFs) to regulate gene expression causing photomorphogenesis that in turn enables adaptation of the plant to changing environmental conditions (Nagatani, 2004). In order to examine the physiological relevance of the Pfr in the glyphosate response, wt and gre1 plants were treated with 20 μM glyphosate and grown under high R:FR and low R:FR conditions (Figure 3a). Both wt and

![Figure 2. The glyphosate resistant phenotypes of gre1 are conferred by the knock-out mutation of PHYB gene.](image)
showed the same resistance response under high R:FR light conditions, whereas only the wt was hypersensitive under low R:FR. This FR light-specific (low R:FR) glyphosate hypersensitivity of wt plants is reflected in the glyphosate-induced shikimate accumulation. Glyphosate treatment caused a seven-fold increase in shikimate accumulation in shoots at low R:FR as compared with the control plants, but only a three-fold increase at high R:FR (Figure 3b). In gre1, both high and low R:FR light caused significantly low amounts of shikimate accumulation in the glyphosate treatment if compared with wt (Figure 3b).

We also performed transcript analyses of all six genes that encoded enzymes of the shikimate pathway (Figure S1) under dark (D), white light (W) and high and low R:FR light conditions 2 h after the end of the night cycle (Figure 3c) and observed a hyper-accumulation in the response to high R:FR light. As compared with W light, high R:FR light caused a significant increase in DeHydroQuianate Synthase (DHQS), 3-DeHydroquinate Dehydratase-Shikimate DeHydrogenase (DHD-SOH) and Shikimare Kinase 1 (SK1) transcripts in the wt. In the mutant this increase was slightly reduced. The high R:FR light-dependent transcript level of Deoxy d-Arbino Heptulsonate 7-Phosphate Synthase 1 (DAHPS1) was more pronounced in gre1 than in the wt. Except for DAHPS1, there was hardly any difference between the expression values during the 2 h exposure to D and W in the wt as well as in gre1 (Figure 3c).

Roles of phyB and the circadian clock in the regulation of the shikimate pathway

Given the phyB-dependent glyphosate hypersensitive response (Figures 2 and 3), we looked for light-regulated transcription factor-binding sites (TFSs) in the promoters of the shikimate pathway genes. We found that the first and the fourth genes of the pathway, DAHPS1 and SK1, harbored circadian clock-related TFS motifs such as the Evening Element (EE) motif (AAAATATCT), and circadian clock associated 1 (CCA1) binding site (AAMAAATCT), while the second and fifth genes, DHQS and Enol Pyruvyl Shikimate Phosphate Synthase 1 (EPSPS1), harbored the Phytochrome-Interacting Factor (PIF) binding G-motif promoter elements (CACGTG) (Table S2). The presence of both phytochrome and circadian clock associated TFSs suggested

Figure 3. Low R:FR light causes glyphosate hypersensitivity. (a) Four-day-old wt and gre1 plants were tested under high (3.8 μmol m⁻² sec⁻¹) and low (0.09 μmol m⁻² sec⁻¹) R:FR light enriched with white light (total LI = 22 ± 2 μmol m⁻² sec⁻¹) in a 16 h light regime with (20 μM) or without (control) glyphosate treatments for 9 days. White scale bars are 10 mm. (b) Shikimate assay was done on glyphosate treated plants treated under the same conditions described above. Data represent the mean of five plants with ± standard error (SE). Asterisk indicates P-values < 0.05. (c) Shikimate pathway regulatory gene expression was performed by qPCR on 12-day-old, agar plate grown plants treated under white (W), high or low R:FR light conditions, or dark (D) condition for 2 h after the 8 h dark period. Relative transcript abundance was calculated using W light treated wt mRNA as the calibrator and normalized with respect to βACTIN7 gene transcript level. Error bars represent ± SE of the mean of three samples. Ratio of relative transcript numbers in high R:FR/W of both genotype are written next to the corresponding gene. Asterisks indicate a significant increase.

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an expression analysis on shikimate pathway genes and genes that encode components of the circadian clock (Early Flowering 4 - ELF4 and CCA1) and phyB-dependent signaling molecules (Phytochrome-Interacting Factor 4 and 5 - PIF4 and PIF5). These analyses were performed on plants that were grown on both agar plates under 22 ± 2 μmol m⁻² s⁻¹ white light and in soil under 75 ± 5 μmol m⁻² s⁻¹ white light. Under both growth conditions the gre1 phenotype was obtained in response to glyphosate (Figures 1 and S5). Quantitative PCR (qPCR) revealed a diurnal expression pattern of the shikimate pathway genes under both conditions with only slight differences (Figures 4a and S6a).

