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Immediate Activation of Respiration in *Petroselinum crispum* L. in Response to the *Phytophthora megasperma* f. sp. *Glycinea* Elicitor[1]

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Treatment of parsley (*Petroselinum crispum* L.) cell cultures with the *Phytophthora megasperma* elicitor isolated from the fungus *Phytophthora megasperma* f. sp. *Glycinea* caused an immediate increase in the rate of respiratory CO_2 evolution in the dark. The respiratory response was biphasic, showing a rapid enhancement in the first 20 min and then a slower increase until a steady rate was attained 60 min posttreatment. The enhanced rate of CO_2 evolution corresponded to the activation of phosphofructokinase and glucose-6-phosphate dehydrogenase, key enzymes in the regulation of carbohydrate flow to glycolysis and the oxidative pentose phosphate (OPP) pathway, respectively. The increased rate of CO_2 evolution and the activation of phosphofructokinase and glucose-6-phosphate dehydrogenase were maintained for the duration of the experiments, indicating long-term stimulation of respiration through both glycolysis and the OPP pathway. A 23% decrease in the C_6:C_1 ratio of $^{14}CO_2$ evolution from labeled glucose 60 min after the addition of *Phytophthora megasperma* elicitor is consistent with an increased contribution of the OPP pathway to cellular respiration. Long-term activation of the OPP pathway following elicitation could serve to maintain the pools of substrates necessary during activation of the shikimic acid pathway, leading to the production of defensive compounds.

Plants respond to stress such as wounding, UV irradiation, and fungal infection by increasing the activity of the OPP, shikimic acid, and phenylpropanoid pathways to synthesize defensive and protective compounds. An important role for respiratory metabolism, including increases in both glycolysis and the OPP pathway, has been demonstrated in the long-term response of a wide variety of plant tissues to wounding (Kahl, 1974; Daly, 1976). The increase in the activity of the OPP pathway coincides with the lignification associated with wounding in *Coleus blumei* and *Helianthus tuberosus* (Pryke and ap Rees, 1976) and *Pisum sativum* (Wong and ap Rees, 1971) and provides much of the reductant required for lignification (Pryke and ap Rees, 1977). In other types of stress the overall response resembles that to wounding, but the role of respiration has not been elucidated.

Response to pathogenic attack can be studied in vitro using parsley (*Petroselinum crispum* L.) cell-suspension cultures and an elicitor purified from the fungus *Phytophthora megasperma* f.sp. *glycinea*, Pmg. Following treatment with Pmg elicitor, transcription and enzyme activity associated with the shikimic acid pathway (Henstrand et al., 1992), phenylpropanoid metabolism (Hahlbrock and Scheel, 1989), and the production of defensive furanocoumarins (Hauffe et al., 1986; Kawalleck et al., 1992) increase. Furanocoumarin biosynthesis depletes the substrates of the shikimic acid pathway, the biochemical pathway linking respiratory metabolism to phenylpropanoid metabolism (Fig. 1), which places a significant demand on respiratory metabolism to supply E4P and PEP. In addition, the immediate intracellular acidification (Kneusel et al., 1989), extracellular alkalinization (Scheel et al., 1991), influx of Ca^{2+} and H^+ and efflux of Cl^- and K^+ (Conrath et al., 1991; Sacks et al., 1993), and Ca^{2+}-dependent phosphorylation/dephosphorylation of proteins (Dietrich et al., 1990; Renelt et al., 1993) place a considerable demand for ATP and reductant on the plant cell, which should be reflected in the response of primary carbon metabolism during the initial phases of elicitor recognition by parsley. Thus, primary carbon metabolism should be activated during parsley elicitation to provide the substrates for both the immediate and long-term responses.

Research on the response of parsley to the Pmg elicitor has focused on the transduction of the elicitor's signal to the nucleus and the activation of genes coding for enzymes involved in the production of furanocoumarins and other minor phenolics, but the role of primary carbon metabolism in the defensive response remains poorly understood. In this study, we have examined the activation of respiratory carbon metabolism in elicited parsley cell-suspension cultures. Results are discussed in terms of a role for both the immediate and long-term activation of glycolysis and the OPP pathway in the defensive response.

