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The pleiotropic effects of the *bar* gene and glufosinate on the *Arabidopsis* transcriptome

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Summary

The *Arabidopsis* transcriptome was studied using the Affymetrix *Arabidopsis* ATH1 GeneChip in wild-type plants and glufosinate-tolerant transgenic plants expressing the bialaphos resistance (*bar*) gene. Pleiotropic effects were specifically generated in the transcriptomes of transgenic plants by both the *bar* gene and glufosinate treatments. In the absence of glufosinate, four genes were differentially expressed in the transgenic lines and another 80 genes were differentially expressed in the presence of glufosinate, 29 of which were specific to transgenic plants. In contrast, the number of differentially expressed genes specific to wild-type plants was 194 during the early response at 6 h of glufosinate treatment, and increased to 3711 during the late response at 48 h. Although the wild-type plants undergo extensive transcriptional reprofiling in response to herbicide-induced stress and, finally, plant death, the transgenic plants appear to activate other detoxification processes to offset the toxic effects of the residual herbicide or its derivatives. This study provides the first description of the pleiotropic effects of the *bar* gene and glufosinate on the plant transcriptome.

Introduction

Phosphinothricin (L-PPT), also known as glufosinate-ammonium, is the active ingredient in several non-selective herbicide products, such as Basta, Liberty, Finale, Buster and Herbiace. Because L-PPT is structurally similar to L-glutamate, it is able to bind to the active site of glutamine synthetase (GS) and act as a competitive inhibitor of the enzyme (Block *et al*., 1987; Wohlleben *et al*., 1988). GS is an essential enzyme in plant nitrogen metabolism, because it assimilates ammonium through the conversion of L-glutamate to L-glutamine. Ammonium is generated in a variety of metabolic processes and serves as the nitrogen donor for the biosynthesis of all nitrogenous organic compounds needed for plant growth and development (Crawford and Arst, 1993; Daniel-Vedele *et al*., 1998). The mechanism of L-PPT toxicity is believed to involve ammonia accumulation to phytotoxic levels, followed by the impairment of photosynthesis causing plant death (Wendler *et al*., 1990; Wehrmann *et al*., 1996; Dan Hess, 2000).

The bialaphos resistance (*bar*) and phosphinothricin acetyltransferase (*pat*) genes from *Streptomyces hygroscopicus* and *S. viridochromogenes*, respectively, encode the enzyme phosphinothricin acetyltransferase (PAT) which inactivates L-PPT by transferring the acetyl group from acetyl-coenzyme A (acetyl-CoA) to the free amino group of L-PPT, yielding *N*-acetyl-L-PPT (Thompson *et al*., 1987; Wohlleben *et al*., 1988; D'Halluin *et al*., 1992). The enzymes perform comparably in plants and are highly specific for L-PPT (Wehrmann *et al*., 1996). Crops resistant to L-PPT have been created with the *bar* and *pat* genes using *Agrobacterium*- and particle bombardment-mediated transformation, and are now widely grown. In addition to *Arabidopsis*, these include transgenic maize, rice, barley, oat, wheat and cotton (Fromm *et al*., 1990; Christou *et al*., 1991; Somers *et al*., 1992; Weeks *et al*., 1993; Akama *et al*., 1995; Cheng *et al*., 1997; Keller *et al*., 1997; Tingay *et al*., 1997). Furthermore, both the *bar* and *pat* genes are preferred plant-selectable marker genes in several plant species (Wehrmann *et al*., 1996). Among the herbicide-resistance genes, they are the most extensively

used as selectable marker genes in the scientific literature (Miki and McHugh, 2004).

An understanding of the unintended effects of genes (Cellini *et al*., 2004), such as *pat* or *bar*, is essential for a true comparison of transgenic plants with their non-transgenic progenitors. The functional analyses of unknown genes in transgenic plants in which *pat* or *bar* have been co-transformed and co-expressed as selectable marker genes are dependent on this knowledge. Studies with other selectable marker genes and reporter genes have shown that the insertion and expression of neomycin phosphotransferase type II (*nptII*) for kanamycin resistance and *UidA* for β-glucuronidase (GUS) reporter activity do not produce unintended effects on the transcriptome of *Arabidopsis* plants under normal growth conditions or under abiotic stresses, such as heat, cold, salt and drought (El Ouakfaoui and Miki, 2005). These data show that transgenic plants created with these marker genes are transcriptionally identical to non-transgenic plants. Comparable data are needed for each marker gene used in the study of transgenic plants.

The effects of the *pat* and *bar* genes on the transcriptome have not yet been investigated in the presence or absence of L-PPT. Therefore, a large number of questions related to the detailed mechanism of action, the range of pleiotropic effects and the progression of events following exposure to L-PPT remain largely unanswered, despite the widespread use of the genes and herbicides containing L-PPT. In this work, we used the Affymetrix ATH1 GeneChip to profile global gene expression patterns associated with the insertion and expression of the *bar* gene in the model plant *Arabidopsis thaliana*. We found that the expression and insertion of the *bar* gene produced changes in global gene expression in both the absence and presence of L-PPT. We report the identification of genes that are differentially expressed between wild-type (WT) and *bar*-expressing plants in response to 6-h and 48-h treatments with glufosinate-ammonium, providing new knowledge on the mechanisms behind L-PPT toxicity and plant responses to its presence.

Results

The *bar* gene has limited effects on the transcriptome

Three transgenic *Arabidopsis* lines (BAR1, BAR2, BAR3) with single insertions of the *bar* gene (*35S-bar-35S*) were generated by *Agrobacterium*-mediated transformation using the pCAMBIA3300 transformation vector (CAMBIA, Canberra, Australia; http://www.cambia.org). They were compared with the progenitor WT line by percentage seedling germination, average leaf number, root length and fresh weight. There was no significant difference between the three transgenic lines and the WT plants (data not shown), indicating that the *bar* gene did not impart an obvious visible phenotype. The data confirmed several previous studies of transgenic plants containing *bar* or *pat* genes (Wehrmann *et al*., 1996; Oberdoerfer *et al*., 2005).

Plants were grown in Murashige and Skoog (MS) medium for 7 days, and total RNA was extracted from 200 individual seedlings per transgenic line and non-transformed controls. Microarray analysis using the Affymetrix ATH1 GeneChip was performed in triplicate, and the data were normalized within each group using the Robust Multichip Average (RMA) (Millenaar *et al*., 2006). Quality control was performed using the AffylmGUI package of BioConductor (Gentleman *et al*., 2004; Wettenhall *et al*., 2006). Using a *P* value cut-off of 0.05 and a criterion of 1.5-fold change, the data revealed a very small number of differentially expressed genes between each of the three transgenic lines (BAR1, BAR2 and BAR3) and the non-transformed control (7, 18 and 32 genes, respectively, representing 0.028%, 0.065% and 0.128% of the *Arabidopsis* genome). Four genes [At3g30720, At5g45820 (CIPK20), At5g02810 (PRR7) and At2g23840] were found to be significantly repressed only in the three transgenic lines (mean decrease of –1.7- to –3.2-fold).

Inhibition of seedling growth and development by glufosinate

In preparation for microarray experiments designed to examine the effects of L-PPT on the transcriptome, we first determined the most suitable level of glufosinate (see 'Experimental procedures') needed to inhibit seedling growth, and the duration of treatment. Seven-day-old seedlings of WT and transgenic lines (BAR1, BAR2, BAR3) were germinated on MS medium and transferred to glufosinate-containing medium (concentration range, 0.1–100 μg/mL). The total fresh weight was measured after 2 weeks. Figure 1a shows that the growth of WT seedlings was inhibited at concentrations of glufosinate above 1 μg/mL, whereas all of the transgenic lines were resistant to concentrations up to 50 μg/mL glufosinate. At 100 μg/mL glufosinate, the transgenic lines showed partial inhibition of growth (Figure 1a) accompanied by root growth inhibition and yellowing (data not shown). These results clearly show that 25 μg/mL glufosinate is the optimal concentration required to distinguish between glufosinate-tolerant and WT phenotypes under our experimental conditions.

