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# THE CHLOROPLAST AS MEDIATOR OF PHENOLIC INDUCTION

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Plant Physiology

by

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## ABSTRACT

The degree to which insect herbivores and plant pathogens elicit accumulation of phenolic defenses in plants is variable in nature. Because light and leaf development, factors which can alter resource levels in chloroplasts, influence enemy responses to plants, I was interested in the degree to which chloroplast carbon resources constrain phenolic induction responses. To address the central role of the chloroplast in supplying carbon resources for induced phenolic accumulation, I devised the Chloroplast Control Hypothesis: constraints on phenolic induction occur when stromal substrates are limited. Levels of carbon resources were manipulated by shading plants and monitoring phenolic accumulation responses to Jasmonic acid (JA), a wound signal that elicits phenolic metabolism in plant leaves much as does wounding or herbivory. Phenolic accumulation was prevented in darkened tobacco plants, and low light constrained phenolic accumulation, but photosynthetic carbon assimilation was a poor predictor of resource investment in phenolic defenses. While young leaves which are carbon resource sinks were more responsive to JA, metabolite import was not enhanced in these leaves. In fact, activity of the chloroplast phosphoenolpyruvate/ phosphate transporter was not required for Arabidopsis plants to mount strong shikimate-based defense responses that deterred an insect herbivore. These results suggest that chloroplasts must possess alternate means of acquiring substrates for induced phenolic synthesis, possibly via starch breakdown. I was unable to disprove the chloroplast control hypothesis, as mechanisms of resource allocation to induced phenolics are much more complex than I originally envisioned. Chloroplasts may possess at least three avenues to acquire carbon resources for the shikimate pathway, allowing plants to compensate for deficiencies in one route when others are limiting. Such redundancy in resource supply may not be surprising since products of this pathway play central roles in plant physiology.

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# CHAPTER 1

# THE CHLOROPLAST'S ROLE IN SUPPLYING CARBON RESOURCES FOR INDUCED PHENOLIC ACCUMULATION

## Rationale for Overall Hypothesis

Herbivorous insect and plant pathogens frequently induce plants to accumulate a variety of phenolic defenses, including lignin, flavonoids, caffeoylquinic acids, hydroxycinnamic acids, and furanocoumarins (Karban and Baldwin, 1997). Considering that 20% of net primary carbon assimilation by plants is allocated to constitutive phenolic synthesis (Walton, 1995), it is remarkable that additional resources are allocated to induced phenolics, resulting in 10 to 20-fold induction of some phenolic compounds (Berenbaum and Zangerl, 1999; Hartley and Lawton, Hauffe et al., 1986; Keinänen et al., 2001). Phenolic induction responses often incur a cost to competing resource sinks within the plant, but a cost is not always evident and in spite of extensive research, there is no clear explanation for this phenomenon (Heil et al., 2002; Strauss et al., 2002).

Resource allocation to phenolic metabolism begins in the chloroplast, where 3-deoxy-D-arabino-7-heptulosonate phosphate (DAHP) is synthesized from erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) in a reaction catalyzed by DAHP synthase, the first enzyme of the shikimate pathway (SP) (**Fig. 1**) (Herrmann and Weaver, 1999). To address the central role of the chloroplast in supplying carbon resources for phenolic synthesis, I developed the **Chloroplast Control Hypothesis**: constraints on phenolic induction occur when stromal substrates are limited. While heterogeneity in phenolic induction responses may be controlled by either resource supply or pathway activation, I focused on resource supply for the following three reasons. First, enhanced gene expression and activities of enzymes in the chloroplast SP (Bischoff et al., 1996; Dyer et al., 1989; Görlach et al., 1995; Keith et al., 1991; McCue and Conn, 1989; Muday and Herrmann, 1992; Sharma et al., 1999) and the cytoplasmic phenylpropanoid pathway (PP) (Hermsmeier et al., 2001; Nemestothy and Guest, 1990; Pellegrini et al., 1994; Reymond et al., 2000; Schmelzer et al., 1985), the

core reactions of phenolic metabolism, are frequently measured in plants responding to enemies. Second, photosynthesis, which can supply carbon resources to the SP (Kleiner et al., 1999; Schulze-Siebert and Schultz, 1989), can be decreased by insect herbivores and plant pathogens (Hibberd et al., 1996; Larson, 1998; Zangerl et al., 2002). Finally, decreased PEP import from the cytoplasm via the PEP:phosphate translocator limits constitutive phenolic synthesis (Streatfield et al., 1999). The extent to which induced phenolic synthesis is supplied by resources from the Calvin Cycle or metabolite import remains to be determined.

**Research Hypotheses**

*Hypothesis 1.* Light intensity constrains induced phenolic accumulation.
*Hypothesis 2.* Leaf development constrains induced phenolic accumulation.

Rationale for Hypotheses 1 and 2.

Given the location of induced SP activity in the chloroplast (Herrmann and Weaver, 1999), we would expect to find constrained phenolic induction responses in environments that limit carbon resources in this organelle. For example, plants are often more susceptible to enemies in low light, when Calvin Cycle activity is likely to be reduced (Pennypacker et al., 1994; Wiltshire, 1956). Whether increased vulnerability is caused by lower concentrations of constitutive defenses produced in low light or by constrained phenolic induction was unknown when I began my dissertation research (Buttery et al., 1992; Conti et al., 1983; Vergeer and van der Velde, 1997). During my research one study appeared that investigated light constraints on phenolic induction (Nabeshima et al., 2001). While fewer condensed tannins were induced by *Quercus crispula* saplings growing in forest shade, these authors were not able to show a causal relationship between phenolic induction and light intensity since understory light differs from open gaps in both light intensity and light quality (lower red to far red ratio), the latter of which also decreases phenolic metabolism (Huub et al., 1997; McClure, 1974). Enemy success is also influenced by leaf development (Carnegie and Colhoun, 1982; Kogel et al., 1997; Quiring, 1992; Reuveni et al., 1986; Reynolds and Smith, 1985), but susceptibility to enemies and phenolic induction responses in developing or

old leaves vary between species (Karban and Baldwin, 1997). Sources of carbon resources change through development; young leaves are resource sinks whereas mature leaves are sources (Turgeon, 1989). Higher sink strength in developing leaves mediated by invertases promotes greater phenolic induction (Arnold and Schultz, 2002), but it is unclear if imported leaf resources are transported to the chloroplast stroma to serve as SP substrates. I designed my first experiments to address hypotheses 1 and 2 while at the same time establishing a model research system in tobacco to investigate resource supply constraints on induced phenolic accumulation (see Hypotheses 3 and 4).

*Hypothesis 3.* Photosynthetic carbon assimilation, as influenced by light and leaf development, constrains induced phenolic accumulation.

Rationale for Hypothesis 3.

$^{14}$C-labeled phenylalanine can be synthesized from $^{14}$C-$CO_2$ in isolated chloroplasts (Schulze-Siebert and Schultz, 1989), consistent with the Calvin Cycle providing substrates for the chloroplast shikimate pathway. In fact, E4P is an intermediate of the Calvin Cycle, but PEP must by synthesized from 3-phosphoglycerate by the action of two additional enzymes, phosphoglycerate mutase and enolase. We would expect photosynthetic carbon assimilation (PCA) to be enhanced in plants undergoing phenolic induction responses if Calvin Cycle activity is the source of additional substrates for induced phenolic synthesis. While photosynthetic responses to insect and pathogen enemies have been recorded in many instances, results are largely equivocal perhaps resulting from differences in damage patterns, leaf development or light intensities employed in these studies (Bassham and Dickmann, 1982; Morrison et al., 1995; Oleksyn et al., 1998). In the few instances where photosynthesis and induced phenolic responses to real or simulated herbivory were recorded in the same study, photosynthesis was either unresponsive to (Arnold and Schultz, 2002) or decreased by treatment (Zangerl et al., 1997; Zangerl et al., 2002). In wild parsnip, surprisingly, $^{14}$C-labeled $CO_2$ incorporation into induced furanocoumarin synthesis was actually decreased by herbivory (Zangerl et al., 1997), indicating that the Calvin Cycle may not be the primary carbon source for induced phenolic synthesis. To investigate possible limitations of the Calvin Cycle in supplying resources for induced phenolic synthesis, I wanted to investigate

photosynthetic responses of induced plants under limiting light conditions and in leaves in different developmental states, both of which alter chloroplast resource levels. Experiments addressing Hypothesis 3 were designed to investigate the role of the Calvin Cycle in providing carbon resources for induced phenolic accumulation.

*Hypothesis 4*. Additional resource substrates for induced phenolic accumulation are provided by enhanced metabolite import from the cytoplasm.

Rationale for Hypothesis 4.

Chloroplasts can augment stromal carbon resources by importing metabolites from the cytoplasm. A family of closely related phosphate translocators, located within the chloroplast inner envelope, transports specific metabolites across the chloroplast envelope in counter-exchange with phosphate (Flügge, 1999). Two of these translocators import metabolites which could serve as substrates for phenolic metabolism. Activity of the PEP:Pi translocator (PPT), which imports PEP into the chloroplast stroma, has recently been linked to constitutive phenolic metabolism (Streatfield et al., 1999), indicating that cytoplasmic PEP is a substrate for stromal shikimate metabolism (**Fig. 1**). Further support was provided by experiments with isolated chloroplasts demonstrating that $^{14}$C-phenylalanine could be synthesized from $^{14}C$-$CO_2$, but that $^{14}$C-phenylalanine synthesis increased dramatically when $^{14}$C-PEP was additionally supplied to the bathing chloroplast solution (Schulze-Siebert and Schultz, 1989). Whether PEP import is enhanced during induced phenolic synthesis has not been investigated. The glucose-6P:Pi translocator (GPT) imports glucose-6P into amyloplasts in support of starch synthesis but its role in chloroplast metabolism is unclear (Batz et al., 1995; Quick et al., 1995). Once inside the chloroplast, glucose-6P could be converted to E4P via reactions of the chloroplast oxidative pentose phosphate (OPP) pathway. Glucose-6P dehydrogenase, the first enzyme in the OPP pathway which uses glucose-6P as its substrate, is activated in the chloroplast in response to enemies (Sindelar et al., 1999), but whether glucose-6P is imported for induced OPP activity is unknown. Experiments addressing Hypothesis 4 were designed to investigate whether metabolite import is enhanced to provide substrates for induced phenolic metabolism when resources are limited by low light or constrained by leaf development.

*Hypothesis 5.* Induced phenolic accumulation that decreases insect fitness requires PEP:phosphate translocator activity.

Rationale for Hypothesis 5.

The *Arabidopsis ppt1-1* mutant lacks PPT activity and is unable to import PEP into chloroplasts by activity of the PPT (Streatfield et al., 1999). Constitutive phenolic synthesis is constrained by the *PPT* mutation, as *ppt1-1* mutants contained fewer hydroxycinnamic acids, flavonoids and simple phenolics when compared to wild type plants. Mutants also display constrained anthocyanin accumulation in high light growth conditions. These results are consistent with PEP imported via the PPT serving as a substrate of the chloroplast shikimate pathway, leading to constitutive phenolic accumulation. Whether PPT activity is required to supply PEP for induced phenolic metabolism has not been determined. I investigated potential ecological costs of the *PPT* mutation by subjecting wild type plants and *ppt1-1* mutants to JA treatment followed by an insect bioassay. Previous research has demonstrated variable fitness responses of *Pieris rapae* larvae to plants treated with stimuli that commonly induce phenolic metabolism. In wild radish plants, for example, JA treatment had no influence on *P. rapae* fitness (Agrawal, 1999). But UV light treatment of *Arabidopsis* plants led to increases in flavonoids and hydroxycinnamic acids with corresponding decreases in *P. rapae* growth (Grant-Petersson and Renwick, 1996). To address Hypothesis 5, I designed experiments to investigate involvement of the PPT in supplying PEP substrates for phenolic induction responses against insect herbivores.

**Research Systems and Methods Development**

*Model System: Tobacco and phenolic metabolism*

I elicited phenolic accumulation in tobacco plants with Jasmonic acid (JA), a wound hormone produced in response to insect chewing (McCloud and Baldwin, 1997). JA elicits phenolic metabolism in plant leaves much as does wounding or herbivory (Creelman and Mullet, 1997; Parthier, 1991; Rickauer et al., 1992; Thaler et al., 1996). The predominant phenolics in tobacco plants (*Nicotiana* spp.) are rutin, caffeoylquinic acids (CQA), and

caffeoylputrescine (Stitt et al., 2002; Keinänen et al., 2001). In tobacco, JA enhances gene expression for enzymes of both the SP (Suzuki et al., 1995) and PP (Ellard-Ivey and Douglas, 1996; Lee and Douglas, 1996; Sharan et al., 1998; Taguchi et al., 1988). Phenolic responses to jasmonates are idiosyncratic, with no effect on rutin levels, slight increases in CQAs, and substantial accumulations of caffeoylputrescine (Keinänen et al., 2001). Alkaloids are also induced by JA in tobacco plants, with nicotine being the dominant compound (Baldwin, 1988). Nicotine and caffeoylputrescine share a common biosynthetic precursor, putrescine, the synthesis of which depends on ornithine decarboxylase (Biondi et al., 2001) and putrescine methyltransferase (Winz and Baldwin, 2001), which are also activated by jasmonates. Tobacco growth, photosynthesis, carbohydrate metabolism, and vascular interconnections between source and sink leaves are well characterized, providing a solid foundation for this research.

To address Hypotheses 1 and 2, I designed an experiment to investigate the effects of light intensity and leaf development on induced phenolic accumulation in seven-week old (pre-flowering) tobacco (*N. tabacum)*. Following JA treatment, plants were either maintained in ambient light or shaded for three days, and young (developing) and mature leaves were collected for phenolic analyses. For Hypothesis 3, the same experimental design was employed and photosynthetic parameters (carbon assimilation, chlorophyll, and Rubisco activity) were measured on the first and last days of the experiment. To address Hypothesis 4, experiments were modified by using plants which were one week younger to increase chloroplast yield. Metabolite uptake experiments with isolated chloroplasts were conducted between 24 to 48h after JA treatment.

*Isolating intact chloroplasts from tobacco leaves*

Tobacco plants in my research system were relatively mature, contained high levels of starch, and were induced to accumulate phenolic chemicals, all factors which compromise chloroplast envelope intactness (Walker, 1980). I developed a chloroplast extraction protocol to counter the damaging potential of each of these factors. Because initial 'raw' leaf extracts contained chloroplasts with varying degrees of envelope intactness, I used a Percoll

step gradient (21%-45%-80%) to separate chloroplasts with 70-100% intact envelopes (migrating to the 45/80 interface) from damaged chloroplasts, which remained in the 21/45 interface. To reduce starch levels, plants were removed from the growth chamber early in the morning, effectively extending the night period. To counteract damage from plant lipids, oxidation, and induced phenolics, bovine serum albumin, polyvinylpyrrolidine, polyethylene glycol, iso-ascorbic acid, and diothiolthreitol were included in the Grinding Buffer (Loomis, 1974). Chloroplast yield from starting material generally decreases with plant age (Walker, 1980), so I employed two methods to increase chloroplast yield from tobacco leaves. First, I extracted multiple leaves per treatment and combined the extracts. Second, chloroplasts adhering to the sides of the centrifuge tube in the final centrifugation step of the extraction method were carefully dislodged with gentle mixing and tubes were spun an additional time, enabling a two-fold increase in chloroplast yield.