MATERIALS AND METHODS

Cell Culture and Elicitor

Cell-suspension cultures of parsley (*Petroselinum crispum* L.) (Schmelzer et al., 1985) were grown in the dark (150 rpm,

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Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate; ddH_2O, distilled deionized water; E4P, erythrose-4-phosphate; G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); OPP, oxidative pentose phosphate; 6-PG, 6-phosphogluconate; PFK, phosphofructokinase (EC 2.7.1.11); Pmg, *Phytophthora megasperma*; TCA, tricarboxylic acid.

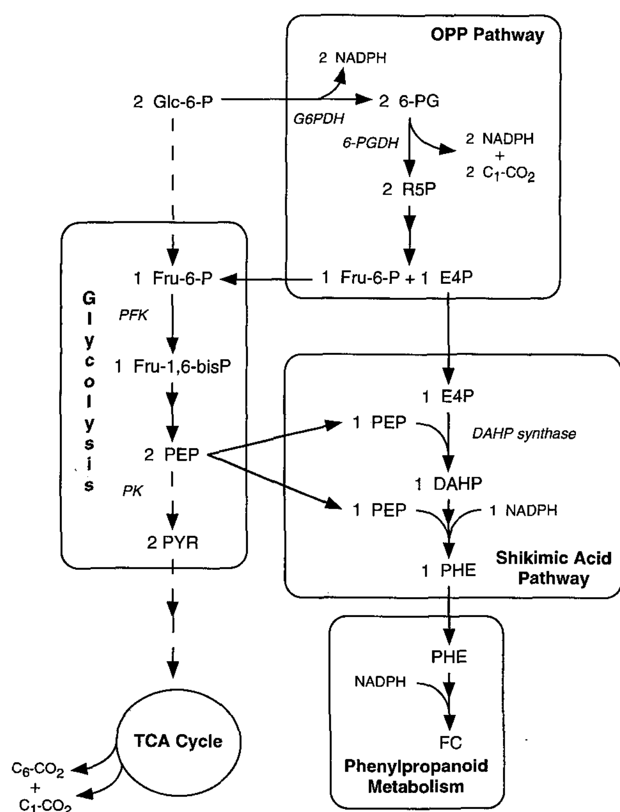

Figure 1. Diagrammatic representation of the respiratory pathways participating in the biosynthesis of furanocoumarins. (Abbreviations not previously defined: FC, furanocoumarins; 6-PGDH, 6-phosphogluconate dehydrogenase; PHE, phenylalanine; PK, pyruvate kinase; PYR, pyruvate; R5P, ribulose-5-phosphate.)

25°C) in 250-mL or 1-L Erlenmeyer flasks as previously described (Ragg et al., 1981) and were subcultured in fresh modified B5 medium (Hahlbrock, 1975) every 7 d. Experiments were performed on 6-d-old cultures that had reached a density of 120 to 140 mg mL^{-1} fresh weight as determined after vacuum filtration using Whatman filter paper (No. 1) for 10 s. The Pmg elicitor, a crude cell wall extract of the fungus *Phytophthora megasperma* f. sp. *Glycinea*, isolated as described by Ayers et al. (1976), was kindly donated by Dr. Carl J. Douglas (University of British Columbia), and aliquots of a stock solution (5 mg mL^{-1}) were used in all elicitation experiments to give a final concentration of 30 μg mL^{-1}. In control experiments, Pmg solution was replaced by an equivalent volume of ddH_2O.