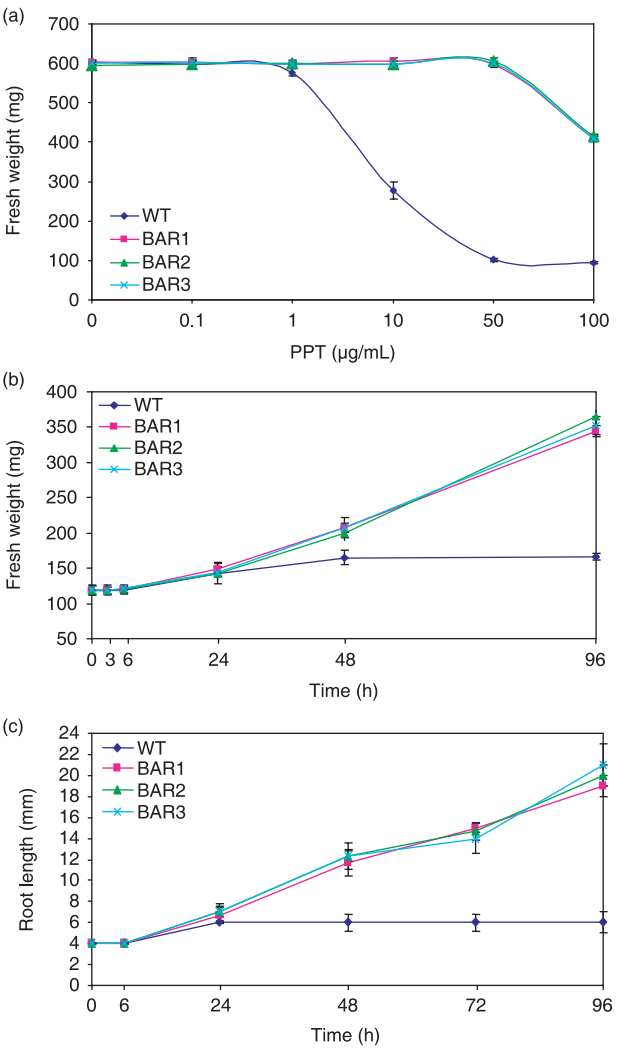

Figure 1 Inhibition of growth by glufosinate (PPT). One-week-old seedlings from the three transgenic lines BAR1, BAR2 and BAR3 and the wild-type (WT) plant were transferred to medium supplemented with glufosinate (concentration range, 0.1–100 µg/mL) and the total fresh weight of 10 seedlings was measured after 10 days (A). The fresh weight (B) and root length (C) of groups of 10 seedlings transferred to medium containing 25 µg/mL glufosinate were measured at intervals up to 96 h. Error bars indicate standard errors.

To determine the optimal duration of glufosinate treatment, 5-day-old WT and transgenic lines (BAR1, BAR2, BAR3) were treated with 25 µg/mL glufosinate, as described previously, followed by measurements of fresh weight and root length at intervals up to 96 h. As shown in Figure 1b,c, both parameters revealed differences between the WT and transgenic lines, starting at 24 h. These differences were significant by 48 h, as the WT seedlings ceased root development and failed to gain fresh weight, whereas the transgenic seedlings continued to develop and grow. Furthermore, the data indicated that 6-h and 48-h treatments should be adequate to distinguish between early and late molecular events related to glufosinate toxicity.

Responses of WT transcriptome to glufosinate

Early responses

For microarray experiments, WT samples consisted of approximately 200 seedlings, treated or untreated with 25 µg/mL glufosinate for 6 and 48 h. Three replicates were performed for each sample, which were grown in the same growth chamber to minimize experimental and sample-to-sample variation. Microarray analysis was performed using the Affymetrix ATH1 GeneChip and the data were normalized using RMA (Millenaar *et al.*, 2006). Using a *P* value cut-off of 0.05 and a two-fold change, the data revealed an early transient response of the transcriptome to glufosinate at 6 h, and more widespread changes that subsequently occurred by 48 h.

Although the glufosinate-induced phenotypes were not apparent at 6 h in WT plants, previous studies have shown that glufosinate induces more rapid changes at the molecular level. For example, it is well known that glufosinate is absorbed in the first hour by leaves and roots, and that inhibition of GS activity reaches 90% within 30 min, followed by a ≥ 10-fold accumulation in ammonia within a few hours (Ullrich *et al.*, 1990; Lacuesta *et al.*, 1992; Steckel *et al.*, 1997). As shown in Figure 2, our microarray data revealed that the

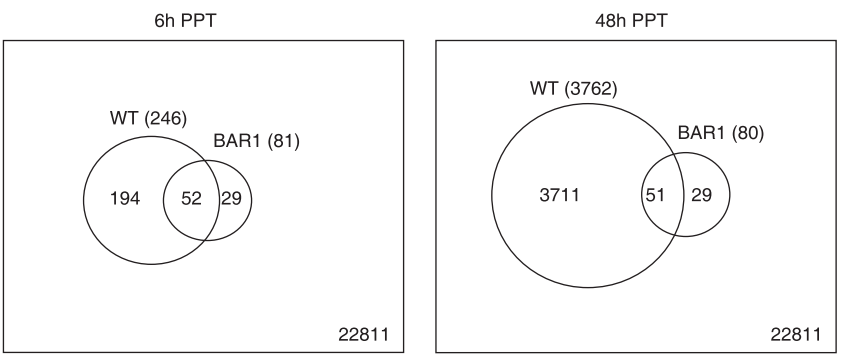

Figure 2 Differential expression of genes in the early response (6 h) and late response (48 h) to glufosinate (PPT) in wild-type (WT) and transgenic BAR1 plants. The total number of differentially expressed genes is shown in parentheses. Those which are specific to each condition are shown in the individual circles and those which are expressed under both conditions are shown in the overlapping areas. RNA was extracted from 10-day-old WT and BAR1 plants untreated and treated with 25 mg/mL glufosinate at 6 and 48 h.

early effect on the transcriptome can be seen by 6 h by the differential expression of 246 genes, or 0.86% of the total genes annotated in the *Arabidopsis* genome, of WT plants. Of these, 194 differentially expressed genes were specific to WT plants (70% were up-regulated and 30% were down-regulated) and only 52 genes were also differentially expressed in the treated transgenic BAR1 plants (Table S1, see 'Supporting Information'). Of the 194 genes, the differential expression of 84 genes (43%) was transient at 6 h and not observed at 48 h.

Based on FunCat assignments available through the Munich Information Centre for Protein Sequences (MIPS) *Arabidopsis* database (mips.gsf.de/proj/thal/db/), the 194 differentially expressed genes were classified into functional categories, the most extensive being metabolism (16.6%), protein with binding function or cofactor requirement (15.3%), cell rescue (defence and virulence) (10.1%), interaction with environment (8.1%), cellular transport (7.8%) and transcription (6.2%). Almost 5% of the genes could not be classified into known functional categories (Figure 3a). As previous studies have reported the transient inhibition of nitrate reductase following GS inhibition (Trogisch *et al*., 1989), the repression of genes involved in nitrate metabolism and assimilation, i.e. NIA1 (nitrate reductase; At1g77760) and NIR1 (ferredoxin-nitrite reductase; At2g15620), was expected. The expression of several stress-related genes was also affected, including six heat shock proteins and four oxidative stress response-related genes. The primary response included the rapid induction of transcription factors: eight transcription factors were up-regulated, including members of ethylene-response factor/APETALA 2 (ERF/AP2) (At1g64380, At4g32800, At1g77640, At4g17490 and At2g44840), MYB transcription factor (At2g23290), basic helix–loop–helix (bHLH) (At1g51140) and scarecrow-like protein (At4g17230). Expression profiling did not reveal the activation of selective pathways at this early time point, but rather the induction of several regulatory proteins that could be essential to drive the secondary and subsequent responses that follow in time. To verify the microarray results, 10 genes that were significantly up-regulated by glufosinate treatment were tested for transcription levels by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. As shown in Figure 3b, the induction of the selected genes was confirmed, providing confidence in the microarray data.

Late responses

At 48 h of glufosinate treatment, extensive changes in gene expression were observed, including the differential expression of 3762 genes, or 13.5% of the total genes annotated in the *Arabidopsis* genome. Of these, 3711 genes were specific to WT plants and, again, only 51 were also differentially expressed in the treated BAR1 plants (Figure 2; Table S1, see 'Supporting Information'). Of the 3711 genes, 1765 (47.5%) were up-regulated and 1946 (52.5%) were down-regulated. Only 111 genes of the 3711 were also differentially expressed at 6 h; however, the expression level increased by 20–120-fold in some cases. The other 3600 genes reflected the late response at 48 h in WT plants.