When we studied the agar plate grown plants, in the wt, the peak level of DAHPS1, SK1 and Chorismate Synthase (CS) transcript accumulation occurred after 1 h light exposure and the lowest levels occurred at the onset of dark. In contrast, transcript accumulation of DHQS, DHD-SDH and EPSPS1 reached a peak at the onset of dark and the lowest level was observed at midday (8 h). In gre1 plants, the oscillations of DAHPS1 and SK1 were altered, with DAHPS1 peaking at midday (8 h) and SK1 at the onset of the dark period (16 h), the lowest transcript levels of these genes shifted to the onset of the light period. The pronounced reduction of transcripts seen in the wt plants at midday did not occur in gre1 (Figure 4a).

In soil, all genes of the pathway were highly transcribed during the dark period, and immediately down-regulated at the onset of light period (Figure S6a). Transcript accumulation of wt genes, except for DAHPS1, appeared to increase continuously from the second half of the day and reached a peak at the onset of the light period. DAHPS1 transcripts started accumulating after 1 h light. In gre1 plants, the maximum transcript accumulation of these genes appears to be shifted to the end of the light period (16 h) and extended into the dark period in gre1. This shift in relative transcript amounts in gre1 is seen clearly between 8 and 16 h. In comparison with the wt, all mRNA ratios of 16 h/8 h in gre1 were higher, significantly (≥2-fold) in DAHPS1 and EPSPS1. The transcript peak times for both DAHPS1 and SK1 in wt were at the end of the light period and the peak times were not altered in gre1 (Figure S6a). The different transcript accumulation patterns of DAHPS1, SK1 and CS genes under different fluence-rates implicated light intensity as a factor in the regulation of the shikimate pathway (Figures 4a and S6a).

Furthermore, we have analysed transcript accumulation of the circadian clock regulatory elements: CCA1, and ELF4, and phyB downstream signaling components, PIF4...
and PIF5 under the same conditions as described above. qPCR revealed that the expression profiles of these TFs were similar in both genotypes, however, transcript accumulation of each gene was more pronounced in gre1 (Figures 4b and S6b).

We also checked the maintenance of diurnal patterns of the pathway under continuous light in order to detect whether this oscillation is controlled by the internal time keeping mechanism. qPCR revealed that the transcript accumulation pattern of all shikimate pathway genes still oscillated in a rhythm similar to the one of the circadian clock component ELF4 under continuous light in both wt and gre1 (Figures 4c and S6c). The peak value of ELF4 was two times more pronounced in agar and six times more in soil for gre1 as compared with wt. The oscillation patterns during the light period – ZT (Zeitgeber Time) – were identical in both genotypes on the agar plate but more pronounced in gre1 than in wt in soil grown plants. The gre1 mRNA ratios (32 ZT/24 ZT) were 4–11.4 times bigger than those of the corresponding wt ratios (Figure S6c). As light has a strong inhibitory effect on shikimate pathway gene expression (Figures 4a and S6a), it is conceivable that continuous dark might increase the amplitude of the circadian oscillation of these genes. To test this hypothesis, the circadian oscillation effect was studied under continuously dark conditions in the soil grown plants and revealed a response that was similar to that under continuous light. In the dark, transcript abundance of all pathway genes in gre1 was more pronounced (1.7–3.6 times) at 32 ZT when compared with wt and also follows ELF4 oscillation (Figure S7).

**Glyphosate effects depend on shikimate pathway activity**

Our previous results revealed that both the phyB-dependent mechanism and the circadian clock regulatory components are essential parts of the transcriptional regulation of shikimate pathway genes, and that defective phyB causes transcript accumulation in major components of the shikimate pathway in gre1 (Figures 4 and S6). Thus, enhanced transcript accumulation is the most likely reason for the development of glyphosate resistance in gre1. In order to test this hypothesis, we performed glyphosate experiments on greenhouse grown plants that had been sprayed at different times. The growth inhibitory potential of the herbicide varied according to the time of day the spray was applied. The plant survival rates were determined based on the development of stem elongation (Figure 5). In comparison with gre1 plants, growth of wt plants was arrested completely when they were sprayed 200 μM glyphosate (0.2535 mg m⁻²) during the first half of the day, whereas glyphosate spraying in late afternoon caused only a partial inhibition (Figure 5a, b). When glyphosate was applied either at 5 or 9 o’clock (am) it was lethal to wt plants, however, all wt plants survived if the application occurred later.