*Model System: Arabidopsis and phenolic metabolism*

I used JA to elicit phenolic accumulation in *Arabidopsis* plants, as I did for tobacco. *Arabidopsis* responds to JA treatment with enhanced gene expression for both SP (McConn et al., 1997) and PP enzymes (McConn et al., 1997; Van Poecke et al., 2001). A variety of phenolics have been detected in *Arabidopsis* seedlings, the main ones being anthocyanins (Martin et al., 2002), flavonoid glycosides and aglycones (Pelletier et al., 1999), and sinapic acid esters (Chappel et al., 1992). Anthocyanins are often the predominant phenolic class in rosette leaves, and they are also induced by JA (Feys et al., 1994). However, because *Arabidopsis* rosettes containing elevated levels of flavonoid glycosides were shown to decrease *P. rapae* fitness (Grant-Petersson and Renwick, 1995), phenolic extraction and chromatographic separation procedures were selected which were optimized for measuring these chemicals (Pelletier et al., 1999). I uncovered a disparity between the main phenolics in my extracts and published phenolic profiles. Unexpectedly, there were few, if any, flavonoid glycosides in the wild type and *PPT* mutant plants. Two factors which could explain these disparities are ecotype differences (*PPT* mutants were in the Columbia and Bensheim backgrounds while flavonoid glycosides were measured in Landsberg) and plant age. Plants in the Pelletier et al. (1999) study were young seedlings and flavonoid glycosides were the predominant phenolics in this case, but Grant-Petersson and Renwick (1995) used

plants that were 6-weeks old, comparable to our plants. While sinapic acid esters were detected, confirming their presence in Columbia plants (Chapple et al., 1992), levels were significantly lower than anthocyanins. Because mutant tissues were limited, we focused on anthocyanin responses of wild type and *PPT* mutant plants to JA treatment.

To answer Hypothesis 5, I designed an experiment to investigate constraints of the *PPT* mutation on shikimate-based responses in *Arabidopsis* plants. Two separate *PPT* mutants, one from the Columbia background (*cue1-6*, Li et al., 1995) and the other from Bensheim (*ppt1-1*, Streatfield et al., 1999) were used in this experiment to assess ecotype constraints on induction responses. Both mutants lacked *PPT* gene expression, precluding chloroplast PEP import via this translocator. Following JA treatment, plants were fed to first and second instars of *P. rapae* and insect growth responses were monitored. I also measured the accumulation of an unidentified indole compound, possibly a breakdown product of indole glucosinolates, in experiments with *Arabidopsis*. Indole glucosinolates are induced by JA in *Arabidopsis* (Brader et al., 2001), and since they are synthesized from tryptophan (Brader et al., 2001), imported PEP should serve as a substrate for their synthesis. Indole glucosinolates can influence insect feeding behaviors (Renwick and Lopez, 1999) and thus induction of indole glucosinolates could contribute to growth responses of *P. rapae* larvae.

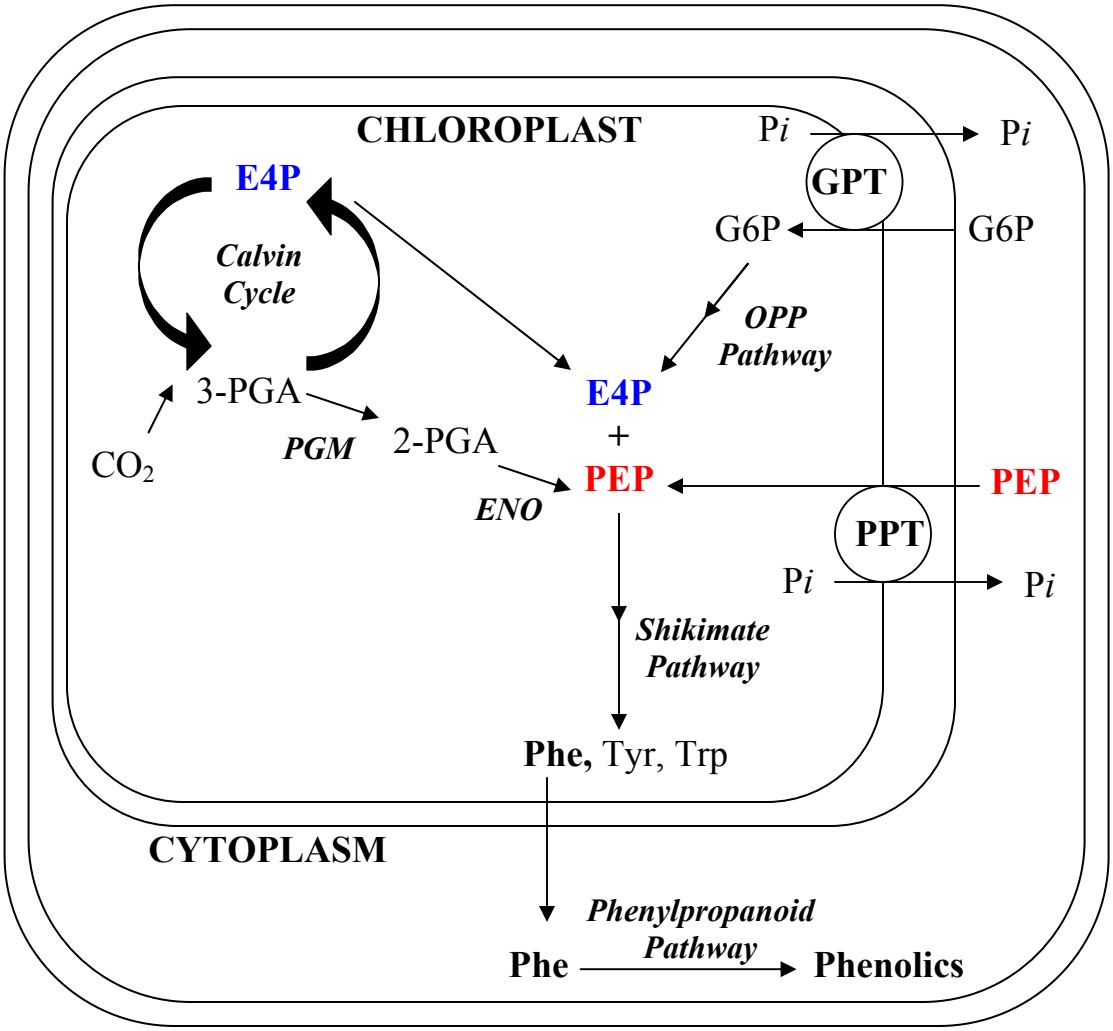


Figure 1. Possible routes of carbon resource supply to phenolic metabolism. E4P, erythrose-4-phosphate; ENO, enolase; G6T, glucose-6-phosphate; GPT, glucose-6P:P*i* translocator; OPP, oxidative pentose phosphate; PEP, phospho*enol*pyruvate; PGM, phosphoglycerate mutase; P*i*, inorganic phosphate; PPT, PEP:*Pi* translocator; Phe, 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; phenylalanine; Trp, tryptophan; Tyr, tyrosine.

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# CHAPTER 2

# LIGHT AND LEAF DEVELOPMENT CONSTRAIN PHENOLIC INDUCTION

## INTRODUCTION

Herbivorous insects and plant pathogens commonly induce plants to accumulate phenolic compounds (Karban and Baldwin, 1997). Phenolic induction responses to enemies generally require activating enzymes of the shikimate and phenylpropanoid pathways (SP and PP, respectively, see Chapter 1 for references), distinct pathways which reside in separate cellular compartments (Herrmann and Weaver, 1999, Hrazdina and Wagner, 1985). Phenolic synthesis begins within the chloroplast, where phenylalanine is synthesized in the SP from the substrates erythrose-4-P (E4P) and phosphoenolpyruvate (PEP). Following its synthesis, phenylalanine is transported to the cytoplasm, where it serves as the substrate for the PP, the core pathway from which all phenolics are synthesized (**Fig. 2.1**). During phenolic induction responses, the demand for substrates of the SP is likely to increase, however the source of these substrates is unknown. Experiments with isolated chloroplasts have demonstrated that $^{14}C$ label from photoassimilated $^{14}C$-$CO_2$ can be detected in newly synthesized phenylalanine (Schulze-Siebert and Schultz, 1989), indicating that the Calvin Cycle is a source of substrates for the SP. If the Calvin Cycle is also a source of additional substrates for induced phenolic synthesis, then Calvin Cycle activity should increase during phenolic induction events. In addition, conditions which regulate Calvin Cycle activity, including light intensity and leaf development, should also modulate resource investment in phenolic defenses.

To the best of our knowledge, only one study has investigated the ability of light to influence phenolic induction responses. Chewing damage inflicted by three species of lepidopteran larvae induced greater levels of condensed tannins (polyphenolic compounds) in leaves from *Quercus crispula* saplings growing in open gaps compared to shaded understory (Nabeshima et al., 2001). Repressed phenolic induction responses in this study could not be attributed solely to Calvin Cycle constraints imposed by lower light quantity in the shade,

however, as understory light differs from gap light in quality as well as quantity. The ratio of red to far red light is lower in the forest understory because canopy leaves preferentially absorb red light. Because red light activates chalcone synthase (Neumann and Schwemmle, 1994), a regulatory enzyme leading to condensed tannin synthesis, phenolic induction responses constrained by light quality in Nabeshima et al. (2001) could not be separated from responses modified by light quantity and hence carbon supply. To manipulate light intensity while maintaining the same ratio of red to far red light, light quantities can be altered with shade cloth and/or darkening treatments.

Responses of leaves in different developmental states are commonly considered in phenolic induction studies. Young leaves are often more inducible than old leaves (Karban and Baldwin, 1997), and enemy fitness is frequently influenced by the developmental status of target leaves (Carnegie and Colhoun, 1982; Kogel et al., 1997; Quiring, 1992; Reuveni et al., 1986; Reynolds and Smith, 1985). However few studies, if any, have elucidated a mechanistic explanation for this phenomenon. Calvin Cycle constraints imposed by leaf development are well characterized in a variety of plants. In general, carbon assimilation rates are low in the youngest and oldest leaves, peaking in mid-aged leaves which have nearly finished expanding (Peterson and Zelitch, 1982; Turgeon, 1989). We predicted that leaves with either inherently higher rates of carbon assimilation or enhanced capability to increase carbon assimilation, would provide more substrates to the SP and thus accumulate more phenolics during induced defense responses.

To clarify the roles of light and leaf development in mediating carbon resource allocation to phenolic defenses, we developed two research hypotheses. First, light intensity and leaf development constrain induced phenolic accumulation and second, photosynthetic carbon assimilation, as influenced by light and leaf development, constrains induced phenolic accumulation. We addressed these hypotheses by inducing phenolic metabolism in *Nicotiana tabacum* plants with Jasmonic acid (JA), a wound hormone commonly produced by plants in response to insect chewing (McCloud and Baldwin, 1997). Jasmonates are used routinely to induce phenolic accumulation in many plant species (Creelman and Mullet, 1997; Parthier, 1991; Rickauer et al., 1992; Thaler et al., 1996), including tobacco (Ellard-

Ivey and Douglas, 1996; Lee and Douglas, 1996; Sharan et al., 1998; Suzuki et al., 1995; Taguchi et al., 1988). We investigated the effects of JA and light availability on phenolic induction and carbon assimilation responses of young and old leaves. Specific phenolic compounds responded differently to JA treatment, however total phenolic induction was constrained in darkened plants and in low light. While young leaves were more responsive to JA, carbon assimilation was a poor predictor of resource investment in phenolic defenses, consistent with an alternate source of additional substrates for induced phenolic metabolism.

## METHODS

### Temporal Induction Experiment

*Plant Growth Conditions*

Tobacco plants (*Nicotiana tabacum* cv petite Havana SR1) were grown in a temperature-controlled greenhouse (22ºC day, 19ºC night). Natural light was supplemented with high-pressure sodium greenhouse lamps (400-watt, G3 Series, Ruud Lighting, Racine, WI), providing plants with a minimum light intensity of 165 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a 12h photoperiod. Seeds were sown in sterilized soil (Metro-Mix 200, Scotts-Sierra Horticultural Products Co., Marysville, OH) and seedlings were transferred to 10 cm by 13 cm pots containing soil supplemented with $120 g \cdot m^{-3}$ Osmocote 14:14:14 (The Geiger Companies, Harleysville, PA) 14 days after planting. Plants were watered daily and repositioned semi-weekly to randomly designated locations established with Minitab Statistical Software (Minitab Inc., State College, PA).

*Experimental Design*

We randomly assigned seven week-old plants to one of two temporal treatment times, either sunrise (07:00) or sunset (19:00). Solvent [3% (v/v) ethanol] or Jasmonic acid (5mM JA dissolved in solvent) was applied in a fine mist to the adaxial side of all leaves of randomly assigned plants. Lengths of developing (leaf 5, counting up from the plant base)

and old leaves (leaf 2) were recorded at 07:00 on the first day of treatment and again three days later. Seventy-two hours after initiating day-treatments, we ended the experiment by flash-freezing leaves in liquid nitrogen. Leaves were stored at -20ºC for subsequent chemical analysis.

**Dark Induction Experiment**

We conducted the remaining experiments in a room of a controlled atmosphere building on the main campus of The Pennsylvania State University. A 16h photoperiod created with three 1000-watt high pressure sodium greenhouse lamps (G3 Series, Ruud Lighting, Racine, WI) provided an average of 204 ± 24 SD $\mu mol \cdot m^{-2} \cdot s^{-1}$ light at plant height. Chamber temperature was maintained at 24ºC during the day and 20ºC at night. Plants were sown from seed and seedlings were transferred to pots as described above. We began the experiment when plants were seven weeks old. Treatments were initiated at the end of the light period on the first day of the experiment (day 0). JA and solvent treatments were applied in the dark to randomly selected plants as described previously. Lights remained off for the duration of the experiment. Stem height and lengths of young and old leaves were recorded on day 0 and 24, 48, and 72h after treatment. A small amount of tissue was removed from each leaf to convert leaf fresh weight to dry weight, and leaves were subsequently flash-frozen in liquid nitrogen and stored at -20ºC.

**Shade Induction Experiment**

Six-to seven-week old plants were treated with JA or solvent between 11:00 and 13:00 as previously described and were subsequently placed into unshaded (204 ± 24 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or shaded sections of the growth chamber. Shade tents constructed from PVC pipe (Lowe's, State College, PA) and 80% shade cloth (PAK Unlimited, Inc., Cornelia, GA) covered 36 plants at a time and transmitted 26 ± 4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light at plant height. Leaf lengths and stem heights were recorded 24h before treatments, immediately after treatments, and again after 72h. Old leaves (position 6-7 from bottom of plant) and young leaves (position 10-11) were flash-frozen in liquid nitrogen and stored at -20ºC. Remaining leaves,

stems, and roots (soil particles carefully removed with water) were separated and weights recorded after heated drying for one week at 75ºC. This experiment was repeated three times with different plants. Regressions between lengths (independent variable) and dry weights (dependent variable) were used to estimate beginning dry weight values for individual plant organs on day 0 of the experiment. Using these starting parameters, we estimated the specific growth rates (in mm or g dry weight) of leaves, stems, and roots over the 72h experimental period.