As shown in Figure 4, the most abundant functional categories of genes were metabolism (17.8%), protein with binding function or cofactor requirement (17.4%), protein fate (7.6%), cellular transport (6.6%) and transcription (4.8%). Almost 12.9% of the late-response genes could not be classified into a known functional category. The differential expression of several genes involved in amino acid metabolism, glutamine metabolism and ammonium assimilation was observed during the late response, including members of the GS1 family and GS2 gene. Furthermore, several other genes involved in detoxification processes were induced. These included five genes previously described as detoxification-related genes (At1g28480, At1g56650, At2g29420, At2g29490 and At2g30540) and others that have been hypothesized to function in detoxification processes, including members of glutathione transferase (GST), cytochrome P450, ABC transporters and uridine diphosphate (UDP)-glucose transferase (see Table 1) (Schaeffner *et al*., 2002; Baerson *et al*., 2005; Pilon-Smits, 2005). The expression levels of UDP-glucosyl transferase (At1g05680) and cytochrome P450 (At4g37370) increased up to 74-fold and 36-fold, respectively, at 48 h (Table 1). Six other transcription factors were also up-regulated, including members of WRKY (WRKY46 and WRKY25), no apical meristem (NAM) family protein (At5g63790, At1g77450 and At1g01720), ERF/AP2 (At1g43160), MYB57 (At1g56650) and the heat stress transcription factor (Hsf) family (At4g18880). The up-regulation of genes known to be involved in the biosynthesis of auxin, such as indoleacetic acid (IAA)-amino acid hydrolase 1 (ILL5) (At1g51780), and jasmonic acid biosynthesis, such as 12-oxophytodienoic acid reductase 2 (OPR2) and lipoxygenase 3 (LOX3), was observed. The differential expression of senescence-related genes and photosynthesis-related genes was also observed. These data demonstrate the coordination and integration of several specific pathways following GS inhibition, as a secondary reaction to the initial response to glufosinate seen at 6 h (Figure 5).

Downstream effects of GS inhibition by glufosinate

In plants, there are two isoforms of GS: the cytosolic form (GS1), encoded by a multigene family, and a plastidic form

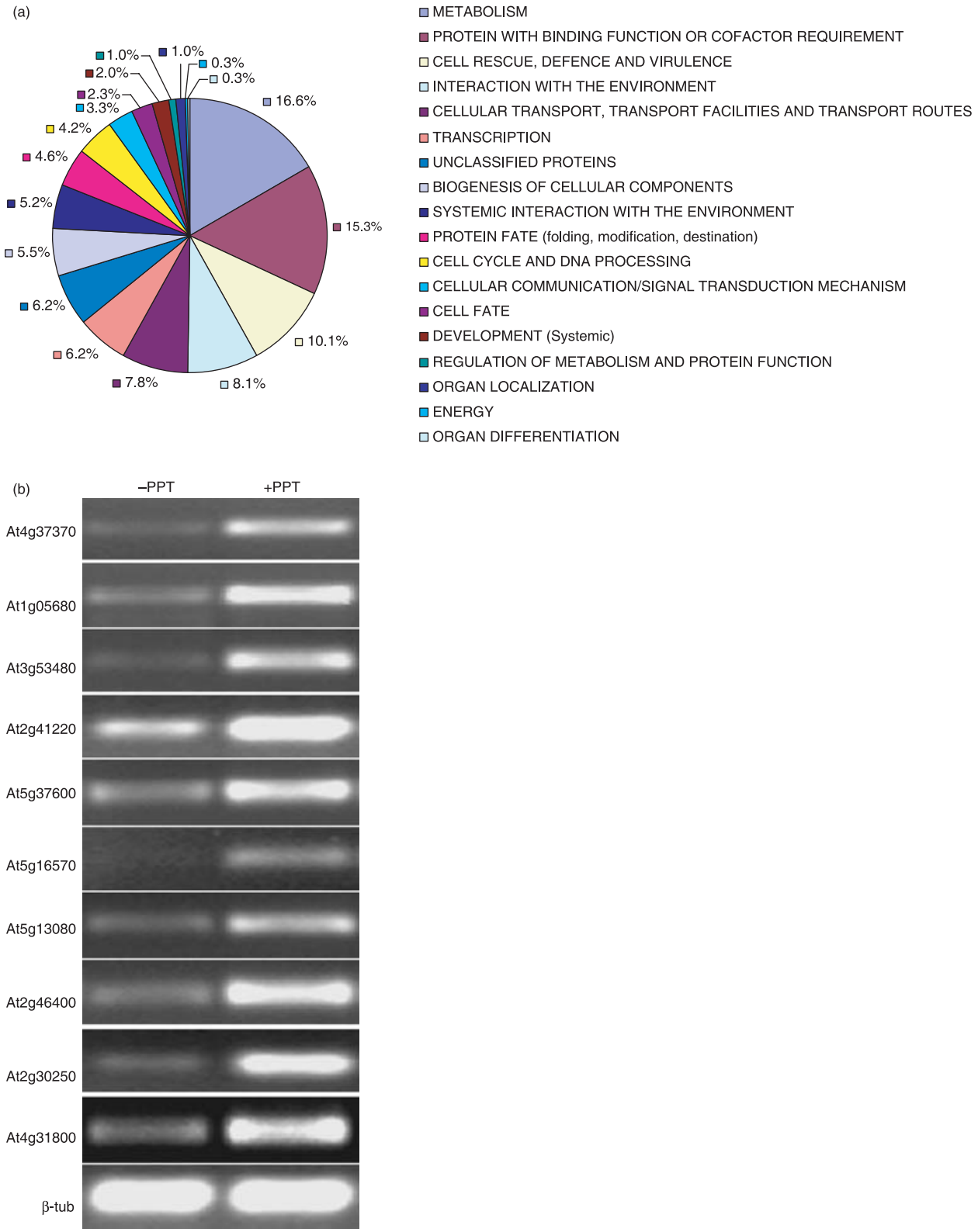

Figure 3 Functional classification of glufosinate (PPT)-responsive genes in the wild-type *Arabidopsis* plant. (A) Functional categories of genes differentially expressed at 6 h. (B) Confirmation of microarray results. Reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted using specific primers for the 10 genes indicated and β-tubulin (β-tub) as a control. RNA was extracted from untreated and treated wild-type plants at 6 h.

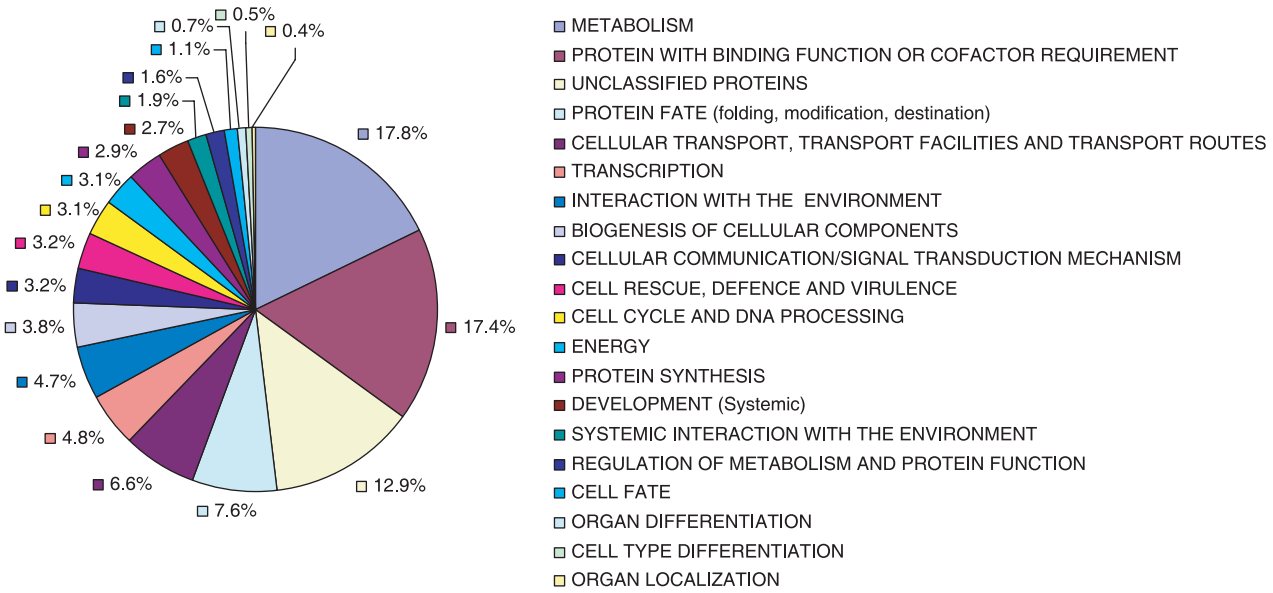

Figure 4 Functional classification of glufosinate-responsive genes in the wild-type *Arabidopsis* plant at 48 h.