Gas-Exchange Experiments

All experiments were performed in the dark at 25°C in a sterile 160-mL water-jacketed chamber attached to an IRGA (The Analytical Development Co. Ltd., Hoddesdon, Hertfordshire, UK) in an open gas-exchange system. Sterile CO_2-free air was channeled from the IRGA to the bottom of the chamber at 200 mL min^{-1} and then returned to the IRGA. The IRGA, calibrated with 90 μL L^{-1} CO_2 in compressed air, was used to monitor the CO_2 concentration in the air returning from the cell suspension. Cells (40–50 mL) were transferred aseptically to the chamber through a sealed sampling port at the base of the chamber using 60-mL syringes capped with 18.5-gauge (wide bore) needles and buffered with 25 mM Mes, pH 5.7. Cell samples were removed with syringes capped with 18.5-gauge (wide bore) needles through the sampling port. Cells were allowed to equilibrate for 30 min prior to treatment to obtain a steady rate of CO_2 evolution. Pmg or ddH_2O was added, and the responses were monitored for 100 min. Both elicitation and control experiments were performed in triplicate. Three 1-mL samples were taken at the end of the experiment for protein determination. Rates of CO_2 evolution were expressed as μmol CO_2 mg^{-1} protein h^{-1}.

Metabolite Experiments

Metabolite experiments were performed in triplicate under the same conditions described above for gas-exchange experiments except 1-mL metabolite samples were withdrawn through the sampling port at points throughout the experiment and three 1-mL samples were removed prior to the experiment for protein determination. Samples were removed, plunged into 167 μL of 70% $HClO_4$, giving a final concentration of 10% (v/v), and rapidly frozen in liquid N_2. The time course of metabolite sampling included 10 min prior to treatment and 100 min following treatment.

Metabolite samples were thawed and centrifuged ($15g \times 10^3$, 4 min), and the supernatant was neutralized with 5 N KOH/1 M triethanolamine. The samples were again centrifuged, and the supernatants were brought to 1.5 mL with ddH_2O and stored in liquid N_2 until analyzed. Metabolites were measured in enzymatic assays coupled to the reduction/oxidation of pyridine nucleotides using a dual-wavelength UV-VIS spectralline-photometer (ZFP-22; Sigma Instruments, Berlin, Germany). Glc-6-P, Fru-6-P, Fru-1,6-bisP, and 6-PG assays were performed as previously described (Wirtz et al., 1980; Quick et al., 1989). Metabolite recoveries of 88.0% (Glc-6-P), 82.4% (Fru-6-P), 95.4% (Fru-1,6-bisP), and 81.2% (6-PG) were determined by extracting known amounts of metabolites along with the samples. Corrections for any changes in protein concentration during the experiment were accounted for in the calculations.

C_6:C_1 Ratios: Analysis Of $^{14}CO_2$ Evolution

Parsley cells were prepared and equilibrated as previously detailed (see above). Aliquots of cells (5 mL) were removed immediately before and 60 min following Pmg or ddH_2O addition. Samples were placed in 25-mL Warburg flasks containing 1.0 mCi (5.0 μL) of either D-[1-^{14}C] or D-[6-^{14}C]Glc (60 mCi $mmol^{-1}$) with a center well containing 1 mL of the basic CO_2 trap β-phenylethylamine. The flasks were incubated for 5 min with continuous shaking, and all reactions were stopped by the addition of 0.72 mL of 70% $HClO_4$. Flasks were shaken for an additional 24 h to ensure complete CO_2 release from the medium and effective trapping of CO_2. The center well contents were removed and counted in ACS scintillation cocktail using a Beckman LS 60001C scintillation counter, and the results were used to calculate the C_6:C_1 ratios of $^{14}CO_2$ evolution.

Other Methods

Protein was measured utilizing the Bradford assay (Bradford, 1976) with γ-globulin as the standard. The stimulation of furanocoumarin synthesis was routinely confirmed by monitoring UV fluorescence of the furanocoumarins 24 h postelicitation (Kombrink and Hahlbrock, 1986).