*Photosynthetic Carbon Assimilation Measurements*

We collected photosynthetic measurements in two of the shade induction experiments. Beginning at 10:00 on day 0 and again 72h later, we measured photosynthetic carbon assimilation (PCA) rates in young and old leaves from randomly chosen plants using a LICOR 6400 Portable Photosynthesis System (LI-COR, Lincoln, Nebraska). We used a LICOR external LED light source to provide either high light (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or shade (26 $\mu mol \cdot m^{-2} \cdot s^{-1}$), where appropriate. A section of leaf material of known area was removed from each leaf and dried. Using this conversion factor, we converted PCA rates to nmol $CO_2 \cdot g^{-1}$dry weight$\cdot h^{-1}$.

Rubisco activities were estimated from $CO_2$ response curves (A/Ci) in a subset of these plants using methods described by von Caemmerer and Farquhar (1981). We collected $CO_2$ response curves using the A/Ci program included in the LICOR 6400 software. Light levels were adjusted to reflect the average light intensity provided to the plants during the experiment, either high light (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or shade (26 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Acquiring $CO_2$ response curves required 6h for each light treatment, so we conducted these assays over two days, measuring unshaded plants 72h after treatment and shaded plants 96h after treatment. Following Rubisco measurements, leaves were flash-frozen in liquid nitrogen and stored at -20ºC.

*Chemical Analyses*

Reserved leaves stored at -20ºC were lyophilized and ground to a fine powder in a UDY Cyclone Mill (UDY Corporation, Fort Collins, CO). We measured levels of phenolics, chlorophyll, and protein from samples of this leaf powder. For each assay, values of samples and standards were collected in triplicate using a SPECTRAMax Plus microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA).

Protein was extracted from leaf samples following the procedures cited in Jones et al. (1989), using BSA as the standard. Chlorophyll was measured in 80% acetone leaf extracts according to Arnon (1949). Total phenolics were assayed by the Folin Denis assay, which was adapted for small sample size using methods described by Appel et al. (2001). Lithium sulphate (8% w/v) was added to the Folin reagent to prevent precipitate formation (Singleton and Rossi, 1965). Total phenolics were measured in 50% (v/v) MeOH leaf extracts (partitioned against hexane) using native tobacco standards extracted in a similar manner.

*HPLC Method*

Phenolic extraction and HPLC procedures were adapted from Keinänen et al. (2001). Leaf powder (10mg) was extracted twice into a total volume of 1mL of 40% (v/v) MeOH containing 0.5% (v/v) acetic acid. Each extraction involved vortexing, a 10min sonication, and centrifugation at 11,000 x g for 1min. Extracts were filtered through 0.2 µm nylon membrane SpinX Tubes (CoStar, 11,000 x g, 1min) and stored in brown glass HPLC vials at 4ºC. Our HPLC system (Waters, Bedford, MA) consisted of a WISP 710 autosampler, a photodiode array detector, a SIM control module, and Waters Millenium3.2 software. We followed the separation procedures of Keinänen et al. (2001), using acidified water (adjusted to pH 2.2 with phosphoric acid) and acetonitrile. We separated phenolics and nicotine on an Inertsil ODS-3 RP column, 3µm, 150x 4.6mmm i.d., monitoring eluent between 190-400nm. Nicotine and caffeoylquinic acid identities (chlorogenic acid, cryptochlorogenic acid, and neochlorogenic acid) were confirmed with nicotine and chlorogenic acid standards in addition to comparing our elution times with those published in Keinänen et al. (2001).

Caffeoylputrescine was confirmed by LC/MS using a Quattro II mass spectrometer (Micromass, Beverly, MA). Analyses were performed using atmospheric pressure chemical ionization (APCI) in positive ion mode. The following ions were observed: m/z 251 ($[M+H]^+$), 234 ($[M+H-NH_3]^+$), and 163 (caffeoyl cation). Nicotine was quantified at 254nm and phenolics (caffeoylputrescine, chlorogenic acid, cryptochlorogenic acid, neochlorogenic acid) at 320nm.

*Statistical Analyses*

We used the general linear model procedure (SAS Institute, Cary, NC) to assess the influence of various treatment factors on phenolic and photosynthetic response variables. When models produced a significant result ($p<0.05$), we used the Tukey Lines procedure to assign significant differences across treatment groups. Regressions were conducted with the Correlation procedure.

## RESULTS

### Temporal Treatments and Phenolic Induction

In this experiment, we measured phenolic induction responses in plants treated either in the light or dark and subsequently maintained on a diurnal light/dark cycle in a greenhouse. Phenolic responses in this experiment pertain to the final levels measured three days after treatment. Phenolics were strongly induced by JA (**Fig. 2.3A**, F=116.6, $p<0.0001$). Induction responses were constrained by leaf development; young leaves were more inducible than old leaves, attaining final levels which were 35% greater than controls (young) versus 19-29% (old) (**Fig. 2.3A**, F=155.8, $p<0.0001$). Phenolic induction responses were similar for both light- and dark-treated plants (**Fig. 2.3A**, F=3.19, p=0.078). Young leaves were growing (in length extension) eight times faster than old leaves at the start of the experiment (data not shown). JA decreased leaf growth rates of young leaves by 30% during the three-day treatment period but old leaf growth rates did not change in response to treatment (**Fig. 2.3B** , F=16.59, p=0.0001).

**Dark Treatment and Phenolic Induction**

In this experiment, plants were reared in a growth chamber (16h photoperiod) for seven weeks. At the end of the light period on the first day of the experiment, plants were treated with JA and solvent in the dark. Induced responses are represented by the final phenolic levels attained 72h after treatments were initiated. JA treatment resulted in small elevations in total phenolic levels in darkened plants ($F=10.68$, $p=0.0038$, **Fig. 2.2A**), but in contrast with the temporal induction experiment, phenolics were induced to a similar extent in young (14%) and old leaves (11%) over the control levels ($F=0.41$, $p=0.53$). During the experiment, leaf and stem growth (extension rates) continued even in the absence of light (**Fig. 2.2B**). JA decreased stem height growth 60% (**Fig. 2.2C**, $F=47.79$, $p<0.0001$) but had no effect on leaf length extension (**Fig. 2.2B**, $F=1.32$, $p=0.26$).

**Shading and Phenolic Induction**

We carried out three replicate experiments with different groups of plants to investigate the effects of light intensity and leaf development on JA-induced phenolic accumulation in tobacco. Because the quantitative levels of phenolics and photosynthetic parameters varied between replicates, data for individual experiments are presented separately. Qualitative responses to JA treatment were comparable between replicate experiments. In these experiments, phenolic induction was measured as a change in the levels of phenolics over the duration of the experiment (72h). In addition, we measured photosynthetic parameters to investigate relationships between light and developmentally-regulated carbon assimilation rates and phenolic induction responses.

*Starting leaf characteristics*

At the start of the experiment, plants were 25-30cm tall and had 10-12 leaves which were 10cm in length or longer. Young leaves were 40-45% smaller (length), weighed 70% less (dry weight), and were growing 12 times faster than were old leaves (data not shown). Photosynthetic carbon assimilation (PCA) rates were developmentally regulated (**Fig.**

**2.4A,D**). The young leaves had not reached peak PCA rates by the first day of the experiment; whereas the older leaves were photosynthetically mature. Leaves that were more mature than the older leaf displayed lower rates of PCA, consistent with leaf senescence (Smart, 1994). Rubisco activity was lower in young leaves (**Fig. 2.4B,E**). Chlorophyll levels increased with maturity and declined with age, peaking in leaves which were just past peak PCA rates (**Fig. 2.4C,F**). Constitutive phenolic levels were lowest in young leaves and increased with leaf age (**Fig. 2.5A,B**).

*Phenolic responses*

High light promoted greater levels of phenolic induction than shade over the three-day treatment period (**Fig. 2.6A**, $F=46.4$, $p<0.0001$; **Fig. 2.6B**, $F=10.2$, $p=0.0026$; **Fig. 2.6C**, $F=41.8$; $p<0.0001$). Development also influenced phenolic accumulation; young leaves accumulated more phenolics than old leaves (**Fig. 2.6A**, $F=50$, $p<0.0001$; **Fig. 2.6B**, $F=35.7$, $p<0.0001$; **Fig. 2.6C**, $F=5.05$, $p=0.0269$). JA treatment strongly induced phenolic accumulation in all three replicates of this experiment (**Fig. 2.6A**, $F=153.7$, $p<0.0001$; **Fig. 2.6B**, $F=61.5$, $p<0.0001$; **Fig. 2.6C**, $F=54.4$; $p<0.0001$); however, the magnitude of phenolic induction was strongly influenced by light intensity and leaf development. Phenolic induction was greater in plants maintained in high light conditions compared to shaded plants in two out of three replicates of this experiment (**Fig. 2.6A**, $F=3.05$, $p=0.0824$; **Fig. 2.6B**, $F=29.47$, $p<0.0001$; **Fig. 2.6C**, $F=16.29$; $p=0.0001$). When root growth was included as a covariate in the model, phenolic induction in the third replicate experiment was greater in high light than in shade ($F=5.81$, $p=0.0169$). Young leaves were more sensitive to JA and accumulated higher levels of phenolics than were old leaves (**Fig. 2.6A**, $F=34.8$; $p<0.0001$; **Fig. 2.6B**, $F=4.32$, $p=0.043$; **Fig. 2.6C**, $F=5.00$, $p=0.027$).

Of the two phenolic groups present in leaves of *N. tabacum*, caffeoylputrescine (CP) was the only phenolic to be induced by JA in two replicates of this experiment (**Fig. 2.7A**, $F=46.13$, $p<0.0001$; **Fig. 2.7B**, $F=27.18$, $p<0.0001$). Young leaves accumulated more CP than did old leaves, similar to the total phenolic pattern (**Fig. 2.7A**, $F=27.8$, $p<0.0001$; **Fig. 2.7B**, $F=15.5$, $p=0.0003$). CP induction was greater in high light for one replicate but similar

between high light and shaded plants in the other experimental replicate (**Fig. 2.7A**, F=11.3, p=0.0017; **Fig. 2.7B**, F=0.23, p=0.63), a result which was consistent with total phenolic responses. Caffeoylquinic acids (CQAs: chlorogenic, cryptochlorogenic, and neochlorogenic acid) comprised the second major phenolic group in *N. tabacum* leaves. Chlorogenic acid, the most abundant CQA, was not induced by JA but high light stimulated its accumulation (**Fig. 2.8A**, F=26, p<0.0001; **Fig. 2.8D**, F=22.7, p<0.0001). Cryptochlorogenic acid levels were repressed by JA in one replicate of this experiment (**Fig. 2.8B**, F=0.12, p=0.73; **Fig. 2.8E**, F=4.82, p=0.034). Similarly, neochlorogenic acid was also decreased by JA (**Fig. 2.8C**, F=3.18, p=0.082; **Fig. 2.8FH**, F=14.8, p=0.0004), with losses in older leaves being marginally greater than in younger leaves in the one replicate (**Fig. 2.8C**, F=1.06, p=0.31; **Fig. 2.8F**, F=3.11, p=0.0856).

*Nicotine*

Young leaves accumulated more nicotine than did old leaves in both replicates of this experiment (**Fig. 2.9A,** F=22.99, p<0.0001; **Fig. 2.9B**, F=26.25, p<0.0001). While JA induced nicotine in only one experimental replicate (**Fig. 2.9A**, F=2.31, p=0.1366; **Fig. 2.9B**, F=64.83, p<0.0001), young leaves accumulated higher levels of nicotine over the 72h treatment period than did old leaves in both experimental replicates (**Fig. 2.9A**, F=6.78, p=0.0129; **Fig. 2.9B**, F=7.15, p=0.0108).

*Growth responses*

JA decreased rates of leaf extension in young leaves, but old leaf growth was insensitive to treatment (**Fig.2.10A**, F=27. 9, p<0.0001; **Fig. 2.10B**, F= 18.7, p<0.0001). Leaf dry mass accumulation was unaffected by JA treatments (**Fig. 2.10C**, F=1.28, p=0.27). JA strongly decreased rates of stem extension (**Fig. 2.10D**, F=115, p<0.0001; **Fig. 2.10E**, F=44.9, p<0.0001) and dry mass accumulation (**Fig. 2.10F**, F=27.7, p<0.0001). While low light only moderately repressed extension rates of leaves (**Fig.2.10A**, F=9.69, p=0.0021; **Fig. 2.10B**, F= 13.27, p=0.0004) and stems (**Fig. 2.10D**, F=5.69, p=0.0189; **Fig. 2.10E**, F=7.92,

p=0.007), dry mass accumulation was strongly inhibited in shaded leaves (**Fig. 2.10C**, F=99.9, p<0.0001) and stems (**Fig. 2.10F**, F=85.1, p<0.0001).

*Photosynthetic responses*

Shading reduced PCA by 80-85% (Fig. **2.11A**, F=161.8, p<0.0001; Fig. **2.11D**, F=234.3, p<0.0001), and Rubisco activity by 80-85% (**Fig. 2.11B**, F=267.5, p<0.0001; **Fig. 2.11E**, F=383.8, p<0.0001). Chlorophyll levels, however, were enhanced by 15-50% in the shade (**Fig. 2.11C**, F=43.4, p<0.0001; **Fig. 2.11F**, F=63.6, p<0.0001). On the last day of the experiment, PCA rates in young and old leaves were not statistically different (**Fig. 2.11A**, F=0.11, p=0.74; **Fig. 2.11D**, F=0.93, p=0.34), but Rubisco activity was slightly higher in young leaves in one replicate of this experiment (**Fig. 2.11B**, F=7.13, p=0.0168; **Fig. 2.11E**, F=1.04, p=0.3228). Old leaves had higher levels of chlorophyll than did younger leaves (**Fig. 2.11C**, F=27.7, p<0.0001; **Fig. 2.11F**, F=98.1, p<0.0001).

In response to JA treatment, PCA decreased 15-35% in one experimental replicate (**Fig. 2.11A**, F=5.84, p=0.0197; **Fig. 2.11D**, F=0.97, p=0.327) and in this case only unshaded leaves were sensitive to JA (**Fig 2.11A**, F=6.67, p=0.0137). Rubisco activity was also repressed by JA treatment (**Fig. 2.11B**, F=21.52, p=0.0003; **Fig. 2.11E**, F=8.93, p=0.0087), again only in unshaded leaves (**Fig. 2.11B**, F=20.19, p=0.0004; **Fig. 2.11E**, F=11.88, p=0.0033). JA decreased chlorophyll levels (**Fig. 2.11C**, F=25.5, p<0.0001; **Fig. 2.11F**, F=30.3, p<0.0001), and this effect was more pronounced in young leaves (**Fig. 2.11C**, F=7.02, p=0.011; **Fig. 2.11F**, F=16.91, p<0.0001). Phenolic accumulation in young unshaded leaves was negatively correlated with PCA (**Fig. 2.12A**, p=0.0007; **Fig. 2.12B**, p=0.027).