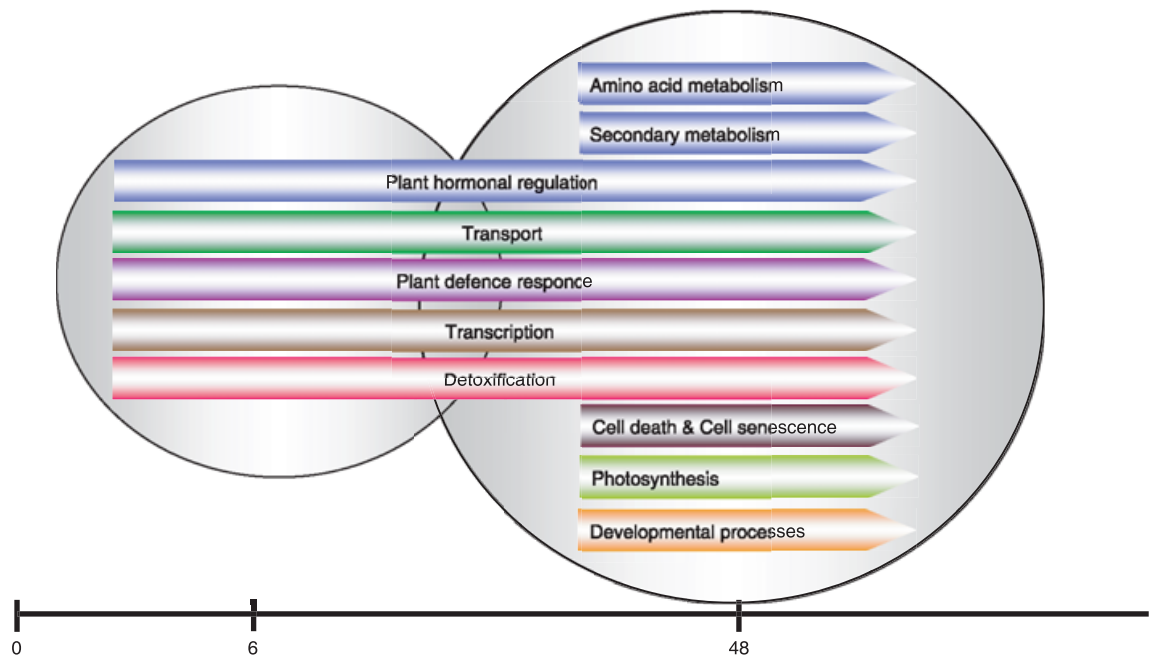

Figure 5 Early and late events after glufosinate treatment in the wild-type plant.

(GS2), encoded by a single gene (Hirel and Gadal, 1980). In the late response of Arabidopsis, the GS1 family (*GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4*, *GLN1;5*; Ishiyama *et al*., 2004), excluding *GLN1;5*, was up-regulated, whereas GS2 was repressed. Furthermore, 14 other genes hypothesized to be linked to this pathway were also differentially expressed (Table 2). The underlying signal transduction and mechanistic pathways are unclear at this time, but presumably result from the stress caused by the inhibition of GS activity, subsequent ammonia accumulation and the lack of essential amino acids.

As GS plays a major role in ammonium assimilation, which then serves as the nitrogen donor for the biosynthesis of all nitrogenous organic compounds in plants (Crawford and Arst, 1993; Daniel-Vedele *et al*., 1998), it would be expected that the inhibition of GS would have profound downstream effects on pathways leading to the metabolism of nitrogenous

Table 1 List of selected glufosinate-responsive genes hypothesized to be involved in detoxification processes

			Fold change	
Locus ID	Name	Description	6 h	48 h
ABC transporter				
At3g60160	ATMRP9	ABC transporter family protein		14.5
At1g15520		ABC transporter family protein		14.3
At3g59140	ATMRP14	ABC transporter family protein		14.2
At1g71330	ATNAP5	ABC transporter family protein	2.1	9.1
At3g13100	ATMRP7	ABC transporter family protein		7.7
At3g47780	ATATH6	ABC transporter family protein		5.4
At4g18050		ABC transporter family protein		4.7
At2g36380		ABC transporter family protein		3.6
At3g47730	ATATH1	ABC transporter family protein		3.3
At3g53480		ABC transporter family protein	2.2	3.2
At1g67940	ATNAP3	ABC transporter family protein		3.1
At2g26910		ABC transporter family protein		–3.3
At1g51500	CER5	ABC transporter family protein		–3.3
At2g13610		ABC transporter family protein		–3.4
At1g17840		ABC transporter family protein		–4.2
Cytochrome P450				
At4g37370	CYP81D8	Cytochrome P450	15.3	36.6
At2g45570	CYP76C2	Cytochrome P450 76C2, putative		28.6
At3g26830	PAD3	Cytochrome P450 71B15, putative (CYP71B15)		27.6
At2g34500	CYP710A1	Cytochrome P450 family protein		17.6
At5g67310	CYP81G1	Cytochrome P450 family protein; member of CYP81G		11.2
At3g28740		Cytochrome P450 family protein		10.8
At3g26210	CYP71B23	Cytochrome P450 71B23, putative		7.2
At2g30770	CYP71A13	Cytochrome P450 71A13, putative		6.9
At3g14680	CYP72A14	Cytochrome P450, putative		6.3
At1g64950	CYP89A5	Cytochrome P450, putative		5.3
At3g03470	CYP89A9	Cytochrome P450, putative		5.1
At5g36220	CYP81D1	Cytochrome P450 81D1		3.7
At4g15110	CYP97B3	Cytochrome P450 97B3, putative		–3.0
At3g44970		Cytochrome P450 family protein		–3.1
At4g00360	CYP86A2	Cytochrome P450, putative		–3.4
At4g39510	CYP96A12	Cytochrome P450 family protein		–4.3
At4g13770	CYP83A1	Cytochrome P450 family protein		–4.6
At2g34490	CYP710A2	Cytochrome P450 family protein		–4.9
At3g53130	LUT1	Cytochrome P450 family protein		–5.3
At5g45340	CYP707A3	Cytochrome P450 family protein	5.8	
At5g63450	CYP94B1	Cytochrome P450, putative	2.4	
UDP-glucosyl transferase				
At1g05680		UDP-glucuronosyl/UDP-glucosyl transferase	15.8	74.4
At3g11340		UDP-glucuronosyl/UDP-glucosyl transferase		27.7
At3g46660		UDP-glucuronosyl/UDP-glucosyl transferase	3.0	23.3
At2g15490		UDP-glucuronosyl/UDP-glucosyl transferase	2.8	18.4
At2g36750		UDP-glucuronosyl/UDP-glucosyl transferase	3.4	17.8
At4g34135		UDP-glucuronosyl/UDP-glucosyl transferase	4.1	13.3
At1g22400		UDP-glucuronosyl/UDP-glucosyl transferase	3.1	12.5
At2g15480		UDP-glucuronosyl/UDP-glucosyl transferase	6.0	11.9
At2g30140		UDP-glucuronosyl/UDP-glucosyl transferase		11.4
At4g15490		UDP-glucuronosyl/UDP-glucosyl transferase		10.0
At1g05560	UGT1	UDP-glucose transferase (UGT75B2)	3.4	9.4
At4g10960		UDP-glucose 4-epimerase, putative		9.4
At5g49690		UDP-glucuronosyl/UDP-glucosyl transferase		9.2
At2g36770		UDP-glucuronosyl/UDP-glucosyl transferase		8.3
At2g36790		UDP-glucuronosyl/UDP-glucosyl transferase	6.0	6.9
At3g46670		UDP-glucuronosyl/UDP-glucosyl transferase		6.0
At2g36970		UDP-glucuronosyl/UDP-glucosyl transferase		5.2