RESULTS

Respiratory CO_2 Evolution

The rate of CO_2 evolution increased 61% from 2.46 to 3.96 μmol mg^{-1} protein h^{-1} (Fig. 2B) within 20 min of elicitor treatment. The rate increased more slowly during the next 40 min, reaching a steady rate of 4.42 μmol mg^{-1} protein h^{-1}, which represents an overall increase of 80%. This biphasic pattern of CO_2 evolution was observed in all experiments. The enhanced rate of CO_2 evolution was maintained for the duration of the experiment. The rate of CO_2 evolution in the control experiments increased from 2.19 to 2.37 μmol mg^{-1} protein h^{-1} after 100 min, an insignificant increase of 8% (Fig. 2A), indicating that transfer of cells from the growing conditions to an aerated environment did not significantly affect the rate of dark respiratory CO_2 evolution throughout the duration of the experiment.

Metabolites

Elicitation had no effect on the levels of Fru-6-P, the substrate of PFK, throughout the experiment (Fig. 3A). The levels of Fru-1,6-bisP, the product of PFK, decreased within the first 6 min of elicitation and then increased to a level 2 times the control (Fig. 3B). Likewise, the ratio of Fru-1,6-bisP:Fru-6-P began to increase within 7 min following elicitation (Fig. 3C) and reached a maximum 2 times that of the control within 20 min. The rapid and sustained increase in the Fru-1,6-bisP:Fru-6-P ratio is consistent with the activation of PFK for the duration of the experiment.

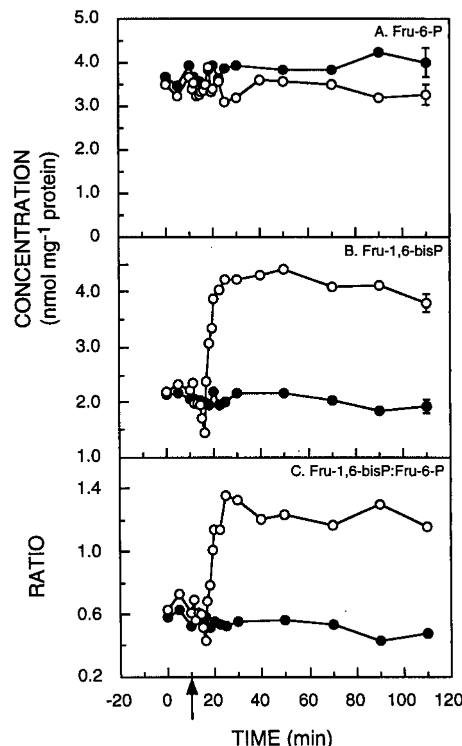

Figure 3. The intracellular levels of Fru-6-P (A) and Fru-1,6-bisP (B) and the ratio of Fru-1,6-bisP:Fru-6-P (C) in cell suspensions of *P. crispum* treated with Pmg elicitor (30 μg mL^{-1}) (○) and water (●). Symbols represent the means for three separate experiments. The arrow indicates the time of treatment. The error bars on the last time points represent the average SE of the three experiments for each treatment.

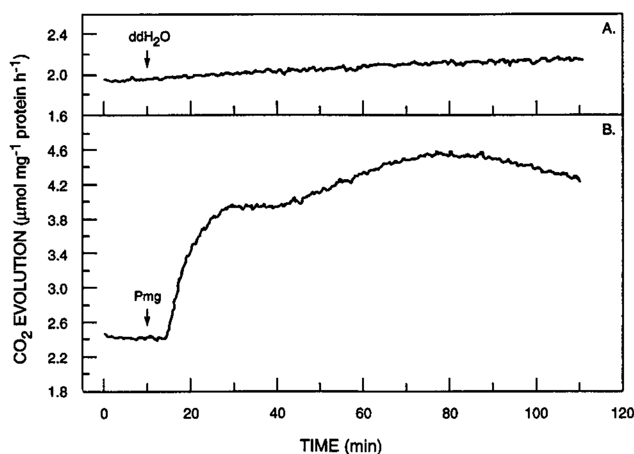

Figure 2. Representative traces of the changes in the rate of CO_2 evolution in cell suspensions of *P. crispum* treated with water (A) or Pmg elicitor (30 μg mL^{-1}) (B). Traces are from two individual experiments and do not correspond to the average values for each treatment obtained from three separate experiments. The increase in the rate of CO_2 evolution in A is insignificant at 100 min following treatment (paired-sample *t* test, $P > 0.05$) and in B is significant at 20 and 60 min following treatment (paired-sample *t* test, $P < 0.001$).