# DISCUSSION

## Phenolic Induction is Inhibited in the Dark

Tobacco plants induced very low levels of phenolics, if any, when plants were treated with JA in the dark and subsequently maintained in the dark for three days. In a similar period of time, JA promoted significant phenolic induction in plants treated in either the light or dark but maintained on a diurnal light/dark cycle. Young soybean seedlings were similarly incapable of accumulating phenolics (anthocyanins) in response to jasmonates in the dark (Franceschi and Grimes, 1991). To investigate the possibility that low phenolic induction in our system resulted from an inability of plants to perceive JA in the dark, we compared growth responses of darkened plants with those receiving light following JA treatment. In darkened plants, JA repressed stem growth by 60%, similar to growth decreases experienced by plants maintained in low light after JA treatment. These results are consistent with the ability of darkened plants to perceive JA and elicit physiological responses in the dark which are comparable to plant responses in the light (He et al., 2002). However, phenolic induction responses following JA perception are severely limited by the absence of light.

In the temporal phenolic induction experiment, plants were treated with JA either in the dark (after dusk) or in the light (at dawn) and subsequently maintained under diurnal conditions of 12h light/ 12h dark. This experiment differed from the dark-induction experiment by allowing dark-treated plants to receive light during the day. Results from this experiment showed that phenolic induction and growth repression responses to JA were the same independent of the time of day in which JA treatments were applied. These results indicate that perception of JA in the dark can be temporally uncoupled from a strong phenolic induction response, which requires light. While induction responses occur slowly in some species, often requiring days before induced phenolics are detected (Karban and Baldwin, 1997), other plants are quite capable of accumulating defenses within hours of an insect attack (Zangerl et al., 1997). Thus, nocturnal feeding behaviors (De Moraes et al.,

2001) may be an adaptation allowing insects to temporally escape defense responses in plants which normally would accumulate toxic phenolics within hours of a daytime attack.

**Shading Constrains Phenolic Induction**

Compared to plants growing in high light, shaded plants are often more susceptible to enemy damage (Vergeer and van der Velde, 1997; Wiltshire, 1956), which has been attributed to greater carbon resource investment in constitutive phenolic defenses in high light (Buttery et al., 1992; Conti et al., 1983; Vergeer and van der Velde, 1997). We hypothesized that induced phenolic synthesis would be similarly constrained by light intensity. To the best of our knowledge, we are presenting the first definitive data showing that low light restricts carbon resource investment in induced phenolic compounds. Thus, for plants employing either a strong constitutive phenolic defense strategy or for plants relying on phenolic induction, light can play a critical role in modulating the defense response. For plants growing near neighbors, light limitations may be even more restrictive, as lower levels of red light may additionally constrain activities of key enzymes in phenolic metabolism leading to flavonoid synthesis (Huub et al., 1997; McClure, 1974). It is interesting that oaks and maples (Jack Schultz, personal communication) induce hydrolysable tannins, phenolic compounds that do not require activity of enzymes of the PP or flavonoid metabolism, perhaps as an adaptation to low light quantity and quality conditions in the understory.

**Leaf Development Constrains Phenolic Induction**

While leaf age commonly influences insect success and pathogen virulence, a clear preference for developing or old leaves is not evident from the literature (Carnegie and Colhoun, 1982; Kogel et al., 1997; Quiring, 1992; Reuveni et al., 1986; Reynolds and Smith, 1985). How enemies choose a target leaf age class is species-dependent and can be influenced by levels of other chemicals that are also influenced by leaf age, including protein (Mattson, 1980). We hypothesized that leaf development would constrain induced phenolic accumulation. Developmental complexities of phenolic defenses were confirmed by our measures of both constitutive and induced phenolics. On the first day of the experiment, the

youngest leaves contained the lowest levels of constitutive phenolics and the highest levels of proteins, characteristics which could make these leaves highly palatable to insects (Mattson, 1980). However, young leaves were also the most inducible, mobilizing resources into both phenolic and nicotine defenses more readily than older leaves. These data indicate that even though leaves may lose the ability to be induced as they age, higher investment of resources in constitutive defenses may afford an alternate defense mechanism. Phenolic induction responses in young leaves were strongly inhibited by shading, however, showing that for tobacco plants, at least, defense responses in developing leaves can be constrained by low light resources. These results are inconsistent with the optimal defense theory of carbon allocation to defenses, which states that plants allocate resources toward defending the most valuable organs, in terms of the plant's future fitness (Rhoades, 1979).

**Phenolic Induction Responses do not Require Enhanced Photosynthetic Rates**

Demand for substrates of induced phenolic metabolism is likely to increase when plants are confronted by enemies. We hypothesized that photosynthesis would increase in response to JA treatment if the Calvin Cycle was the source of additional substrates for phenolic induction responses in tobacco. On the contrary, none of the photosynthetic parameters that we measured was enhanced by JA treatment, and the greatest decreases in PCA, Rubisco activity, and chlorophyll levels occurred in the most inducible (young, unshaded) leaves. In these leaves, JA decreased $CO_2$ assimilation to 0.17 $nmol \cdot mg^{-1}$dry $weight \cdot h^{-1}$, which is equivalent to 0.15 µg $C \cdot mg^{-1}$dry weight for the 72h following treatment. In the same time period, caffeoylputrescine (CP) levels were induced to 5.5 $\mu g \cdot mg^{-1}$dry weight (or 22 nmol caffeic acid $equivalents \cdot mg^{-1}$dry weight), which is equivalent to 2.4 µg carbon in the caffeic acid backbone. At this level of induction, PCA could support only 6% of the additional carbon resources required for induced caffeic acid synthesis, assuming that all assimilated carbon was allocated to induced caffeic synthesis associated with caffeoylputrescine. In shaded young leaves, PCA rates would support only 1% of induced caffeoylputrescine synthesis. These data indicate that the Calvin Cycle is not the primary source of additional substrates for induced phenolic metabolism. In fact, carbon resources allocated to induced phenolics may actually be decreased during phenolic induction

responses, when compared to resources allocated to constitutive phenolics (Zangerl et al., 1997).

PCA commonly decreases when plants are confronted with insects or plant pathogens and viruses (Hibberd et al., 1996; Larson, 1998; Zangerl et al., 2002), a response which may be attributed to either decreased synthesis or enhanced degradation of Rubisco and chlorophyll (Almasi et al., 2000; Cavalcante et al., 1999; Kombrink and Halbrock, 1990; Tsuchiya et al., 1999). JA decreased Rubisco activity and chlorophyll levels in tobacco; however responses were modulated by light and leaf development. Responses of the light reactions (chlorophyll) and dark reactions (Calvin Cycle) of photosynthesis to JA treatment in shaded plants were uncoupled in our system. The lower activities of PCA and Rubisco in shaded plants were not decreased further by JA. In contrast, chlorophyll levels increased in the shade, but JA prevented this increase in young leaves. Increases in chlorophyll levels may facilitate greater light harvesting as plants adapt to low light (Shainberg et al., 1999); thus our results indicate that shade acclimation may be compromised when plants are under enemy attack.

**What is the Source of Substrates for Induced Phenolics?**

Phenylalanine is the precursor for phenolics synthesized via the PP (**Fig.2.1**). The fact that phenylalanine is also used to build proteins leads to the suggestion that metabolic pathways for phenolic and protein synthesis compete for the same pool of phenylalanine (Jones and Hartley, 1999). If this were the case in our system, then an alternate substrate source for induced phenolic synthesis could be created by increasing phenylalanine allocation to phenolic metabolism at the expense of its allocation to protein metabolism and hence growth-related processes. We conducted linear regression analyses and found no consistent relationships between leaf growth parameters (changes in leaf extension rates, leaf dry mass or protein levels) and phenolic induction responses (changes in phenolic levels) during the 72h following JA treatment (data not shown). These results may be interpreted in one of two ways. First, phenylalanine was not being allocated to the synthesis of phenolic compounds at the expense of leaf growth processes. Second, it is possible that cytoplasmic

phenylalanine levels were being replenished from stored reserves. Phenylalanine can be transported into the vacuole after its synthesis in the chloroplast (Asami et al., 1985; Homeyer et al., 1985). Increased phenylalanine transport from the vacuole to the cytoplasm could facilitate maintenance of sufficient phenylalanine for both growth and defense metabolism. Because the vacuole also stores sucrose (Asami et al., 1985), it could be informative to investigate the role of the vacuole as a source of carbon resources for plant stress responses in general.

The predominant phenolics in *N. tabacum* are caffeoylputrescine (CP) and caffeoylquinic acids (CQAs) (Keinänen et al., 2001), which are comprised of chlorogenic acid and its two isomers, cryptochlorogenic acid and neochlorogenic acid. It is noteworthy that CP and CQA responded quite differently to JA treatment; CP was induced while CQAs were either unaffected by JA or decreased in shaded leaves. We measured variable levels of both total phenolic induction and CP induction in shaded young leaves, while at the same time the degree of CQA decreases in shaded old leaves was also inconsistent. These variable responses in shaded leaves complicated our interpretations of the role of light intensity in constraining phenolic induction. Because CP and CQAs share a common chemical moiety, caffeic acid, we wondered if CQAs could be serving as a source of caffeic acid for CP synthesis. We conducted linear regressions and measured a strong negative relationship between CQA levels in old leaves and CP levels in young leaves of shaded plants (data not shown). CQAs may serve as a substrate for induced phenolic synthesis in young leaves, giving plants a flexibility to induce phenolic metabolism in the shade.

At the beginning of the shade induction experiment, young leaves were only half the length of old leaves and had a high specific growth rate. Young leaves were also photosynthetically immature, having less chlorophyll and lower PCA rates and Rubisco activities than old leaves. In contrast, old leaves were nearly finished growing and had just attained photosynthetic maturity. These characteristics are consistent with young leaves in our experiment being carbon resource sinks and old leaves being sources (Turgeon, 1989). In addition, the location of both the source and sink leaf on the same side of the plant in our experiment makes it likely that there were direct vascular connections between these leaves

in tobacco (Jones et al., 1959; Shiroya et al., 1961). Support for our hypothesis is provided by the results from a recent study in poplar. Arnold and Schultz (2002) demonstrated that source leaves provided carbon resource to sink leaves during phenolic induction events, and that the imported carbon ended up in condensed tannins which were induced in the sink leaves. It is possible that phenolics in source leaves were transported to supply substrates for induced CP accumulation, as has been suggested to occur for phenolic induction responses in wild wheat (Gianoli and Niemeyer, 1997). While we are unaware of caffeic acid or CQA transport in plants, quinic acid (Kluge, 1964) and hydroxamic acid glucoside (Givovitch et al., 1994) have been detected in phloem, setting a precedent for transport of shikimate pathway products between source and sink leaves. Carbon resource transport may be a mechanism allowing plants to overcome shade and developmental limitations on *de novo* phenolic synthesis in plant-enemy interactions.

In the experimental replicate in which CP induction in young leaves was not constrained by shade, we also detected JA-induced nicotine accumulation. Nicotine and CP metabolism share a common substrate in their synthesis. Putrescine synthesis via putrescine methyltransferase (PMT) occurs in roots and is one of the regulatory steps in nicotine biosynthesis. Because PMT activities increase when tobacco plants are treated with jasmonates (Winz and Baldwin, 2001), we would expect to see enhanced levels of putrescine during inducible nicotine responses. While we did not measure putrescine levels in our experiment, we would imagine that nicotine induction in young shaded leaves reflected a higher abundance of putrescine, which also may have allowed greater levels of CP induction in this replicate. Alkaloids are the predominant inducible chemical defense in tobacco plants, so the phenolic defenses adopted by *Nicotiana* spp. appear to build upon a foundation of N-based chemical defenses. Recent evidence demonstrates that constitutive levels of chlorogenic acid and nicotine are tightly linked in tobacco plants and are affected by both the carbon and nitrogen economy of the plant (Matt et al., 2002). Thus, it would not be surprising if induced alkaloid and phenolic defenses were also coordinately regulated.

**Summary and Conclusions**

The source of carbon resources for induced phenolic compounds remains a mystery. Because phenolic levels are often elevated under high light conditions, it has been assumed that high carbon availability, as modulated by light intensity, regulates resource investment in phenolic synthesis. However, our data suggest that light constraints on phenolic induction responses are not mediated by limitations of PCA imposed by low light. In addition, developing leaves which had not attained photosynthetic maturity at the time of JA treatment were the most inducible. Taken together, these data indicate that additional resources for induced phenolic synthesis can be derived from sources other than the Calvin Cycle. By investigating the qualitative phenolic responses to JA treatment, we have revealed an interesting correlation between decreased levels of caffeic-acid based phenolics (CQAs) in old leaves with increased caffeic-acid based phenolics (CPs) in developing leaves. To determine if CQAs in source leaves can serve as sources of caffeic acid for induced phenolic synthesis in sink leaves, it could be productive to investigate the transport characteristics of caffeic acid derivatives. While imported cytosolic metabolites are potential alternative sources of substrates for phenolic metabolism in the chloroplast, whether they supply resources for induced phenolic synthesis remains unknown.

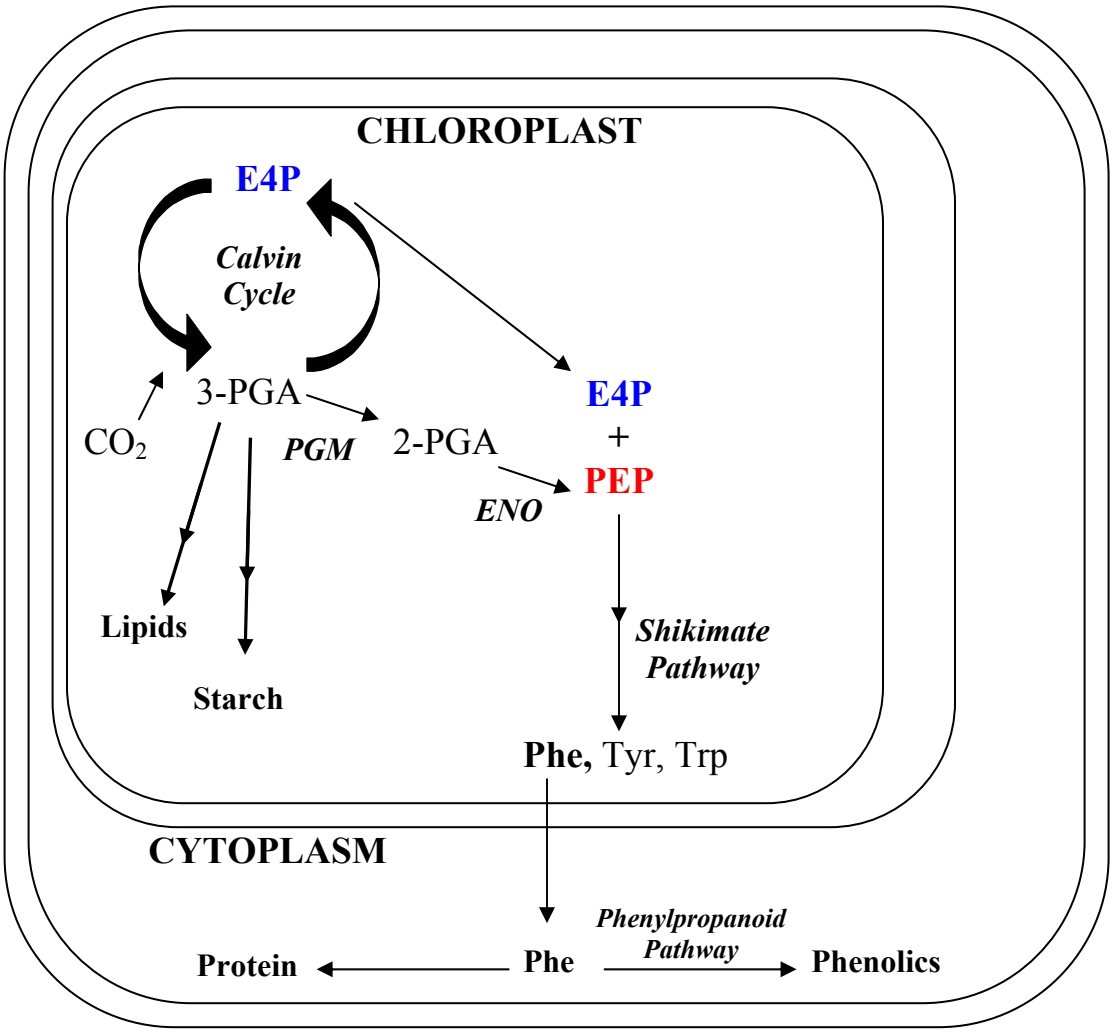


Figure 2.1. Possible routes of carbon resource supply to phenolic metabolism. E4P, erythrose-4-p; ENO, enolase; PEP, phosphoenolpyruvate; PGM, phosphoglycerate mutase; Pi, inorganic phosphate; PPT, PEP:Pi translocator; Phe, 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; phenylalanine; Trp, tryptophan; Tyr, tyrosine; XPT, xylulose-5P; XPT, xylulose-5P:Pi translocator,