**DISCUSSION**

**PhyB mode of action determines the response to glyphosate**

To-date more than 20 GR weed species have been reported, but only in goosegrass (Eleusine indica) and ryegrass species (Lolium rigidum, and Lolium multiflorum) can resistance be attributed to alterations at the binding site of EPSPS (Baerson et al., 2002; Wakelin and Preston, 2003).
with previous reports of dominant (semi-dominant), loss-of-function alleles of phyB-overexpressing lines (Figure 2c). The phenotypic characteristics of the phyB mutation are consistent with those reported for the dominant (semi-dominant), loss-of-function alleles and can be explained by haplo-insufficiency (Veitia, 2002; Exner et al., 2010; Wang et al., 2011; Meinke, 2013). Arabidopsis contains five phytochromes (phyA, B, C, D, and E) classified as type I or type II based on their light stability (Sharrock and Quail, 1989; Furuya, 1993). PhyA is the only light labile type I and is predominant in dark-grown seedlings (Furuya, 1993; Smith, 2000). That the phyA-deficient mutant phyA-201 does not show a GR phenotype further confirms that the glyphosate response phenotypes of gre1 are due specifically to a dysfunction of phyB. Chromophore deficiency could also have a role in the glyphosate response as all phytochrome apoproteins gain functionality only when assembled covalently with phytochromobilin, a light-absorbing linear tetrapyrrole chromophore, by forming 250 kDa dimers in the cytosol (Muramoto et al., 1999; Quail, 2002). A heme-oxygenase, which catalyzes an oxygenation reaction for the formation of most of the phytochromobilins, is encoded by the HY1 locus in Arabidopsis (Muramoto et al., 1999). Our glyphosate response analysis suggested that HY1 locus was also capable of conferring glyphosate resistance as the chromophore biosynthesis-deficient hy1-100 mutant exhibits glyphosate resistance that is similar to phytochrome-deficient mutants (Figure 2c). However, chromophore-related glyphosate responses might be attributed to a dysfunctionality of all phytochromes, while we propose that it is caused specifically by phyB deficiency as phyB is the most abundant phytochrome in light-grown plants (Sharrock and Clack, 2002).

Based on their specific sensitivities to light intensity, three types of modes of action of phytochromes have been suggested: (1) a very-low-fluence response (VLFR); (2) a low-fluence response (LFR); and (3) a high-irradiance response (HIR). PhyB is considered to mediate FR light reversible LFR in R light (Casal et al., 1998). It is therefore reasonable to conclude that phyB-dependent glyphosate sensitivity must be linked to the LFR and this is consistent with the observation that, firstly, the most pronounced phenotype of gre1 in agar plate assays could be seen at light intensities between 15–25 μmol m⁻² s⁻¹ (Figure S3), and secondly, that the wt phenotype under these LFR conditions is reversed by high R:FR light (Figure 3a). Given this dependence of the phenotype on the light regime, the agar plate experiments presented and discussed here were performed at the fluence rate of 22 ± 2 μmol m⁻² s⁻¹ (Figures 1, 2c, S2, S3, and 4). In addition, gre1 plants that are grown in soil under a fluence rate of 75 ± 5 μmol m⁻² s⁻¹ where no SAS occurs, also exhibited a resistant phenotype after spray application of glyphosate (Figure S5). The fluence-rate-dependent glyphosate effect on phyB signaling under different environmental and developmental conditions will need to be elucidated further. Nevertheless, one of our glyphosate spray experiments in the greenhouse demonstrated that the wt and gre1 plants showed increased resistance to glyphosate application when treated in the afternoon or in the late evening (Figure 5). This finding is consistent with the evening LFR response mediated by phyB. There are numerous reports that link glyphosate sensitivity to the time of day at which it is applied, and it was observed that greatest annual weed control effect was obtained when glyphosate was applied between 9 a.m. and 6 p.m. rather than at 6 a.m. or between 9 p.m. and 12 a.m. (Martinson et al., 2005). A diurnal effect on leaf angle change, a phytochrome-dependent process, has also been recognized as one of the major reasons of greater biomass accumulation in broadleaf weeds when glyphosate was applied in the early morning rather than in late afternoon (Mohr et al., 2007). PhyB signaling and the circadian clock affect shikimate pathway activity