Table 1 *Continued*

Locus ID	Name	Description	Fold change 6 h	Fold change 48 h
At3g16520		UDP-glucuronosyl/UDP-glucosyl transferase		–2.4
At2g30150		UDP-glucuronosyl/UDP-glucosyl transferase		–2.4
At2g31790		UDP-glucuronosyl/UDP-glucosyl transferase		–2.7
Glutathione *S*-transferase				
At2g29490	ATGSTU1	Glutathione *S*-transferase, putative	2.2	10.4
At2g29460	ATGSTU4	Glutathione *S*-transferase, putative		52.8
At1g17170	ATGSTU24	Glutathione *S*-transferase, putative		30.0
At2g29480	ATGSTU2	Glutathione *S*-transferase, putative		28.8
At2g29470	ATGSTU3	Glutathione *S*-transferase, putative		19.9
At1g74590	ATGSTU10	Glutathione *S*-transferase, putative		12.0
At1g49860	ATGSTF14	Glutathione *S*-transferase, putative		6.9
At2g29420	ATGSTU7	Glutathione *S*-transferase, putative	3.3	6.0
At1g02930	ATGSTF6	Glutathione *S*-transferase, putative		5.5
At1g69930	ATGSTU11	Glutathione *S*-transferase, putative		4.7
At1g10360	ATGSTU18	Glutathione *S*-transferase, putative		–4.2
At1g78370	ATGSTU20	Glutathione *S*-transferase, putative		–13.6
Glutaredoxin				
At1g28480		Glutaredoxin family protein	4.3	10.4
At4g33040		Glutaredoxin family protein		9.1
At3g62960		Glutaredoxin family protein		5.8
At2g47880		Glutaredoxin family protein		4.7
At1g03850		Glutaredoxin family protein		3.9
At5g40370		Glutaredoxin, putative		2.8
At2g30540		Glutaredoxin family protein	3.5	2.3
At4g15690		Glutaredoxin family protein		–2.4
At5g18600		Glutaredoxin family protein		–2.9
At1g06830		Glutaredoxin family protein	2.1	
MATE family protein				
At2g04040		MATE efflux family protein		29.8
At2g04050		MATE efflux family protein		10.5
At3g23550		MATE efflux family protein		8.7
At1g71140		MATE efflux family protein		7.3
At2g04070		MATE efflux family protein		3.4
At5g17700		MATE efflux family protein		–3.2
At1g15180		MATE efflux family protein		–4.8
Peroxidase				
At4g08770		Peroxidase, putative, identical to class III peroxidase ATP38		6.6
At4g36430		Peroxidase, putative		4.0
At4g37530		Peroxidase, putative, identical to cDNA peroxidase ATP37		3.7
At2g18140		Peroxidase, putative, similar to peroxidase ATP6a		3.5
At4g11290		Peroxidase, putative, identical to peroxidase ATP19a		3.1
At5g05340		Peroxidase, putative, similar to peroxidase P7		3.1
At1g68850		Peroxidase, putative, identical to peroxidase ATP23a		3.0
At5g40150		Peroxidase, putative, identical to peroxidase ATP26a		–2.9
At1g71695		Peroxidase 12 (PER12) (P12) (PRXR6)		–3.7
At5g58390		Peroxidase, putative		–4.1
Transcription factor				
At3g10500	ATAF1	No apical meristem (NAM) family		3.8
At1g01720		No apical meristem (NAM) family protein	2.0	3.5
At5g63790		No apical meristem (NAM) family protein	2.6	3.2
At1g56650	PAP1	Myb family transcription factor (MYB75)	3.1	3.4
At1g62300	WRKY6	WRKY family transcription factor		4.2

MATE, multidrug and toxic compound extrusion; UDP, uridine diphosphate.

Table 2 List of glufosinate-responsive genes hypothesized to be involved in ammonia assimilation and glutamine biosynthesis

Locus ID	Name	Description	Fold change	
			6 h	48 h
At5g18170	GDH1	Glutamate dehydrogenase 1	–2.1	
At2g41220	GLU2	Glutamate synthase		4.3
At3g17820	ATGSKB6	Glutamine synthetase (GS1)		2.4
At5g35630	GS2	Glutamine synthetase (GS2)		–2.4
At5g16570	GLN1; 4	Glutamine synthetase		3.4
At1g66200	ATGSR2	Glutamine synthetase		2.1
At5g37600	ATGSR1	Glutamine synthetase		12.3
At3g22200	POP2	4-Aminobutyrate aminotransferase		2.5
At2g37500		Arginine biosynthesis protein		–2.7
At4g34710	ADC2	Arginine decarboxylase (ADC)		–6.2
At5g11520	ASP3; YSL4	Aspartate aminotransferase.		5.3
At3g55610		δ1-pyrroline-5-carboxylate synthetase B		–2.5
At2g05990	ENR1; MOD1	Enoyl-(acyl-carrier protein) reductase		–3.9
At3g57560		*N*-Acetylglutamate kinase		–2.2
At3g47450		Nitric oxide synthase		–2.4
At5g46180	delta-OAT	Ornithine aminotransferase, putative		4.4
At4g01900	GLB1	P II nitrogen sensing protein		–3.5
At3g30775	ERD5	Proline oxidase		3.8
At5g38710		Proline oxidase, putative		7

molecules, such as amino acids, proteins, nucleic acids and chlorophyll. This was confirmed by the functional analysis of the categories of genes affected by glufosinate treatment. A large set of genes involved in important metabolic pathways (16.6%–17.8%) (see Figures 3a and 4) were affected, including genes involved in nitrogen metabolism, nucleotide/nucleobase metabolism, phosphate metabolism, carbon compound and carbohydrate metabolism, lipid and fatty acid metabolism, metabolism of vitamins/cofactors/prosthetic groups and secondary metabolism.

Amino acid and chlorophyll synthesis. Our list of glufosinate-responsive genes was compared with the list of genes in the AraCyc database of metabolic pathways (http://www.arabidopsis.org/biocyc/) (Mueller *et al*., 2003). Of the genes that are responsive to glufosinate treatment at 6 and 48 h, 455 were identified in the AraCyc list (Table S4, see 'Supporting Information'). Of these, 52 genes belong to the amino acid metabolism category. The early response included the down-regulation of only one gene, i.e. asparagine synthase (At5g65010), involved in asparagine biosynthesis. The extent of repression reached 20-fold after 48 h. The late response included the up- or down-regulation of 52 genes involved in the biosynthesis and degradation of the other amino acids, excluding leucine and histidine. As the effect was not limited to glutamine, the data provided evidence for the existence of cross-talk among genes in different metabolic pathways. As expected, one consequence was the repression of 15 genes involved in chlorophyll and chlorophyllide biosynthesis during the late response.

Photosynthesis. The expression of 32 genes involved in photosynthesis was found to be down-regulated during the late response (see Table 3). These included members of the light-harvesting chlorophyll *a*/*b*-binding family (Cab/LHC), such as LHCA2 (At3g61470), LHCA2*1 (At1g19150), LHCA3*1 (At1g61520), LHCA5 (At1g45474), LHCB2.2 (At2g05070), LHCB2:4 (At3g27690), LHCB3 (At5g54270), LHCB4.2 (At3g08940), LHCB4.3 (At2g40100), LHCB5 (At4g10340) and LHCB6 (At1g15820). The Cab superfamily in *Arabidopsis* consists of 20 different proteins (Jansson, 1999), six of which are associated with photosystem I (PSI) (Lhca1–6) and 14 with photosystem II (PSII) (Lhcb1–6 and their isomers). The primary function of this family is the absorption of light through chlorophyll excitation and the transfer of absorbed energy to photochemical reaction centres (Heddad *et al*., 2006). Consistent with our microarray data, several other studies have linked glufosinate exposure to the inhibition of photosynthesis (Sauer *et al*., 1987). Glufosinate is known to indirectly inhibit the light reaction in photosynthesis (Sauer *et al*., 1987; Wild and Wendler, 1991; Lacuesta *et al*., 1992; Dan Hess, 2000). The mechanism of this inhibition is not yet clear.