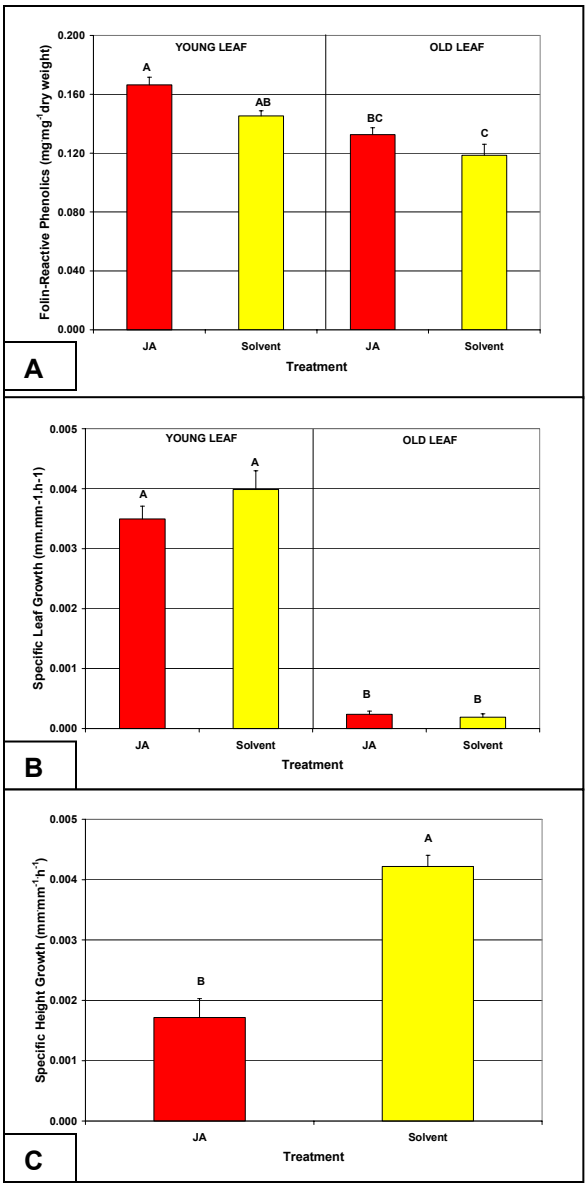


Figure 2.2. Folin reactive (total phenolic) levels (A), leaf growth (B) and stem growth rates (C) of tobacco 72h after Jasmonic acid (JA) treatment. Following treatment, plants were maintained in the dark for 72h. Means (± standard error) with the same letter are not significantly different (p=0.05, Tukey test). Solvent, 3% (v/v) ethanol; JA, 5mM dissolved in solvent. N=5-6 plants per treatment.

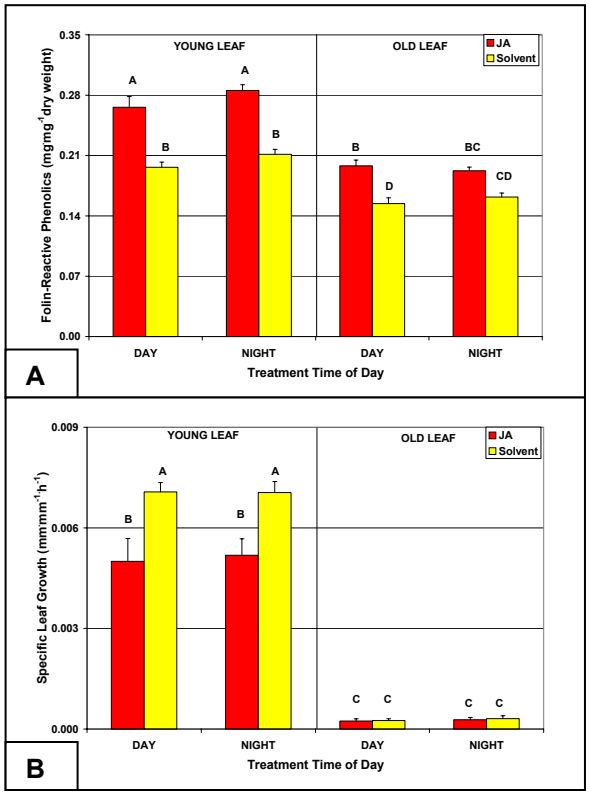


Figure 2.3. Folin reactive (total phenolic) levels (A) and specific growth rates (B) of young and old leaves of seven week-old tobacco three days after treatment. Solvent [3% (v/v) ethanol] or Jasmonic acid (JA, 5mM dissolved in solvent) treatments were applied in the morning or at night and experiments terminated after three days (72h for morning and 60 for night treatments). Means (± standard error) with the same letter are not significantly different (p=0.05,Tukey test). N=9-10 plants per treatment.

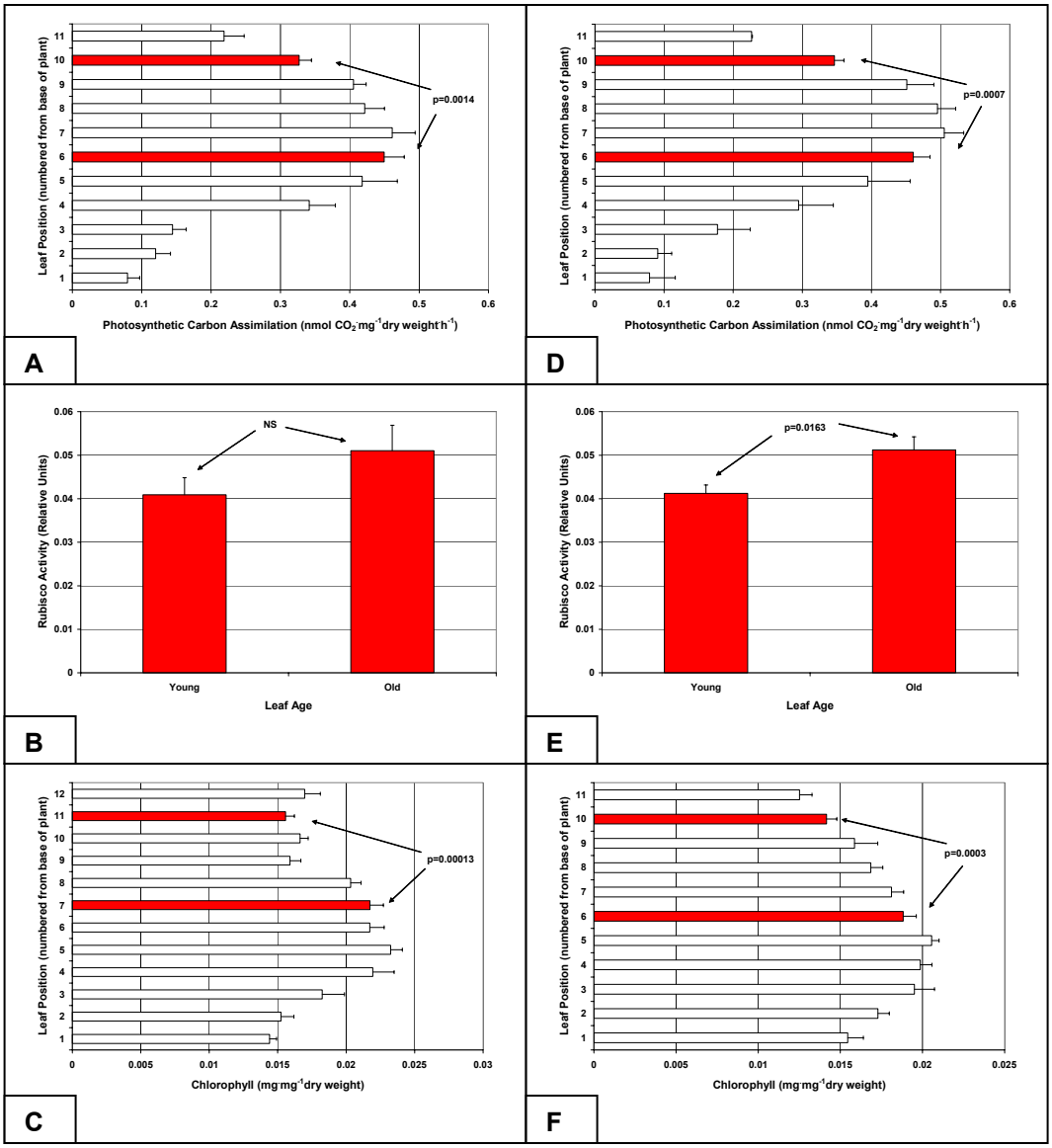


Figure 2.4. Whole plant profiles of photosynthetic carbon assimilation (PCA; A, D), Rubisco (B, E), and chlorophyll (C, F) in seven week-old tobacco plants. Data were collected in replicate experiments (Oct99, A-C; Sept00, D-F). Values represent the mean ± standard error of 5-8 measurements per treatment (PCA, chlorophyll, protein) or three replicates per treatment (Rubisco). Positions 10/11 and 6/7 correspond to young and old leaves, respectively. Statistical differences estimated with one-tailed Student's t-test.

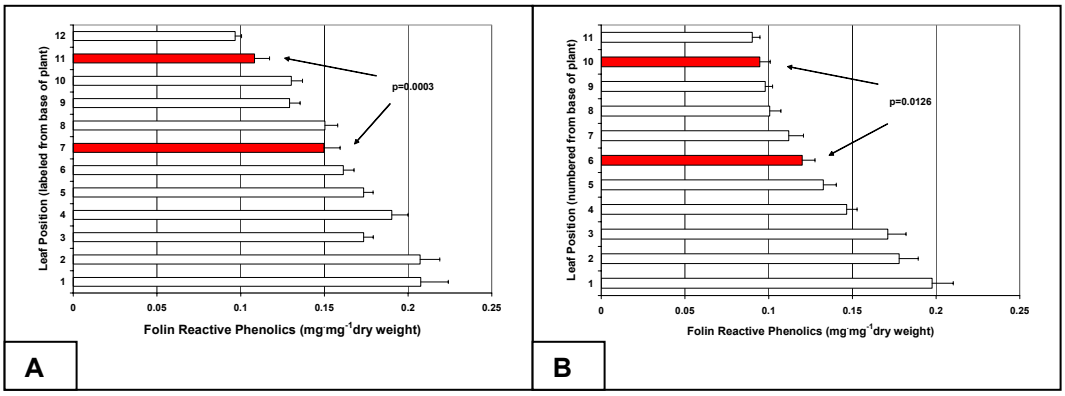


Figure 2.5. Whole plant profiles of Folin-reactive total phenolics in seven week-old tobacco plants. Data were collected in replicate experiments (Oct99, A; Sept00, B). Values represent the mean ± standard error of 5-8 replicates per treatment. Positions 10/11 and 6/7 correspond to young and old leaves, respectively. Statistical differences estimated with one-tailed Student's t-test.

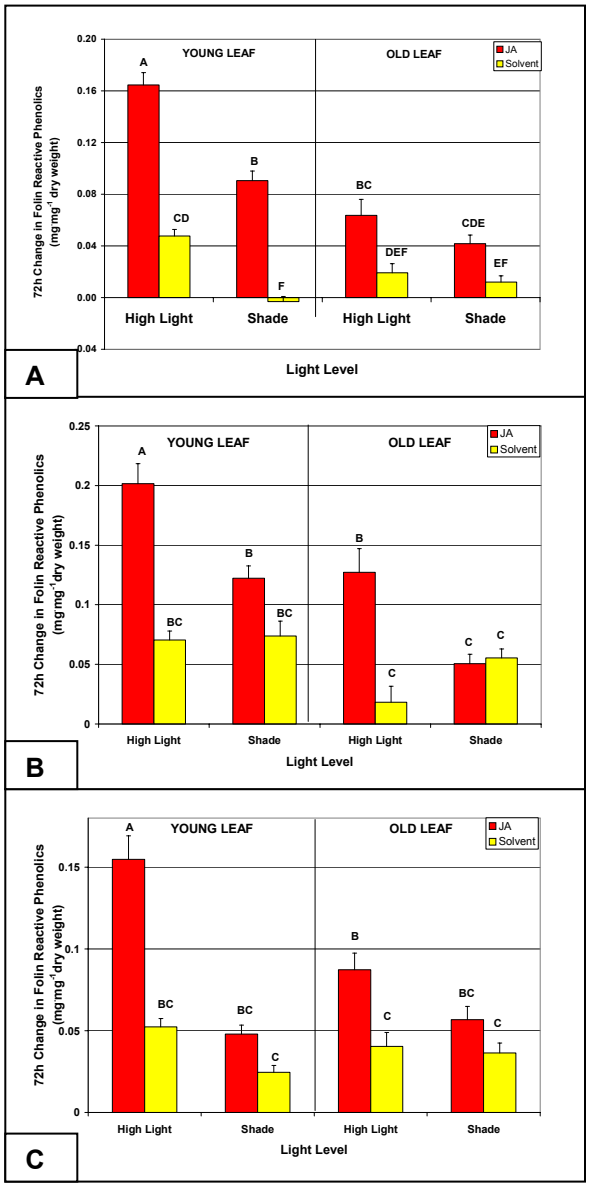


Figure 2.6. Folin-reactive (total phenolic) compounds in young and old leaves from seven week-old tobacco plants. Mean values ± standard error represent the change in phenolic levels three days after treatment. Means with the same letter are not significantly different ($p=0.05$, Tukey test). Solvent [3% (v/v) ethanol], Jasmonic acid (JA, 5mM dissolved in solvent). High light, 204µmol·m$^{-2}$·s$^{-1}$; shade, 26µmol·m$^{-2}$·s$^{-1}$. A. Nov98 (N=22-27 per treatment); B. Oct99 (N=6-7); C. Sept00 (N=10-15).