The shikimate pathway is considered to be a key link between carbohydrate metabolism and the biosynthesis of aromatic amino acids and many secondary metabolites (Singh et al., 1991; Herrmann, 1995a) and consists of a sequence of six metabolic steps that lead to the synthesis of chorismate (Figure S1). The fact that gre1 is a light receptor mutant that affects chorismate biosynthesis makes it a potentially highly useful tool for the study of light effects on the pathway. Our results suggested that the glyphosate resistance of gre1 is not related to any modification of the target enzyme but is the consequence of increased activity of the entire shikimate pathway due to altered regulation of the pathway caused by dysfunctional phyB (Figures 2, 3, 4 and S6). It is noteworthy that the gre1 mutant shows less shikimate accumulation in response to glyphosate under low R:FR light condition and this result is indicative of an active shikimate pathway (Figure 3b). Accumulation of shikimate, an intermediate product of the shikimate pathway, is considered one of the
The regulatory effect of phyB on the shikimate pathway activity cannot account for all transcriptional responses in the regular day–night regime and in continuous light conditions, because the genes responsible for the pathway are also under a diurnal rhythm (Figures 4a, c and S6a, c). Among these, the first and the fourth gene of the pathway, DAHPS1 and SK1, showed the most pronounced and stable oscillation in both the regular day–night regime and continuous light conditions. The distinct expression patterns of these two genes suggested the presence of an additional regulatory mechanism that is under circadian control. Given that both DAHPS1 and SK1 genes contain EE- and CCA1-binding TFSs (Table S2), we propose that the regulation is most likely operated through negative regulation of CCA1 (Figure 6). CCA1 is the central component of circadian clock and it recognizes both EE and CCA1 motifs that are conserved in the promoter regions of light-regulated genes to induce peak expression in the evening (Harmer et al., 2000), or activate R light-dependent transcription (Wang et al., 1997). This explanation is consistent with the up- and down-regulation of the first and fourth genes, DAHPS1 and SK1, of the pathway and constant diurnal oscillation of the shikimate pathway that is inverse to that of the CCA1 expression pattern (Figures 4a, c and S6a, c). The plant circadian clock is composed of many regulatory factors that operate interlinked transcriptional and post-translational positive and negative feed-back loops to enable day and night anticipation (Harmer, 2009; Mäss & Yanovsky, 2009). It has been proven that CCA1 and its close homologue LHY (Late Elongated Hypocotyl) suppress expression of evening-phased genes TOC1 and ELF4 through binding EEs present in their promoter regions. Conversely, both TOC1 and ELF4 promote expression of CCA1 and LHY genes and form a transcriptional feed-back loop, thereby enabling circadian oscillation (Alabadi et al., 2001; Kikis et al., 2005; Pruneda-Paz et al., 2009) (Figure 6). In addition, positive phyB regulation of CCA1 and LHY expression through PIF3 bound to G-boxes in their promoter regions has also been suggested (Martinez-Garcia et al., 2000) (Figure 6). It is this regulatory link that is disturbed in gre1 because of its dysfunctional phyB. However, the overriding system of the circadian clock may, at least in part, compensate this defect in CCA1 (Figure 6).

Another circadian clock related regulatory mechanism that involve EC elements (‘evening complex’ (ELF4-ELF3-LUX)) has been proposed for PIF4 and PIF5 expression in the early morning (Nusinow et al., 2011) as one of the major components of EC, LUX, interacts with the promoter regions of both PIFs in vivo (Nusinow et al., 2011). Dysfunctional phyB is likely to lead to overproduction of EC, because regulation of PIF4 and PIF5 is crucial for the complex function (Nusinow et al., 2011). Therefore, our model proposes a transcriptional feed-back loop in which PIFs