The levels of Glc-6-P, the substrate of G6PDH, decreased within 12 min following elicitor treatment, returning to control levels thereafter (Fig. 4A). A corresponding increase in the levels of 6-PG, the product of G6PDH, was observed within 4 min (Fig. 4B), reaching a level 7-fold higher than the control within 20 to 30 min of elicitation. The ratio of 6-PG:Glc-6-P began to increase within 4 min of elicitation and reached maximum levels, 7 times greater than those of the control, within 20 to 30 min (Fig. 4C). The increase in the 6-PG:Glc-6-P ratio upon elicitation indicates the immediate and sustained activation of G6PDH.

C_6:C_1 Ratios of $^{14}CO_2$ Evolution

The C_6:C_1 ratios determined prior to and 60 min following treatment are presented in Table I. Prior to elicitation, there was no significant difference between control and treatment, with average ratios of 0.46 and 0.43, respectively. After 60 min, the control ratio was not significantly affected, with a value of 0.44, whereas the treatment ratio decreased to 0.33, a 23% decrease.

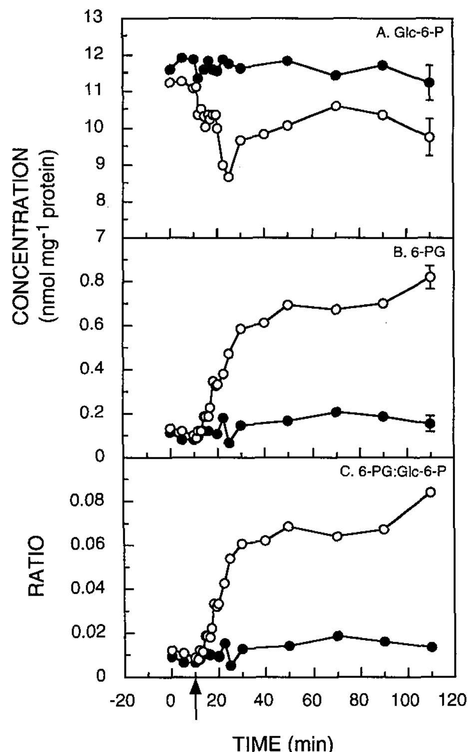

Figure 4. The intracellular levels of Glc-6-P (A) and 6-PG (B) and the ratio of 6-PG:Glc-6-P (C) in cell suspensions of *P. crispum* treated with Pmg elicitor (30 μg mL^{-1}) (O) and water (●). Symbols represent the means for three separate experiments. The arrow indicates the time of treatment. The error bars on the last time points represent the average SE of the three experiments for each treatment.

DISCUSSION

Immediate Events following Elicitor Treatment

In parsley cells elicited with Pmg, there is an immediate increase in the rate of CO_2 evolution (Fig. 2). The increase in the ratios of Fru-1,6-bisP:Fru-6-P (Fig. 3) and 6-PG:Glc-6-P (Fig. 4) within minutes of elicitation indicates the activation of two respiratory enzymes, PFK and G6PDH, respectively. The timing of these changes supports a role for the activation of primary metabolism in the elicitor recognition and signal transduction processes that activate secondary metabolism. During this time frame there are dramatic changes in ion fluxes across the plasma membrane and changes in the phosphorylation status of proteins. Activation of primary metabolism under these conditions would serve to provide ATP and reducing power to meet the increased cellular energy demands arising from elicitor-induced stress.

PFK and G6PDH are key regulatory enzymes in glycolysis and the OPP pathway, respectively, and are finely regulated by a number of factors (Dennis and Miernyk, 1982; Copeland and Turner, 1987; Plaxton, 1990). Immediate changes in intracellular pH could be a key factor in the activation of PFK. Small pH changes can lead to large changes in PFK activity in some plant systems (Kelly and Turner, 1969; Botha et al., 1988). As previously demonstrated by Kneusel et al. (1989), the cytoplasmic pH of aerated parsley cells decreases as much as 0.25 pH unit following treatment with Pmg. The timing of this decrease in pH corresponds to the alkalinization of unbuffered external medium (data not shown), consistent with pH playing a role in the activation of PFK in elicited parsley cells.