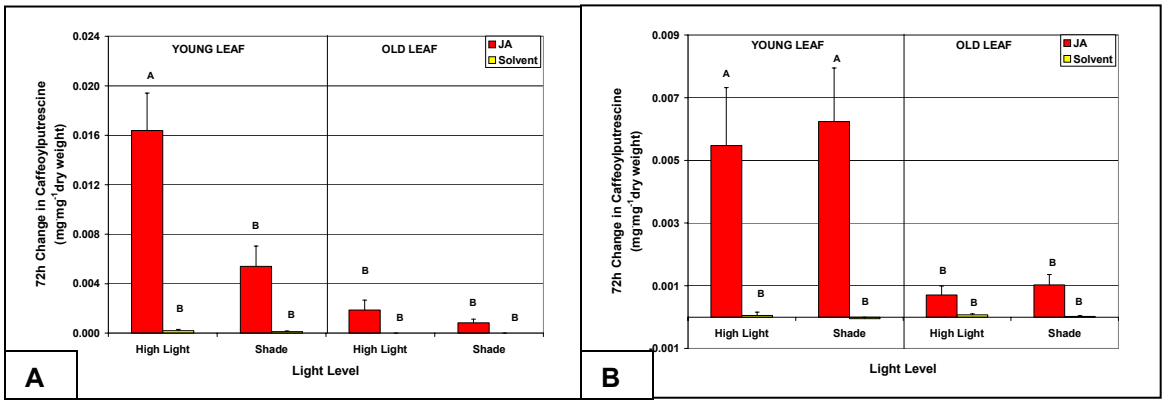


Figure 2.7. Net change in levels of caffeoylputrescine (A,B ) in young and old leaves of seven week-old tobacco leaves over the three days following Jasmonic acid (JA) treatment. Means (± standard error ) with the same letter are not significantly different (p=0.05, Tukey test). Solvent [3% (v/v) ethanol], JA (5mM dissolved in solvent). High light, 204µmol·m$^{-2}$·s$^{-1}$; shade, 26µmol·m$^{-2}$·s$^{-1}$. (A, Nov98; B, Oct99). N=6 plants per treatment.

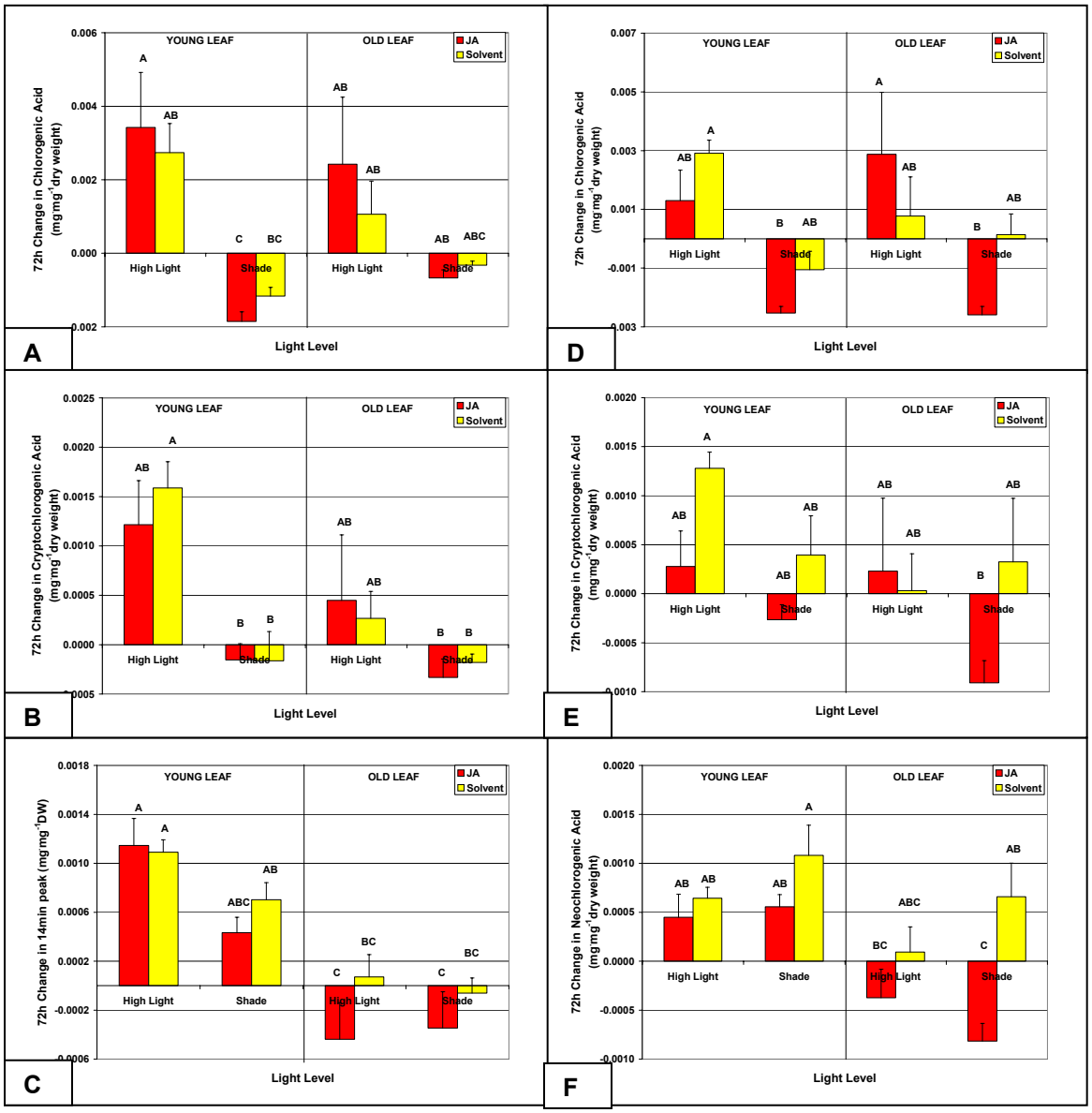


Figure 2.8. Net change in levels of chlorogenic acid (A, D), cryptochlorogenic acid (B, E), and neochlorogenic acid (C, F) in young and old leaves of seven week-old tobacco leaves over the three days following Jasmonic acid (JA) treatment. Means (± standard error ) with the same letter are not significantly different (p=0.05, Tukey test). Solvent [3% (v/v) ethanol], JA (5mM dissolved in solvent). High light, 204µmol·m$^{-2}$·s$^{-1}$; shade, 26µmol·m$^{-2}$·s$^{-1}$. (A-C, Nov98; D-F, Oct99). N=6 plants per treatment.

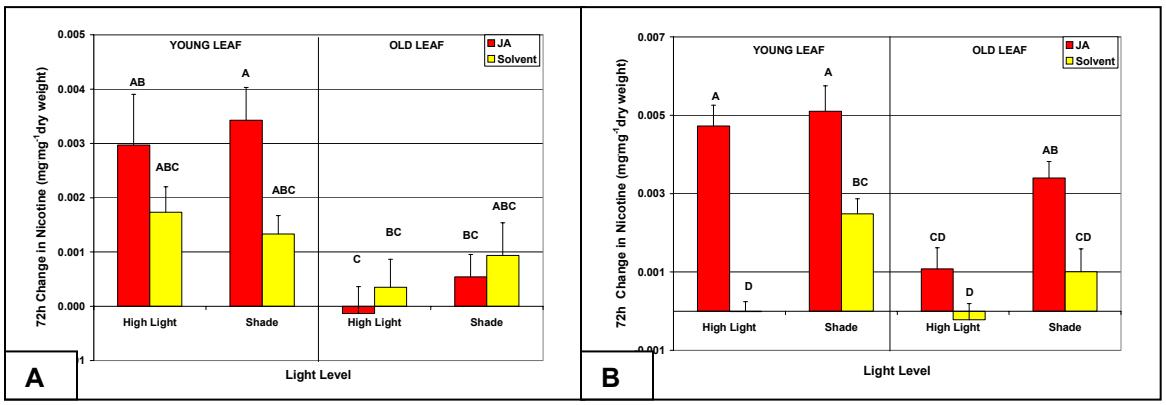


Figure 2.9. Net change in levels of nicotine (A, B) in young and old leaves of seven week-old tobacco leaves over the three days following Jasmonic acid (JA) treatment. Mean values ± standard error represent the net change over the 72h treatment period. Means (± standard error) with the same letter are not significantly different (p=0.05, Tukey test). Solvent [3% (v/v) ethanol], JA (5mM dissolved in solvent). High light, 204µmol·m$^{-2}$·s$^{-1}$; shade, 26µmol·m$^{-2}$·s$^{-1}$. (A-, Nov98; B, Oct99). N=6 plants per treatment.

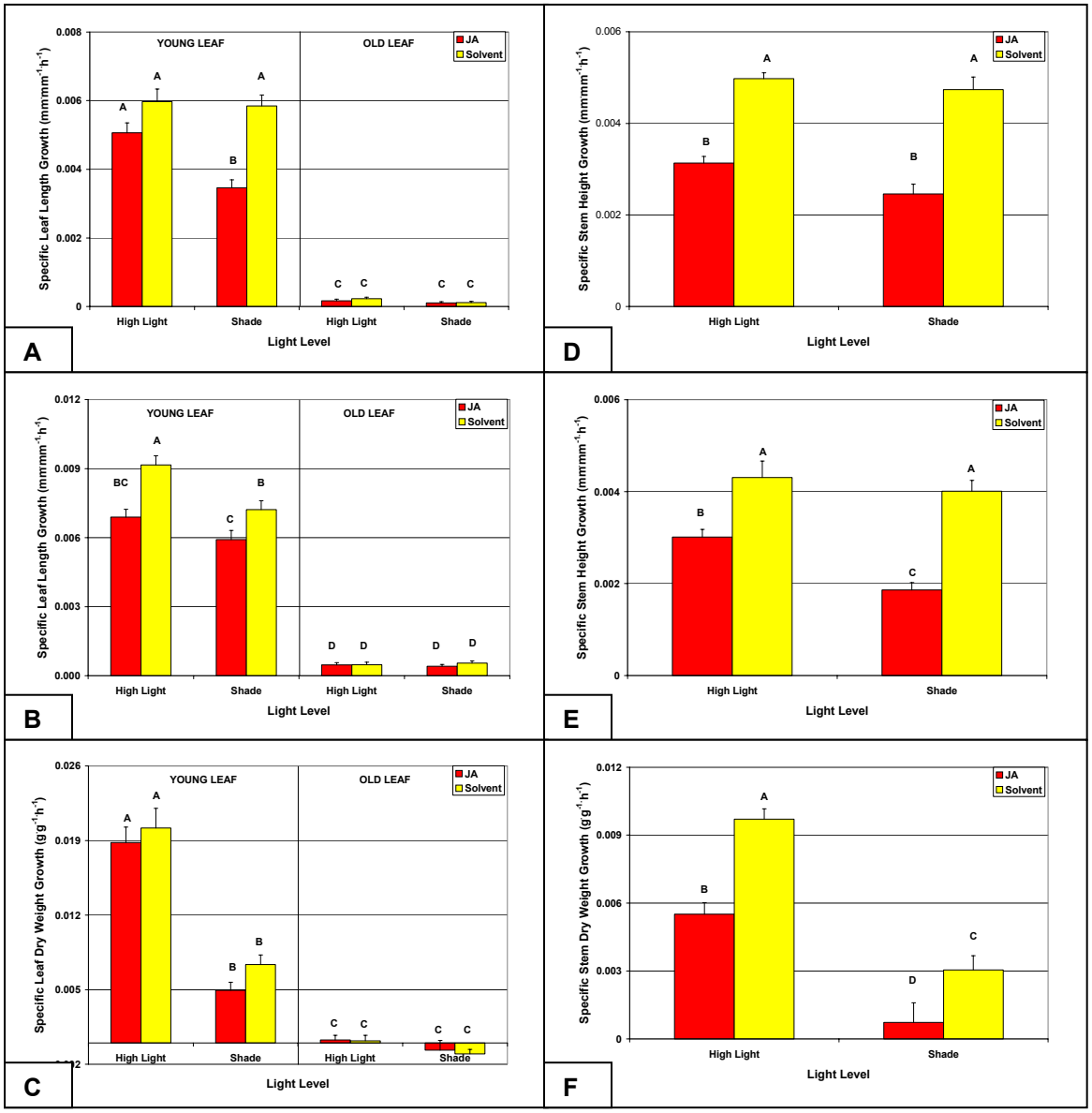


Figure 2.10. Leaf and stem growth of seven week-old tobacco plants. Mean values ± standard error represent the specific leaf growth (A, B, length; C, g dry weight) and specific stem growth (D, E, height; F, g dry weight) rates obtained from two replicated experiments (A, C, D, F, Nov98, N=24-30 per treatment; B,E, Sept00, N=11-15 per treatment). Means with the same letter are not significantly different (p=0.05, Tukey test). Solvent [3% (v/v) ethanol], Jasmonic acid (JA, 5mM dissolved in solvent). High light, 204µmol·m$^{-2}$·s$^{-1}$; shade, 26µmol·m$^{-2}$·s$^{-1}$.