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**Figure 6.** A model proposing controls of the shikimate pathway.
PhyB exerts control over the pathway by changing its active and non-active, Pfr and Pr, conformations according to the light quality (R:FR). The R:FR increases gradually from the onset of light till midday and decreases towards evening. Expression of DAHPS1 and SK1 is repressed by the circadian component CCA1 during the day, while phyB (Pfr) promotes the accumulation of DHQS and EPSPS1 in response to high R:FR through the interaction with PIF4 and PIF5. During the evening, EC elements have a role in stabilizing PIF4 and PIF5. Arrows and blunt end lines indicate stimulatory and inhibitory effects respectively, and dashed arrows indicate transcriptional activation. The proposed feed-back regulation of the circadian clock and EC-dependent regulation are shown as embossed arrows.
play a positive regulatory role in the transcription of LUX in the afternoon (Figure 6). LUX might play a ‘housekeeping’ role in the absence of phyB during the night to control proper transcription of PIFs. This idea might explain the persisting high levels of the shikimate pathway genes at midday and the extended expression of DHQS and EPSPS1 in gre1 (Figures 4a and S6a). Transcript analyses of ELF4, CCA1, PIF4 and PIF5 support the idea that the circadian rhythm and phyB affect the shikimate pathway as the ELF4 appears over-expressed and CCA1 and the two PIWs show reduced transcript accumulation during the afternoon in the gre1 (Figures 4b and S6b). Furthermore, oscillation of all shikimate pathway genes under continuous light indicated that this pathway is under the control of the circadian clock (Figures 4c and S6c). These data therefore link the circadian clock to phyB signaling and also enable integrated regulation of the shikimate pathway.

Based on our transcript accumulation studies of the major components of the shikimate pathway, we propose a regulatory network that involves both the circadian clock and phyB signaling. The circadian clock assures internal time keeping, whereas, phyB plays a stabilizing role and tunes the response to specific environmental conditions. In the case of gre1, hyper-activation of the shikimate pathway affects the entire pathway and overrides the functions normally under the control of circadian clock. The resulting increase of EPSPS1 contributes to an increase in the glyphosate target enzyme, which in turn causes increased resistance to glyphosate. This conclusion is consistent with the phenotype that shows reduced glyphosate sensitivity, increased fresh weight, reduced chlorophyll damage and reduced shikimate accumulation in the presence of glyphosate. Thus, downstream signaling of phyB may shed light on the regulation of the shikimate pathway and increased levels of EPSPS in particular, and this in turn may explain glyphosate resistance in some weed species.

EXPERIMENTAL PROCEDURES

Plant material and growth condition

T-DNA tagged (pGPTV-Bar (http://biotech.unl.edu/pgptv-bar)) mutant population of Arabidopsis thaliana, ecotype Col0, were used in the screen. To select for positive transformed lines, seedlings of each generation were sprayed with 0.01% Basta (bialaphos) solution (Aventis Crop Science Deutschland GmbH, Hattersheim, Germany) repeatedly at 3-day intervals for 2 weeks. Seeds of the bialaphos resistant T0 (transformant 2) plants were pooled and screened for glyphosate resistance by sowing directly on 50 ml of 0.25 N HCl solution followed by incubation at 60°C for 15 min. Shikimate content was determined using a 96-well microtiter plate assay following a method detailed elsewhere (Shaner et al., 2005).

Shikimate content analysis

Shikimic acid was extracted from plantlets by floating them in 100 μL of 0.25 N HCl solution followed by incubation at 60°C for 15 min. Shikimate content was determined using a 96-well microtiter plate assay following a method detailed elsewhere (Shaner et al., 2005).

PCR conditions and gene expression analyses

Sequences of all oligo-nucleotides used for PCRs are described in Table S3. TAIL-PCR was done using three nested primers with the combination of two degenerative primers. For the PHYB gene expression analyses (RT-PCR) expression of UBIQUITIN 10 gene was used for normalizing the band intensity. Total RNA was extracted using the RNeasy plant mini kit (Qiagen, www.qiagen.com) and superscript RT-PCR first strand synthesis kit with oligo(dT) (Invitrogen, www.ift.com) was used for cDNA synthesis. qPCRs were performed using fast SYBR green master mix (Applied Biosystems, www.appliedbiosystems.com) in the AB-7900HT Fast Real-Time PCR machine. In the QPCR, CT values of mRNA expression in the W light, at 1 h light treatment, and at 24 h ZT point were used as calibrators for gene expression analyses in the light quality studies, light and dark period and circadian oscillation studies respectively. Twelve-day-old plants were used for qPCR analyses with three replications. All qPCR data were normalized to the value of mRNA expression of ACTIN7.

Glyphosate spraying of plants in the greenhouse

Plants were grown in the greenhouse under natural light conditions. A 200 μM glyphosate solution (N-phosphonomethyl glycine (Sigma Aldrich) in 10 mM (NH4)2SO4 and 0.01% w/v Tween-20) was applied to 20-day-old soil grown plants at 5:00, 9:00, 13:00, 17:00 and 21:00 h by using a herbicide spray booth. Generation
PhyB signaling affects glyphosate efficiency

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