The early response did not include the alteration of photosynthesis-related genes; however, the expression of a zinc

Table 3 List of glufosinate-responsive genes hypothesized to be involved in photosynthesis

Locus ID	Name	Description	Fold change
At3g27690	LHCB2:4	Chlorophyll *a*/*b*-binding protein	–71.2
At2g39470		Photosystem II reaction centre PsbP family protein	–23.0
At1g19150	LHCA2*1	Chlorophyll *a*/*b*-binding protein	–17.0
At3g08940	LHCB4.2	Chlorophyll *a*/*b*-binding protein	–13.6
At5g54270	LHCB3	Chlorophyll *a*/*b*-binding protein	–12.1
At3g55330		Photosystem II reaction centre PsbP family protein	–9.5
At3g54890		Encodes a component associated with photosystem I	–8.7
At1g52230		Photosystem I reaction centre subunit VI	–7.4
At4g28660		Photosystem II reaction centre W (PsbW) family protein	–7.2
At3g47470	CAB4	Chlorophyll *a*/*b*-binding protein 4	–7.1
At4g05180		Oxygen-evolving enhancer protein 3, chloroplast	–6.4
At2g34430	LHB1B1	Chlorophyll *a*/*b*-binding protein	–6.2
At3g50820		Encodes a protein which is an extrinsic subunit of photosystem II	–5.6
At4g02770		Photosystem I reaction centre subunit II	–5.0
At3g16140		Photosystem I reaction centre subunit VI	–4.9
At1g29910	CAB3	Chlorophyll *a*/*b*-binding protein 2	–4.6
At5g57030	LUT2	Lycopene ε-cyclase	–4.5
At3g15850	FAD5	Fatty acid desaturase family protein	–4.4
At4g15510		Photosystem II reaction centre PsbP family protein	–4.4
At5g66570		Encodes a protein which is an extrinsic subunit of photosystem II	–4.4
At4g30950	FAD6	ω-6 fatty acid desaturase, chloroplast	–4.4
At4g10340	LHCB5	Chlorophyll *a*/*b*-binding protein CP26	–3.9
At1g55670		Photosystem I reaction centre subunit V	–3.8
At2g05070	LHCB2.2	Chlorophyll *a*/*b*-binding protein	–3.8
At2g40100	LHCB4.3	Chlorophyll *a*/*b*-binding protein	–3.6
At1g61520	LHCA3*1	Chlorophyll *a*/*b*-binding protein	–3.5
At5g47110		lil3 protein, putative	–3.3
At1g45474	LHCA5	Chlorophyll *a*/*b*-binding protein	–3.2
At3g61470	LHCA2	Chlorophyll *a*/*b*-binding protein	–3.0
At1g67740	PSBY	Photosystem II core complex proteins psbY	–2.9
At5g01530		Chlorophyll *a*/*b*-binding protein CP29	–2.9
At2g30790		Photosystem II oxygen-evolving complex 23	–2.1
At5g07920	ATDGK1	Diacylglycerol kinase 1 (DGK1)	2.5
At4g14690	ELIP1	Chlorophyll *a*/*b*-binding family protein	2.8
At5g59820	RHL41	Zinc finger (C2H2 type) family protein (ZAT12)	10.1

finger protein DFL2 ('DWARF IN LIGHT 2') involved in high light and cold acclimation was found to be up-regulated at 6 and 48 h, and appears to be one of the first early responses. This gene encodes a GH3-related gene involved in red light-specific hypocotyl elongation. The analysis of sense and antisense transgenic plants suggests that DFL2 is located downstream of red light signal transduction and determines the degree of hypocotyl elongation (Takase *et al*., 2004). Therefore, the early induction of this regulatory protein may provide an early signal in the pathway leading to the inhibition of photosynthesis by glufosinate.

Sugar metabolism. Other differentially expressed genes in the late response included 28 genes involved in sucrose and cellulose biosynthesis. Our data were consistent with the report of decreased levels of sucrose following GS inhibition (Gordon *et al*., 1999). Metabolite profiling analysis of *Medicago truncatula* root nodules also showed a reduction of fructose, fructose-6-phosphate and glucose-6-phosphate after glufosinate treatment (Barsch *et al*., 2006).

Regulatory pathways responding to glufosinate

Hormone synthesis and signalling. Our microarray analysis revealed that glufosinate treatment caused an increase in the transcript levels of genes involved in plant hormone biosynthesis and signal transduction pathways. We observed the early up-regulation of three genes involved in the biosynthesis of auxin and jasmonic acid, i.e. ILL5, OPR2 and LOX3. The late response included the elevation of transcript levels of key enzymes involved in jasmonic acid biosynthesis,

such as lipoxygenase 1 (LOX1, At1g55020) and 12-oxophytodienoic acid reductase 3 (OPR3, At2g06050) (Schaller *et al*., 2000; Ziegler *et al*., 2000). Genes involved in ethylene biosynthesis were also up-regulated, including members of the 1-aminocyclopropane-1-carboxylate (ACC) synthase family (ACS2: At1g01480, ACS8: At4g37770 and At5g59530) (Yang and Hoffman, 1984). Genes involved in abscisic acid biosynthesis and signal transduction pathways, such as aldehyde oxidase 3 (AAO3: At2g27150) and protein phosphatase 2C (ABI2: At5g57050), were found to be up-regulated (Seo *et al*., 2000). The auxin biosynthesis pathway was also affected by glufosinate, in which some genes were induced, such as the IAA-amido synthase (At2g23170 and At1g59500) (over 50-fold), IAA-amino acid hydrolase 1 (ILR1: At3g02875 and ILL5: At1g51780), nitrile aminohydrolase-nitrilase (NIT2: At3g44300, NIT3: At3g44320 and NIT4: At5g22300) and cytochrome P450 (CYP79B2: At4g39950) (Cheng *et al*., 2006). Other genes, such as IAA4 and IAA8, were repressed. The complex profile of differentially expressed genes in plant hormone biosynthetic pathways suggests that they coordinate many of the secondary responses to glufosinate.

Trancriptional regulation. Almost 5% of glufosinate responsive genes belong to the transcription factors and DNA-binding proteins. Examples include the plant-specific WRKY transcription factors, in which 19 genes were up-regulated; NAM/NAC family, in which 24 genes were up-regulated; basic region/leucine zipper motif (bZIP) transcription factors, in which nine genes were up-regulated; ERF/AP2 family, in which eight genes were up-regulated and one was down-regulated; MYB family, in which 12 genes were up-regulated and 12 were down-regulated; and bHLH family, in which four genes were up-regulated and nine were down-regulated. In addition, a number of genes associated with cellular communication and signal transduction mechanisms (6.5%), including receptor-like protein kinases and protein kinases, were also significantly regulated by glufosinate.

Two WRKY transcription factors, i.e. WRKY25 and WRKY46, were up-regulated during the early and late responses, and 17 other members of the WRKY family were up-regulated during the late response. This family of regulatory proteins has been reported to be implicated in different stress responses in many species, and is strongly and rapidly up-regulated in response to wounding, pathogen infection, senescence or abiotic stresses (Eulgem *et al*., 2000). The function of several WRKY genes has already been elucidated. WRKY70 is known to be implicated in salicylic acid-dependent defence responses (Yu *et al*., 2001). WRKY22 and WRKY29 have been identified as important downstream components of a mitogen-activated protein kinase (MAPK) pathway that confers resistance to both bacterial and fungal pathogens (Asai *et al*., 2002). WRKY6 and WRKY53 have been shown to be involved in leaf senescence (Hinderhofer and Zentgraf, 2001; Robatzek and Somssich, 2002). Furthermore, recent microarray analysis reported the up-regulation of several WRKY genes in response to other herbicides and xenobiotic compounds (Baerson *et al*., 2005; Madhou *et al*., 2006; Manabe *et al*., 2007).

Three NAC transcription factors were up-regulated by glufosinate during both early and late responses, and 21 others were induced specifically during the late response. Members of this family have been implicated in various aspects of plant development, including apical shoot, stem and leaf development (Souer *et al*., 1996). More recently, NAC domain genes have also been implicated in defence responses to biotic and abiotic stresses. The potato *StNAC* and *Arabidopsis ATAF1* and *ATAF2* genes have been shown to be induced by pathogen attack and wounding (Collinge and Boller, 2001). Members of the *Brassica napus* NAC (*BnNAC*) family of genes have also been shown to be differentially regulated in response to biotic and abiotic stresses, indicating an important role in the mediation of the transcriptional responses to diverse biotic and abiotic stresses (Hegedus *et al*., 2003).