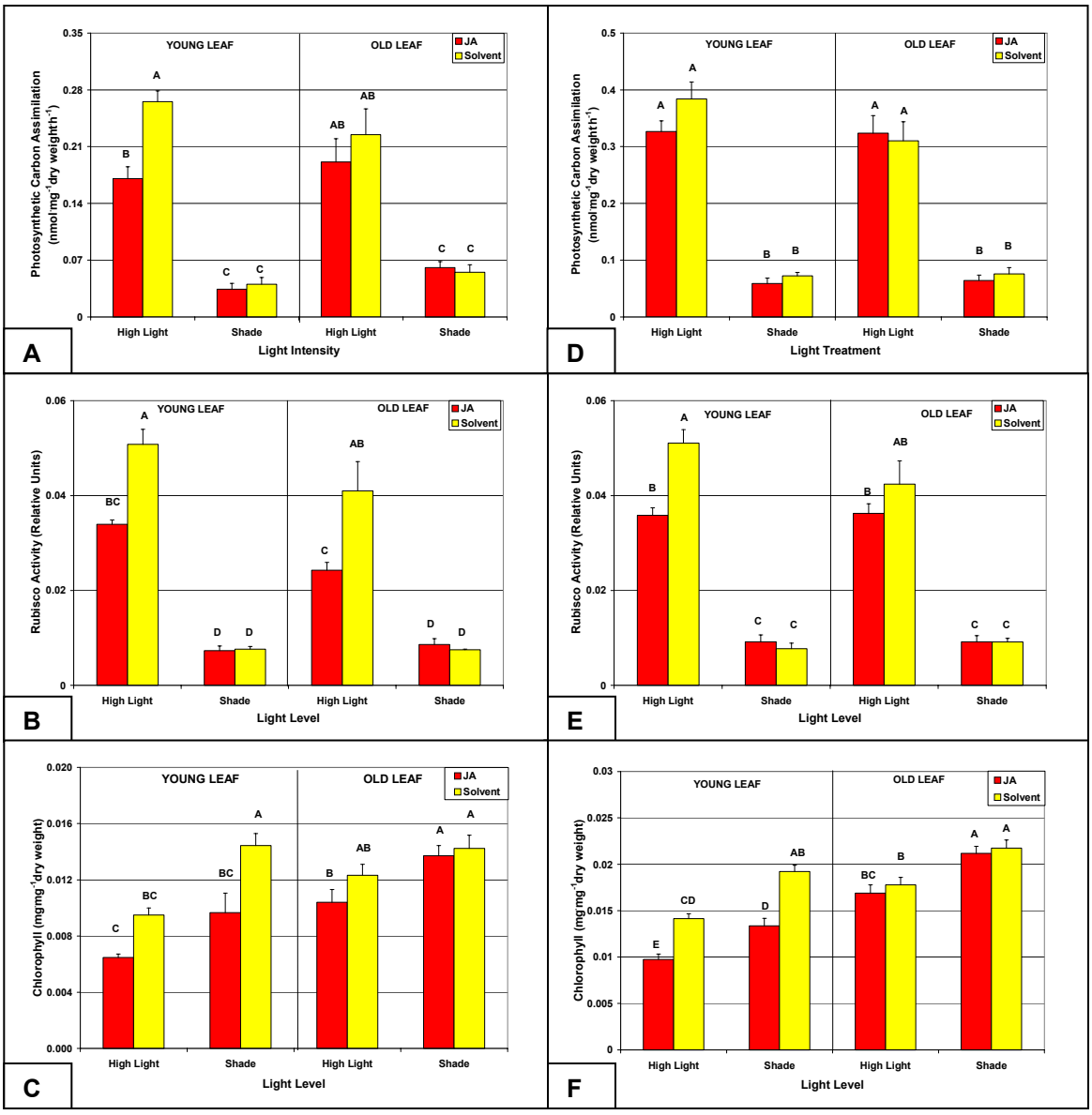


Figure 2.11. Photosynthetic carbon assimilation (A, D), Rubisco activity (B, E), and chlorophyll levels (C, F) in young and old leaves of seven week-old tobacco plants. Measurements were collected in two replicate experiments (Oct99, A-C; Sept00, D-F). Values represent the mean ± standard error of 6-7 (A, C), 9-15 (D, F) or 3 replicates per treatment (B, E). Solvent [3% (v/v) ethanol], Jasmonic acid (JA, 5mM dissolved in solvent). High light, 204 $\mu mol \cdot m^{-2} \cdot s^{-1}$; shade, 26$\mu mol \cdot m^{-2} \cdot s^{-1}$. Means with the same letter are not significantly different (p=0.05, Tukey test).

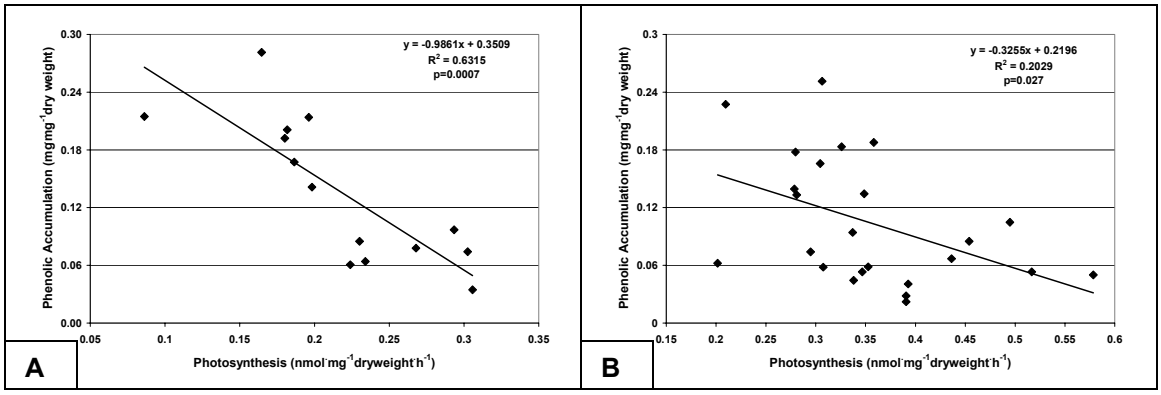


Figure 2.12. Regressions of phenolic accumulation versus photosynthesis in young, unshaded leaves from seven week-old tobacco plants. A. Oct99, B, Sept00. Light, 204µmol·m$^{-2}$·s$^{-1}$. Solvent [3% (v/v) ethanol], Jasmonic acid (JA, 5mM dissolved in solvent).

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# CHAPTER 3

# DOES CHLOROPLAST METABOLITE IMPORT LIMIT PHENOLIC INDUCTION RESPONSES?

## INTRODUCTION

Herbivorous insects and plant pathogens commonly induce plants to accumulate carbon-rich phenolic compounds (Karban and Baldwin, 1997). The phenolic induction response includes enhanced expression of genes encoding a common suite of enzymes involved in phenolic synthesis in the chloroplast shikimate pathway (SP) and cytoplasmic phenylpropanoid pathway (**Fig. 3.1**, see Chapter 1 for references). The ability of plants to increase production of phenolics to defend themselves against attackers may be modulated by leaf development and carbon resource availability (Chapter 2). We previously demonstrated that developing leaves (but not older, mature leaves) of tobacco plants could overcome low resource availability imposed by shading to accumulate phenolic compounds in response to simulated herbivory (Chapter 2). Additionally, the strong phenolic induction responses in young, unshaded leaves were negatively correlated with photosynthetic carbon assimilation, implying that substrates for phenolic synthesis were not being supplied from newly assimilated sugars. If plant leaves do not increase photosynthetic carbon gain when carbon demand is amplified by increased phenolic synthesis, alternative sources of carbon must be used.

Young leaves undergoing phenolic induction responses are able to import carbon-containing substrates from distant source leaves (Arnold and Schultz, 2002). Moreover, the imported carbon serves as a resource for *de novo* phenolic synthesis (Arnold and Schultz, 2002). Dependence of this response on enhanced invertase activities in the induced leaves suggests that sucrose is the imported substrate providing carbon resources for carbohydrate metabolism in the cytoplasm. Activities of the glycolytic and oxidative pentose phosphate (OPP) pathways are activated during plant defense responses (Batz et al., 1998; Norman et al., 1994; Sindelar et al. 1999) and may provide carbon substrates required for induced

phenolic synthesis. Thus, because phenolic synthesis begins in the chloroplast, it is reasonable to hypothesize that chloroplasts from sink leaves import the necessary substrates for phenolic synthesis when there are shortfalls in photosynthetic carbon gain.

The inner envelope of chloroplasts contains several phosphate transporters that mediate exchange of metabolites between the cytosol and chloroplast stroma (Flügge, 1999). Two of these transporters in particular, the phosphoenolpyruvate:phosphate translocator (PPT, Fischer et al., 1997) and the glucose-6P:phosphate translocator (GPT, Kammerer et al., 1998), may provide a means of importing substrates for phenolic synthesis. Activity of the PPT has recently been linked to constitutive and inducible phenolic synthesis (Streatfield et al., 1999). Arabidopsis *ppt1-1* mutants lacking PPT activity were reported to make 57-87% fewer constitutive hydroxycinnamic acids, flavonoids, and simple phenolics than did wild type plants (Streatfield et al., 1999). The same authors reported that these mutants accumulated 80% fewer anthocyanins in response to high light. These results are consistent with the importance of supplying phosphoenolpyruvate (PEP) to the chloroplast for the synthesis of aromatic amino acids and eventually phenylpropanoids (**Fig. 3.1**). The GPT imports glucose-6P as a substrate for starch synthesis in amyloplasts and while glucose-6P is imported into chloroplasts a role for the GPT in chloroplast metabolism is still unclear (Batz et al., 1995; Quick et al., 1995). E4P can be synthesized in the chloroplast from glucose-6P via the reactions of the oxidative pentose phosphate pathway (Stitt and ap Rees, 1980). Because enzymes of the chloroplastic oxidative pentose phosphate pathway are activated in plants undergoing phenolic defense responses, the GPT may be activated to provide glucose-6P for E4P synthesis.

We tested the hypothesis that additional resource substrates for induced phenolic accumulation are provided by enhanced metabolite import from the cytoplasm. Shortfalls in PEP and E4P availability that may arise in leaves not fully engaged in photosynthetic carbon gain either because of developmental or light constraints could be made up by PEP or glucose-6P import mediated by activities of either the PPT or GPT, respectively. We stimulated phenolic synthesis in tobacco plants by treating them with Jasmonic acid (JA), a signal that elicits phenolic metabolism in plant leaves much as does wounding or herbivory

(Creelman and Mullet, 1997; Parthier, 1991; Thaler et al., 1996), in either high light or deep shade. We measured PEP and glucose-6P transport into intact chloroplasts isolated from developing and old leaves, expecting to find enhanced import into chloroplasts from leaves with the highest demand for these substrates.

## METHODS

### Plant Growth Conditions

Tobacco plants (*Nicoticana tabacum* cv petite havana SR1), were grown in a room of a controlled atmosphere building (24ºC day, 20ºC night) on the main campus of The Pennsylvania State University. A 16h photoperiod created with three 1000-watt high pressure sodium greenhouse lamps (G3 Series, Ruud Lighting, Racine, WI) provided an average of 204 ± 24 SD $\mu mol \cdot m^{-2} \cdot s^{-1}$ light at plant height. Seeds were sown in sterilized soil (Metro-Mix 200, Scotts-Sierra Horticultural Products Co., Marysville, OH) and 2-week old seedlings were transferred to 10 by 2.5cm pots containing similar soil supplemented with $120 g \cdot m^{-3}$ Osmocote 14:14:14 (Scotts-Sierra Horticultural Products Co., Marysville, OH). Plants were watered daily and repositioned semi-weekly to randomly-designated positions within the growth room (Minitab Statistical Software, Minitab Inc., State College, PA).

### Experimental Design

When plants were 6-weeks old, solvent [3% (v/v) ethanol] or Jasmonic acid (5mM JA dissolved in solvent) was applied in a fine mist to the adaxial side of all leaves of randomly assigned plants. Plants were either maintained in high light (204 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or positioned under shade tents constructed from PVC pipe (Lowe's, State College, PA) and 80% shade cloth (PAK Unlimited, Inc., Cornelia, GA). Tents enclosed 36 plants and provided 26 ± 4 SD $\mu mol \cdot m^{-2} \cdot s^{-1}$ light.

**Chloroplast Isolation**

Plants were removed from the growth room before sunrise (07:00) and maintained in the dark for seven hours until leaves were removed for chloroplast isolation. We extended the night-period in this manner to decrease levels of starch, which can reduce chloroplast yields during isolation (Riesmeier et al., 1993). After preparing fresh buffers, leaves were cut at the base of the petiole and maintained in the dark at 22ºC in beakers of water. Leaf material (4.6g fresh weight, excluding mid-ribs) was cut into 1cm squares and placed into 50mL conical plastic centrifuge tubes containing 20mL of semi-frozen grinding buffer [adapted from Benková et al., 1999 and Häusler et al., 1998: 340mM D-sorbitol, 2mM EDTA (disodium salt, dihydrate), 1mM KCl, 0.3% (w/v) BSA (fatty acid and globulin-free), 0.3% (w/v) PVP (MW 10,000), 5mM iso-ascorbic acid, 2% (w/v) polyethylene glycol (MW 8000), 4mM DTT, 1mM $KH_2PO_4$, and 50mM HEPES, pH 7.5;] and ground for 10s with a Polytron PT2100 homogenizer (generator PT-DA 2120/EC, speed 19, Brinkmann Instruments, Inc., Westbury, NY). Chloroplast extracts were filtered through two layers of Miracloth (CALBIOCHEM, San Diego, CA) overlaid with two layers of cheesecloth (which were pre-wet with grinding buffer) and the filtrates were collected in 25mL Erlenmeyer flasks submerged in ice.

Percoll step gradient centrifugation was used to isolate intact chloroplasts from crude leaf extracts. Percoll buffer ingredients were adapted from Benková et al., 1999; Häusler et al., 1998; and Mourioux and Douce, 1981: 340mM D-sorbitol, 2mM EDTA, 0.1% (w/v) BSA, 5mM iso-ascorbic acid, 1mM $MgCl_2$ x $6H_2O$, 1mM $MnCl_2$ x 4 $H_2O$, 0.1mM $KH_2PO_4$, 50mM HEPES, pH 7.5 and 21%,45%, or 80% (v/v) Percoll (Amersham Biosciences Corp., Piscataway, NJ). Filtrates were divided in half (7.5mL each) and carefully layered onto pre-chilled Percoll gradients (8mL each of 21%, 45%, and 80% Percoll buffers in 32mL ultracentrifuge tubes). Extracts were centrifuged for 12min at 4ºC and 1200 x *g* in a Beckman L5-75 ultracentrifuge with a swing-out rotor (Beckman Instruments, Inc., Palo Alto, CA). Chloroplasts migrating to the 45%:80% interface were transferred to 12mL centrifuge tubes, carefully mixed with resuspension buffer [1:3 (v/v), chloroplasts: resuspension buffer, adapted from Bagge and Larsson, 1986 and Benková et al., 1999:

340mM D-sorbitol, 2mM EDTA, 0.07% (w/v) BSA, 1mM $MgCl_2$ x $6H_2O$, 1mM $MnCl_2$ x 4 $H_2O$, 1mM $KH_2PO_4$, 50mM HEPES, pH 7.5)] and centrifuged for 60s at 4ºC and 1000 x *g* in a Beckman Accuspin FR centrifuge (Beckman Instruments, Inc., Palo Alto, CA). To double chloroplast yield, chloroplasts sticking to the sides of centrifuge tubes were carefully dislodged with gentle mixing and the 60s spin was repeated. Supernatant was removed from each tube, pellets were suspended in resuspension buffer to a final chloroplast density of 0.5-1.5 mg chlorophyll·$mL^{-1}$, and chloroplasts were maintained at 4ºC in microcentrifuge tubes.

Aliquots of purified chloroplasts were transferred to microcentrifuge tubes containing either 80% (v/v) acetone for chlorophyll analysis or resuspension buffer for chloroplast counting, respectively. Chlorophyll was measured in acetone extracts according to Arnon (1949). Chloroplasts were counted with a hemacytometer and chloroplast abundance per mL of chloroplast suspension calculated with formulae published by Sharpe (1988). Chloroplast envelope intactness was estimated using the ferricyanide method (Lilley et al., 1975). Rates of oxygen evolution were measured at 20ºC with an OXYGRAPH oxygen electrode system (Hansatech Instruments Ltd., England). To measure photosynthetic $O_2$ evolution, aliquots of chloroplasts (15-25 µg chlorophyll) were suspended in photosynthesis buffer (340mM D-sorbitol, 2mM EDTA, 1mM $MgCl_2$ x $6H_2O$, 1mM $MnCl_2$ x 4 $H_2O$, 0.1mM $KH_2PO_4$ 5mM $NaHCO_3$, 1mM ATP, 1mM PGA 50mM HEPES, pH 7.2, Walker, 1980) and rates of photosynthetic $O_2$ evolution at 200µmol·$m^{-2}$·$s^{-1}$ recorded after 15min.