PFK is also under negative regulatory control by PEP and ATP in higher plants (Dennis and Greyson, 1987). The immediate, transient decrease in the levels of Fru-1,6-bisP (Fig. 3B) and triose-phosphate (data not shown) may indicate activation of another rate-limiting glycolytic enzyme, pyruvate kinase, which regulates the flow of PEP to the TCA cycle (Fig. 1). Alternatively, there could be an immediate drain on PEP pools for increased secondary metabolism, committing carbon to the shikimic acid pathway. The pools of ATP may be utilized during elicitor recognition and ion transport at the plasma membrane. A decrease in the levels of either of these metabolites could result in the activation of PFK. Unfortunately, poor yields of PEP and pyruvate in this study prevented reliable measurements of changes in these metabolites, and ATP measurements were inconclusive.

In response to elicitation, the 6-PG:Glc-6-P ratio increases rapidly, indicating the immediate activation of G6PDH (Fig. 4). In many plant systems the principle activator of G6PDH is a low NADPH:$NADP^+$ ratio. Elicitation induces an immediate extracellular oxidative burst that involves the extracellular production of $O_2^{-\cdot}$ and subsequent transient formation of H_2O_2 within 1 to 4 min of elicitation in many plant-pathogen interactions (Doke, 1983; Apostol et al., 1989), including the interaction of parsley cells with the Pmg fungal elicitor (D. Scheel, personal communication). The production of H_2O_2 from $O_2^{-\cdot}$ is thought to involve a membrane-bound NADPH-utilizing oxidase that transfers 2 H^+ and 2 e^- from intracellular NADPH to $O_2^{-\cdot}$ (Bowler et al., 1992). The immediate production of H_2O_2 upon elicitation could cause the levels of NADPH to decline, decreasing the ratio of

Table I. *Effect of Pmg elicitor (30 μg mL^{-1}) on the C_6:C_1 ratios of $^{14}CO_2$ evolution from cell suspensions of P. crispum (means ± SD)*

Numbers in parentheses represent the number of times the experiments were duplicated. Values with different letters are significantly different at the 99% confidence interval using the Kruskal-Wallis test for multiple sample comparisons.

Treatment	Time (min)		Percentage Change
	0	60	
Control	0.46 ± 0.07 (7)a	0.44 ± 0.05 (11)a	−4
Pmg	0.43 ± 0.05 (7)a	0.33 ± 0.05 (8)b	−23

NADPH:NADP$^+$, thereby activating G6PDH. The immediate demand for NADPH would be consistent with the rapid activation of G6PDH prior to the expected long-term demand for OPP pathway carbon skeletons from the shikimic acid pathway.

Long-Term Events following Elicitor Treatment

The activation of PFK and G6PDH during elicitation indicates that both glycolysis and the OPP pathway contribute to the immediate respiratory enhancement in elicited parsley cells. In the long term, an increase in secondary metabolism to supply the substrates for furanocoumarin synthesis has been associated with elicitation by Pmg. Several observations support a continued involvement of both glycolysis and the OPP pathway in this long-term response. The elevated rate of CO_2 evolution (Fig. 2) and the increased product:substrate ratios for G6PDH and PFK after elicitation (Figs. 3 and 4) are maintained for the duration of the experiment. The G6PDH and 6-PG dehydrogenase reactions of the OPP pathway supply the bulk of the reductant required in the shikimic acid pathway and several steps in the formation of furanocoumarins (Fig. 1). The increased oxidation of NADPH via increased secondary metabolism would maintain a low NADPH:NADP$^+$ ratio, thus supporting the activation of G6PDH and consequently OPP pathway activity. Furanocoumarin production also requires ATP and PEP, both inhibitors of PFK. A demand for these metabolites should keep them at low levels in the long term, thus sustaining the activation of PFK and hence glycolytic activity.