We also observed the differential expression of ERFs, which belong to the AP2 family of transcription factors with AP2/ERF domains. Two ERFs were transiently up-regulated during the early response and another was up-regulated during the early and late responses. Another seven were up-regulated and one was down-regulated specifically during the late response. ERF proteins play a key role in the integration of the ethylene and jasmonate signalling pathways to activate ethylene/jasmonate-dependent responses to pathogens and various environmental stresses (Chen *et al*., 2002; Lorenzo *et al*., 2003).

Interestingly, genes belonging to WRKY, NAC and bZIP families were only up-regulated by glufosinate treatment, whereas members of the MYB and bHLH (MYC) families were both up- or down-regulated. Similarities in gene expression patterns between MYB and MYC families may indicate possible cooperative interactions that regulate glufosinate-responsive genes. Several examples of cooperative interaction of bHLH (MYC) and MYB regulatory proteins have been reported in plants (Roth *et al*., 1991; Goff *et al*., 1992; Martin and Paz-Ares, 1997; Grotewold *et al*., 2000; Payne *et al*., 2000; Nesi *et al*., 2001). Similar combinatorial interactions between transcription factors may be involved in controlling and integrating the diverse responses to glufosinate.

Response of the transgenic transcriptome to glufosinate treatment

Our data revealed that the early response of the WT and transgenic transcriptomes involved a small number of genes and did not translate into visible phenotypes. The transcriptomes of the transgenic BAR1 plants after 6 h of treatment revealed only 81 differentially expressed genes in the presence of glufosinate. This was approximately one-third of the number of differentially expressed genes described earlier in WT plants under identical conditions (Figure 2). Of these, 29 genes (36%) were specific to the transgenic plants and 52 genes (64%) were also differentially expressed in 6 h-treated WT plants. Of the 52 common genes, 44 (85%) were transiently expressed at 6 h and eight (15%) had similar or greater expression levels at 48 h. The late response to glufosinate seen in WT plants at 48 h was essentially eliminated in transgenic BAR plants. It was limited to 80 differentially expressed genes, which is 50-fold lower than that in WT plants under identical conditions. Of these, 51 genes (64%) were again common to both transgenic and WT plants, and 29 (36%) were again specific to transgenic plants (Tables S2 and S3, see 'Supporting Information'). Interestingly, eight genes were shown to be up-regulated in the treated WT and transgenic plants at both 6 and 48 h. These genes were found to be induced in response to wounding; four had been described previously as wound-responsive genes, including wound-responsive gene 3 (WR3) (At5g50200), flavin-adenine dinucleotide (At1g30720), serine protease inhibitor (At5g43580) and β-fructosidase (At3g13790). Moreover, the eight genes were found to be induced by at least one biotic stress condition (http://bbc.botany.utoronto.ca) (Toufighi *et al*., 2005). These findings may reveal the activation of general stress responses in both tolerant and non-tolerant plants as a first transient response to the presence of glufosinate. Several recent studies have shown that plants are able to develop nonspecific detoxification systems against chemicals, including certain herbicides and antibiotics (Yuan *et al*., 2007).

It is interesting that 29 differentially expressed genes were specific to transgenic plants at both 6 and 48 h (Table S3, see 'Supporting Information'; Figure 2). Using a *P* value cut-off of 0.05 and a criterion of two-fold change to distinguish differential expression, these appeared to be separate and distinct sets of genes. Lowering the stringency criteria only identified the possible overlap of one or two genes. Genes specific to transgenic plants at 6 h include the following: 10 genes in unknown functional categories; five detoxification-related genes (three peroxidase, oxidoreductase and cytochrome P450); two disease resistance genes, including zinc finger transcription factor; and four genes involved in the biosynthesis and metabolism of carbohydrate [myo-inositol oxygenase (MOX2), At2g19800; aldose 1-epimerase, At3g47800; sugar transporter, At1g08920; fructose-bisphosphate aldolase, At4g26530]. The induction of four genes in transgenic lines BAR1, BAR2 and BAR3 was confirmed by RT-PCR (see Figure 6). Among those specific to transgenic plants at 48 h, 28 of the 29 genes were up-regulated, and included seven genes in functional categories, one detoxification gene (peroxidase PER57), three cytochrome P450 genes potentially involved in detoxification processes, two defence-related proteins, three late embryogenesis protein genes, two lipid transfer protein genes and a DREB transcription factor that appears to stimulate cytokinin biosynthesis. These results show that both unique sets of genes were represented by detoxification-related genes, such as P450, peroxidase and transporter genes, which may be specifically and temporally activated in transgenic plants by glufosinate, the acetylated form of glufosinate (*N*-acetyl-L-glufosinate) or other intermediates in the detoxification pathway. It is not known whether different subsets of genes respond differentially to the different metabolic intermediates.

As expected for WT plants that are experiencing herbicide-induced stress, the differential expression of detoxification genes was more extensive than in the transgenic plants. Twenty-two detoxification-related genes were observed during the early response, and 148 genes were observed

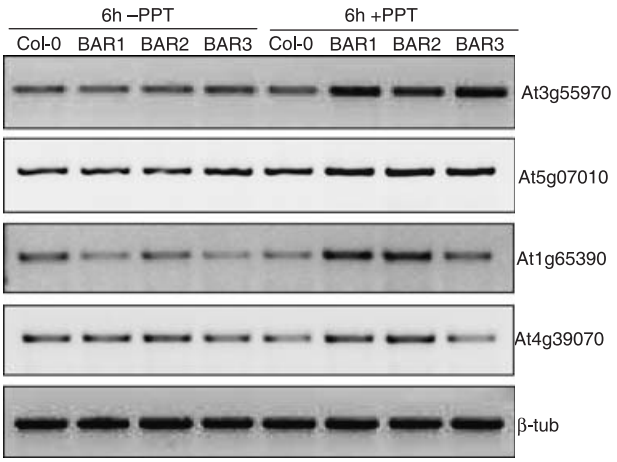

Figure 6 Confirmation of microarray results. Reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted using specific primers for the four genes and β-tubulin (β-tub) as a control. RNA was extracted from untreated (–PPT) and treated (+PPT) wild-type plants and the three transgenic lines BAR1, BAR2 and BAR3 at 6 h.

during the late response (see Table 1). Of the 22, only three genes were transiently up-regulated, whereas the expression of 19 genes was sustained and/or up-regulated into the late response, in some case reaching a 74-fold increase in expression level. As only four of these were up-regulated in both transgenic and WT plants, the majority are probably responding to the widespread stress and damage in WT plants induced by the herbicide in the absence of PAT activity.

The set of detoxification genes expressed in WT plants included the following subfamilies (Table 1): GST, cytochrome P450, ABC transporters, multidrug and toxic compound extrusion (MATE) proteins, alternative oxidase (AOX), steroid sulphotransferase (ST) and ACC-oxidase (ACO). Many of the identified genes have been described previously as detoxification-related genes, such as PAP1 (At1g56650), ATGSTU1 (At2g29490) and ATGSTU7 (At2g29420), whereas others have been hypothesized to function in detoxification processes (Schaeffner *et al*., 2002; Baerson *et al*., 2005; Pilon-Smits, 2005) These data indicate that the early and late responses in the WT plant involve the activation of detoxification processes as expected, but these may differ from the processes activated in the transgenic plants. In the transgenic plants, it is possible that the detoxification genes may include those that are specific to the products of glufosinate metabolism.

Discussion

Bacterial PAT, coded by the *pat* or *bar* genes, has been used extensively for the development of new herbicide-tolerant crops and as a selectable marker in the transformation process (Miki and McHugh, 2004). Yet, the unintended effects of the gene on the plant transcriptome have not been examined in detail using rigorous profiling strategies (Cellini *et al*., 2004). In this study, we have identified pleiotropic effects of the *bar* gene on the *Arabidopsis* transcriptome and document the transcriptome responses to the herbicide glufosinate and its derivatives.

The results reveal that the transcriptomes of the three transgenic plants expressing the *bar* gene differ from their WT counterparts by 7, 18 and 32 genes individually, but only four of these genes are differentially expressed in all three transgenic plants. Other studies that have utilized expression profiling strategies have shown that the insertion and expression of *nptII* has no pleiotropic effects on the transcriptome of *Arabidopsis* plants, including the dramatic transcriptional reprogramming that occurs in response to abiotic stresses (El Ouakfaoui and Miki, 2005). Furthermore, the expression of the *gus/uidA* gene also generates no pleiotropic effects on the transcriptome (El Ouakfaoui and Miki, 2005). These experiments reveal the stability of the *Arabidopsis* transcriptome to T-DNA insertion and show that marker genes conferring novel traits do not generally perturb the global expression of plants.

As expected, differences between the transcriptomes of the WT and transgenic lines were dramatic under the influence of glufosinate. Differences were found that were specific to transgenic lines and did not reflect the normal responses of the WT plants to glufosinate. As these genes were not activated in WT plants, we must consider the possibility that they are responding to other acetylation products of PAT or metabolic derivatives of L-PPT. A previous study has shown that the expression of the *bar* gene can be negatively correlated with the viability of Oregon Wolfe barley dominant hybrids (Bregitzer *et al*., 2007). The authors suggested that the PAT enzyme may acetylate glutamate, a structural analogue of glufosinate. If this is true, the substrate specificity of PAT may be less specific than previously thought, resulting in pleiotropic effects. Alternatively, unknown downstream derivatives of glufosinate may have toxic effects on plants. This is not expected, as studies on several glufosinate-tolerant plants have identified *N*-acetyl-L-glufosinate following glufosinate treatment (Tshabalala, 1993; Burnett, 1994; Rupprecht and Smith, 1994; Thalacker, 1994; Rupprecht *et al*., 1995; Stumpf, 1995; Allan, 1996), and several studies have provided evidence for the stability of these intermediates in the treated plant (OECD, 2002). Although the source of toxicity in transgenic plants is unknown, our microarray data clearly revealed the activation of several stress-related genes, including detoxification-related genes, in glufosinate-tolerant plants that were exposed to the herbicide substrate. It is possible that the sensitivity of the profiling strategy used here revealed key responses to glufosinate and its derivatives that offset any phenotypic effects in the plants. This interpretation seems likely, because our previous research has shown that other herbicides, in particular imidazolinone, do not generate similar pleiotropic effects in tolerant *Arabidopsis* carrying the *csr1-1* mutation, whereas WT *Arabidopsis* shows the activation of detoxification genes as an early response to imidazolinone (Manabe *et al*., 2007).

The early and late responses to glufosinate included many diverse genes known to be implicated in detoxification pathways in the presence and absence of bacterial PAT. Our data demonstrated broad-ranging effects of glufosinate on several metabolic pathways, including nitrogen assimilation and metabolism, that could result in the accumulation of

toxic metabolites, including ammonium. Other microarray studies have also shown the induction of several detoxification genes in response to nitrogen and nitrogen limitation in WT plants (Peng *et al*., 2007). Plants have evolved detoxification systems that can protect them against a broad range of chemicals, including herbicides (Yuan *et al*., 2007). Interestingly, recent studies have shown that more than 300 biotypes of weeds have evolved resistance to one or more of the major groups of herbicides (Yuan *et al*., 2007). Detoxification genes, such as P450 genes, have been linked to naturally occurring herbicide resistance in weeds, and ABC transporters have been used to achieve nonspecific resistance to chemicals as diverse as antibiotics (Yuan *et al*., 2007). The significance of these systems was demonstrated by the finding that several P450 genes might be involved in multiple herbicide resistance, and that a single P450 gene in transgenic plants can confer resistance to up to 13 different herbicides (Robineau *et al*., 1998). The induction of detoxification-related genes by different herbicides now appears to be a common response (Hirose *et al*., 2005; Madhou *et al*., 2006; Manabe *et al*., 2007). Our study showed that many of the detoxification-related genes were not induced in the transgenic plants expressing PAT, and were specific to WT plants. Future studies are needed to understand the different detoxification pathways that occur in WT plants and plants in which glufosinate is metabolized through acetylation.

GS activity is essential for plant growth and development (Limami *et al*., 1999), and the inhibition of GS by glufosinate has been well studied (Wehrmann *et al*., 1996). The consequence is plant death resulting from the coincidental occurrence of ammonia toxicity and the perturbation of pathways downstream of GS. The integration and cross-talk among the pathways that are affected during this process are presumably very complicated and not yet well understood. Gas chromatography-mass spectrometry-based metabolite profiling analysis of *Medicago truncatula* root nodules treated with glufosinate showed a reduction in glutamine, glutamate, asparagine and alanine, but elevated levels of leucine, valine, methionine, threonine and isoleucine (Barsch *et al*., 2006). Our microarray data provide a first step in an understanding of this process by identifying the sequence of transcriptome changes that occur on exposure to glufosinate. The functional classification of genes has revealed the coordinated regulation of several pathways. The initial response includes the regulation of some metabolic pathways other than glutamine biosynthesis, the activation of detoxification processes and the activation of general stress and hormonal regulation pathways. These are directly linked to plant death by the regulation of several metabolic pathways, including the inhibition of the biosynthesis of several amino acids, inhibition of photosynthesis and the alteration of development through leaf senescence-related processes (Figure 5). The effectiveness of glufosinate as a herbicide lies not only its specificity to GS, but also in the broad effects of ammonium toxicity. Transgenic plants may be useful in revealing the pathways that interact with the acetylated derivatives of the herbicide and may shed light on the pleiotropic effects that are now becoming recognized as by-products of the use of the *bar* gene.

In conclusion, this study has shown that the application of glufosinate to *Arabidopsis* progressively and precisely results in the dramatic reprogramming of the transcriptome. The insertion of the bacterial *bar* gene into *Arabidopsis* is responsible for only a small number of changes to the transcriptome; however, exposure to glufosinate activates a specific set of genes that are unique to transgenic plants and not WT plants, raising speculation that glufosinate or a metabolic derivative of glufosinate activates unique detoxification pathways to offset any effects on plant growth and development.

Experimental procedures

Transgenic *Arabidopsis* and vectors

The pCAMBIA3300 transformation vector (CAMBIA), which harbours the *pat* gene driven by a tandem repeat of the cauliflower mosaic virus (CaMV) 35S promoter and the polyA signal of CaMV 35S, was introduced into *Agrobacterium tumefaciens* strain GV3101. This bacterial strain was used for the transformation of *Arabidopsis* WT Col-0 via the floral dip method (Clough and Bent, 1998). The copy number of the inserted gene was determined by Southern blot and segregation analysis.

Glufosinate treatment and growth measurements

T3 seeds were surface sterilized with 25% (v/v) commercial Clorox (final concentration of 1.3% sodium hypochlorite) and 0.05% (v/v) Triton X-100 (Fisher Scientific, Hampton, NH, USA) for 20 min, and then rinsed four times with distilled water. Seeds from independent transgenic lines and the progenitor WT Col-0 line were germinated in MS medium for 7 days. The seedlings were then transferred to either MS medium (control) or MS medium containing glufosinate-ammonium (Sigma-aldrich.com 45520) (concentration range, 0.1–100 μg/mL). The plates were orientated in a vertical (for root growth measurements) or horizontal position and data were collected at intervals up to 2 weeks. Root length measurement was conducted using an image analysis system, as described by Buer *et al*. (2000). Total fresh weight was measured for a group of 10 seedlings. The leaf number was counted under a microscope at the end of the experiment.

Microarray hybridization and analysis

RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA), according to the manufacturer's protocol. The quality of the RNA was determined using a 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). cRNA synthesis, hybridization and scanning were performed at the Botany Affymetrix GeneChip Facility, University of Toronto (Toronto, ON, Canada). Microarray analysis was performed in triplicate and the data were normalized within each group using RMA (Millenaar *et al*., 2006), with the R software packages by Bioconductor (http://www.bioconductor.org/) (Gentleman *et al*., 2004). The lists of differentially expressed genes were generated by the affylmGUI package (Gentleman *et al*., 2004).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 List of differentially expressed genes in response to glufosinate at 6 and 48 h in wild-type *Arabidopsis* plant

Table S2 List of differentially expressed genes in response to glufosinate at 6 and 48 h in wild-type and transgenic BAR1 plants

Table S3 List of differentially expressed genes in response to glufosinate at 6 and 48h in BAR1 plants

Table S4 Categorization of glufosinate-responsive genes based on pathways

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