The decrease in the C_6:C_1 ratio of $^{14}CO_2$ evolution after 60 min (Table I) indicates that the OPP pathway is responsible for some portion of the 80% enhancement of the rate of CO_2 evolution in the presence of elicitor. The decrease in the C_6:C_1 ratio can be explained by the fact that the C_6 carbon of Glc is not oxidized when metabolized through the OPP pathway. In contrast, both the C_6 and C_1 carbons are oxidized when metabolized through the TCA cycle via glycolysis (Fig. 1). If the C_6:C_1 ratio remained unchanged, we could then estimate that the relative contributions of both pathways were unaffected by elicitation, even though the total magnitude may have increased. A C_6:C_1 ratio of 0.24 would be expected if the enhanced respiration (80%) were due solely to the OPP pathway. The observed ratio after 60 min (0.33; Table I) is greater than the theoretical value (0.24) but lower then the initial or control value (0.43). This suggests that the relative contribution of the OPP pathway to carbon oxidation has increased to a greater extent than that of glycolysis following elicitation.

The incomplete oxidation of triose-phosphate would also contribute to the reduction of the C_6:C_1 ratio as PEP is diverted away from the TCA cycle to the shikimic acid pathway (Fig. 1). Demand for PEP in the DAHP synthase and 5-*enol*pyruvylshikimate-3-phosphate synthase reactions of the shikimic acid pathway, leading to the production of furanocoumarins, could lead to the incomplete oxidation of substantial amounts of PEP. The DAHP synthase reaction, which combines one molecule each of PEP and E4P, is the first step committing carbon flow to the shikimic acid pathway, and increased phenylpropanoid metabolism would create a demand for both of these respiratory products. Theoretically, 2 mol of Glc-6-P metabolized through the OPP pathway generate 1 mol each of E4P and Fru-6-P. The Fru-6-P can be metabolized through glycolysis to yield 2 mol of PEP. This PEP can then be used in the DAHP and 5-*enol*pyruvylshikimate-3-phosphate synthase reactions, which lead to the formation of 1 mol of Phe. In terms of a carbon budget, the 2 mol of Glc-6-P that enter the OPP pathway represent precisely the number of carbon skeletons required to satisfy the needs of phenylpropanoid metabolism (Fig. 1).

Evidence linking the increased OPP pathway activity 60 min after elicitation of parsley cells to the production of furanocoumarins includes increased transcription of DAHP synthase (Henstrand et al., 1992) as well as Phe ammonia lyase and 4-coumarate:CoA ligase (Somssich et al., 1986), enzymes of the phenylpropanoid pathway. Increases in the enzyme activities generally have been reported to occur 2 to 3 h following elicitation (Kuhn et al., 1984; McCue and Conn, 1989); however, these activity measurements often represented the first time points taken following elicitation, so any earlier activation would have been missed. We attempted to demonstrate a direct link between increased OPP pathway activity and the production of furanocoumarins by using [U-^{14}C]Phe and [6-^{14}C]Glc to label newly synthesized furanocoumarins. Unfortunately, the elicitor interfered with the uptake of the substrates by parsley cells (data not shown), which is consistent with the membrane changes reported after elicitation and with the inhibition of Asn uptake by elicitor demonstrated in parsley cells (Strasser and Matern, 1986).

CONCLUSION

In parsley, we have shown the immediate effects of the Pmg elicitor upon respiratory metabolism in the dark. Elicitation of parsley cells results in the immediate increase in the rate of CO_2 evolution and the activation of PFK and G6PDH. Enhanced respiratory carbon flow is maintained for the duration of treatment and corresponds to the activation of both glycolysis and the OPP pathway. The decrease in the C_6:C_1 ratio of $^{14}CO_2$ evolution is consistent with a greater contribution of the OPP pathway to the observed respiratory increase, supporting the substrate demands of furanocoumarin production. The possible roles of PEP and ATP as components of the immediate regulatory cascade require further investigation.

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