**Dark Import Assays**

We used the silicone oil centrifugation technique of Heldt (1980) to measure import of $^{14}$C-PEP or $^{14}$C-glucose-6P into isolated chloroplasts. Reaction vessels (400µL centrifuge tubes layered with 20µL 10% (v/v) perchloric acid, 100µL silicone oil (AR200, Wacker-Chemie GmbH, Munich, Germany), and 200µL import buffer [340mM D-sorbitol, 2mM EDTA, 0.07% (w/v) BSA, 1mM $MgCl_2$ x $6H_2O$, 1mM $MnCl_2$ x 4 $H_2O$, 1mM $KH_2PO_4$, 50mM HEPES, pH 7.5, Bagge and Larsson, 1986 and Benková et al., 1999] were equilibrated to 15ºC. Chloroplasts (10-20 µg chlorophyll) were added to the import buffer with gentle mixing and equilibrated for 5min. At time 0, 5µL of either 9 mCi/mmol 1-$^{14}$C-

PEP (Amersham Pharmacia) or 0.8 mCi/mmol U-$^{14}$C-glucose-6P (Sigma) was added to the chloroplast suspension and the reaction allowed to proceed for 1min in the dark. Reactions were terminated by centrifuging for 30s in a Beckman 152 horizontal microfuge (Beckman Instruments, Inc., Palo Alto, CA). Chloroplast and intermembrane space sizes were estimated in double-labeled experiments using 8µCi $^{3}$H-$H_2O$ (Jim Wiggins, Penn State) and 2.5µCi U-$^{14}$C-sorbitol (Sigma), respectively, and similar import assay conditions. Samples were prepared for scintillation counting as described in Heldt (1980). DPMs of $^{14}$C or $^{3}$H in aliquots of processed samples mixed with 10mL EcoScint A (National Diagnostics USA, Atlanta, GA), were measured with a Beckman LS3801 Scintillation counter (Beckman Instruments, Inc., Palo Alto, CA), and $^{14}$C-PEP and $^{14}$C-glucose-6P import rates calculated using published formulae (Heldt, 1980).

**Light Import Assays**

A custom-built water-jacketed glass cuvette (Chemistry Department, The Pennsylvania State University) was used to measure $^{14}$C-PEP import into chloroplasts in the light. Chloroplasts (10-20 µg chlorophyll) were added to 200µL light import buffer (same as dark import buffer but including 5mM $NaHCO_3$, 1mM ATP and 1mM PGA) at 20ºC and adapted to white light (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, LS2 Tungsten-halogen light source, Hansatech Instruments Ltd., England) for 10 min prior to addition of $^{14}$C-PEP (0.8 mCi/mmol, 0.05-1mM). Reactions were terminated after 30s by transferring the 200µL import mixture to 400µL centrifuge tubes containing the layered perchloric acid and silicone oil, centrifuged for 20s, and processed as described above for the dark import assays.

**Phenolic Induction Responses**

Tobacco leaves (2g fresh weight) were ground for 10s in 3mL 50% (v/v) MeOH with the Polytron and a small generator (PT-DA 2112/2EC). Extracts were centrifuged for 4min at 4ºC and 1000 x *g* in a Beckman Accuspin FR centrifuge (Beckman Instruments, Inc., Palo Alto, CA). Aliquots of the supernatant were assayed by the Folin Denis assay (using native tobacco standards), which was adapted for small sample size from Appel et al. (2001).

Lithium sulphate (8%) was added to the Folin reagent to prevent precipitate formation (Singleton and Rossi, 1965). Tobacco standards were extracted from tobacco leaf powder in 50% (v/v) MeOH, partitioned against hexane, lyophilized, and stored in desiccant at -10ºC. Phenolic induction 72h after treatment was expressed per chloroplast number present in 2g (fresh weight) leaf tissue 48h after treatment.

## RESULTS

### Phenolic Induction

We conducted metabolite import experiments with six week old tobacco plants which were one week younger than were plants in our prior phenolic induction experiments (Chapter 2). Using younger plants allowed us to isolate a greater abundance of intact chloroplasts from starting leaf material (Walker, 1980). JA promoted phenolic induction in these plants (**Fig. 3.2**; $F=343$, $p<0.0001$), which was highest in young, developing leaves ($F=221$, $p<0.0001$) and in unshaded plants ($F=204$, $p<0.0001$), similar to phenolic induction responses in older plants (see Chapter 2, Figs. 2.3 and 2.6).

### Characteristics of Isolated Chloroplasts

Chloroplast size was unaffected by treatment (**Fig. 3.3A**, $F=0.06$, $p=0.81$) while the intermembrane space was enlarged in JA-treated chloroplasts (**Fig. 3.3B**, $F=24$, $p<0.0001$). JA left envelope intactness unaltered except in chloroplasts isolated from old unshaded plants (**Fig 3.3C**, $F=12.78$, $p=0.0011$). Chloroplasts isolated from old, unshaded leaves were 26% less intact than were control chloroplasts. JA decreased chloroplast abundance (**Fig. 3.3D**, $F=16.9$, $p=0.0005$), and this effect was most evident in young leaves (**Fig. 3.3D**, $F=5.26$, $p=0.0317$). Chloroplasts from young leaves showed higher rates of photosynthetic oxygen evolution (**Fig. 3.4A,** $F=14.5$, $p=0.001$), similar rates of electron transport (**Fig. 3.4B**, $F=0$, $p=0.98$), and lower levels of chlorophyll when compared to old leaves (**Fig. 3.4C**, $F=40.4$, $p<0.0001$). JA repressed photosynthetic oxygen evolution (**Fig. 3.4A**, $F=27.31$, $p<0.0001$),

electron transport (Fig. **3.4B**, F=74.3, p<0.0001), and chlorophyll levels in isolated chloroplasts (**Fig. 3.4C**, F=7.32, p=0.01).

**Transport of PEP and Glucose-6P into Isolated Chloroplasts in the Dark**

*Chloroplasts from developing leaves*

We conducted *in vitro* transport assays to measure import rates of PEP and glucose-6P in chloroplasts isolated from plants that had been either shaded or maintained in high light following JA treatment. Because phenolic induction responses were detected 72h after treatment, chloroplasts were isolated 24-48h after treatment, since enhanced rates of metabolite import would be expected to precede phenolic synthesis. By 48h after JA treatment, elevated phenolic levels in unshaded plants prevented isolating sufficient numbers of intact chloroplasts for transport experiments with this treatment group. Hence, experiments with young, unshaded leaves were conducted 24h after treatment while chloroplasts from all other treatment groups were extracted 48 after treatment. We carried out replicate experiments with chloroplasts isolated from different groups of plants. Because the quantitative levels of metabolite import varied between replicates, data for individual replicate experiments are presented separately. Qualitative responses to JA treatment in general were comparable between replicate experiments.

JA decreased PEP import rates 50-95% in chloroplasts from shaded leaves (**Fig. 3.5A,B,C**). In contrast, glucose-6P transport was either unaffected (two out of three replicates) or enhanced nearly 28 times over controls in shaded leaf chloroplasts (**Fig. 3.5D,E,F**). PEP import in chloroplasts from unshaded leaves was also decreased by JA treatment, although slightly less than in shaded leaves (45% repression, **Fig. 3.6A**). Glucose-6P transport was not detected in chloroplasts isolated from young, shaded leaves (**Fig. 3.6B**).

*Chloroplasts from mature leaves*

Rates of PEP transport into chloroplasts isolated from old leaves of shaded plants were unaffected by prior treatment with JA (**Fig. 3.7A, B**). Glucose-6P rates were either decreased or unaffected by prior JA treatment (**Fig. 3.7C, D**). We were unable to detect enhanced metabolite import in chloroplasts isolated from unshaded, old leaves. JA had no effect on rates of either PEP or glucose-6P transport rates in this treatment group (**Fig. 3.8 A, B**).

**Light Stimulates PEP Import**

To assess the influence of light on the transport process, we conducted import assays in the light, providing as realistic a "daytime" environment as we could simulate *in vitro*. We provided chloroplasts with $NaHCO_3$, ATP and 3-phosphoglycerate to ensure photosynthetic rates similar to those measured in the oxygen electrode (**Fig. 3.4A**), and after a 10 min acclimation period at 20ºC, we added the $^{14}$C-PEP. Using these procedures, chloroplasts from JA-treated young, unshaded leaves imported 45-65% less PEP than controls (**Fig.3.9A,B,C**). We compared transport rates for chloroplast assays conducted in the dark (**Fig. 3.8A**) and light (**Fig. 3.10**, 1mM point). Presence of light during the transport assay stimulated 20% higher rates of PEP import compared to assays conducted in the dark.

**Substrate Saturation of PEP and Glucose-6P Import**

Substrate saturation experiments were conducted to estimate kinetic transport parameters that would explain enhanced glucose-6P import into chloroplasts from young, shaded leaves 48h after treatment and repressed PEP import from young, unshaded leaves 24h after treatment. JA did not stimulate levels of glucose-6P import in this experimental replicate (**Fig. 3.10A**, $F=1.19$, $p=0.28$) but PEP import was repressed by JA (**Fig. 3.10B**, $F=125.5$, $p<0.0001$). Transport did not saturate at the concentrations of glucose-6P or PEP we used in this experiment, so we were unable to estimate kinetic parameters.

**Does Chloroplast Membrane Integrity Influence Metabolite Import?**

JA treatment may have compromised envelope intactness while increasing the size of the intermembrane space (**Fig. 3.3**). Because the PPT and GPT are located in the inner envelope of the chloroplast membrane, we investigated potential effects of these changes in envelope characteristics on metabolite transport through regression analyses. Combining data from all dark-import experiments, we found a significant negative relationship between intermembrane space size and chloroplast intactness (**Fig. 3.11**). We explored possible impacts of envelope intactness and intermembrane space swelling on metabolite import with additional regressions. For both PEP (**Fig. 3.12A**) and glucose-6P (**Fig. 3.12B**), envelope intactness did not significantly influence metabolite import within the range of intactness values we employed (70-100% intact envelopes). In addition, swelling of the intermembrane space which occurred in response to JA treatment had no significant effect on PEP (**Fig. 3.12C**) or glucose-6P import (**Fig. 3.12D**).

## DISCUSSION

**Isolated Chloroplasts Contained Highly Intact Envelope Membranes**

Like other authors, we used JA to simulate herbivory (Arnold and Schultz, 2002; Kessler and Baldwin, 2002; Cipollini and Redman, 1999; Farmer and Ryan, 1990). JA also elicits senescence-like responses in many plants and recently has been shown to mediate senescence in Arabidopsis (He et al., 2002). While decreased photosynthetic rates and chlorophyll levels in response to insects have been detected in a few instances (Hermsmeier et al., 2001; Ni et al., 2001), it is unclear what role senescence may play during phenolic induction responses. We found that JA also elicited several senescence-like responses in this experiment (diminished photosynthetic carbon assimilation, lower levels of chlorophyll, decreased chloroplast abundance and chloroplasts with increased intermembrane space) which are characteristic senescence responses in leaves (Smart, 1994). Thus, to facilitate isolating chloroplasts with intact membranes from JA-treated plants, we optimized chloroplast extraction buffers to include components which protect membranes from lipids,

phenolics, and oxidizing agents, all of which could additionally compromise membrane integrity during the isolation procedure (Walker, 1980). Our isolation methods allowed us to maintain sufficient membrane integrity such that any membrane degradation caused by JA treatment did not detectably influence PEP and glucose-6P transport rates into isolated chloroplasts.

**PEP and Glucose-6P Import are not Enhanced During Phenolic Induction Responses**

Although we had expected to find higher PEP import rates during phenolic induction responses, none of the treatment groups showed enhanced PEP import. We had demonstrated previously that the Calvin Cycle was unlikely to be the primary source of extra substrates required for phenolic induction (Chapter 2). In the current study, we isolated chloroplasts from tobacco plants undergoing similar phenolic induction responses to test the hypothesis that chloroplasts import carbon resources for induced phenolic synthesis. Young leaves treated with JA in high light exhibited the greatest increase in phenolics and thus we hypothesized that resource substrates for induced phenolic synthesis in this class would be provided by PEP and glucose-6P import from the cytoplasm. Contrary to our predictions, PEP import was decreased substantially by JA treatment and glucose-6P transport was not detected. Old unshaded leaves exhibited the second greatest phenolic increases, but PEP and glucose-6P transport rates were unaffected by JA in this class, too. In young, shaded leaves, phenolic induction responses were variable as were transport measurements. PEP import was consistently repressed by JA, but glucose-6P import was either unaffected or increased by JA in separate experimental replicates. Old, shaded leaves were not inducible, and PEP import was decreased by JA while glucose-6P transport was unaffected by treatment. With the exception of one experiment conducted with young, shaded leaf chloroplasts, glucose-6P transport was unaffected by JA. The transport rates we measured may have been sufficient to supply substrates for enhanced phenolic synthesis or chloroplasts must have alternate means of acquiring PEP during phenolic induction responses.

To examine the possibility that constitutive levels of PEP import are sufficient to support induced phenolic synthesis, we compared import rates with phenolic induction

responses. Since glucose-6P import was not detected in this treatment group, we considered solely PEP import. We used a transport rate measured at a PEP concentration of 50μM (0.17 fmol PEP·chloroplast$^{-1}$·h$^{-1}$, **Fig. 3.10**). This transport rate was calculated using chloroplasts isolated from young, unshaded leaves of JA-treated plants 24h after treatment. Having previously calculated chloroplast abundance per dry weight of leaf tissue at the same point in time after treatment; we used this value to convert transport to 78 nmol PEP·mg$^{-1}$dry weight·72h$^{-1}$. Caffeoylputrescine is the primary phenolic-containing compound induced by JA in these tobacco leaves (Chapter 2); between 5-16 μg·mg$^{-1}$dry weight of caffeoylputrescine was induced in young, unshaded leaves three days after treatment. This translates into 22-65 nmol of caffeic acid, the phenolic component of caffeoylputrescine. Two moles of PEP are used in the synthesis of one mole of caffeic acid; therefore synthesizing 22-65 nmol caffeic acid would require twice the amount of imported PEP (44-130 nmol). We calculated a possible PEP import rate of 78nmol, which falls within the expected range to support caffeoylputrescine synthesis.

These calculations suggest that even the repressed PEP import rates measured in JA-treated chloroplasts may provide sufficient PEP to support induced phenolic synthesis; however, we have a few caveats regarding this conclusion. First, we assumed that all imported PEP is used to synthesize induced caffeoylputrescine. This may be an unrealistic expectation since the shikimate pathway also produces phenylalanine and non-phenolic products (e.g., quinic acid). We were unable to show that protein synthesis was reduced in leaves producing additional phenolics (Chapter 2). In addition, PEP goes into caffeic acid, which is also used to synthesize caffeoylquinic acids (including chlorogenic acid) in tobacco leaves concurrently with caffeoylputrescine (Chapter 2). An additional demand for PEP may be fatty acid synthesis, which also occurs in the chloroplast. Second, our experiments were conducted with static concentrations of PEP. But PEP levels are likely to change with leaf development and in response to enhanced glycolysis during phenolic induction responses. Changes in cytoplasmic concentrations of PEP should influence import into the chloroplast (see **Fig. 3.10**). Repressing PEP import (e.g., by JA) would maintain PEP in the cytoplasm, thus directing carbon flow to mitochondrial respiration, which is commonly increased in plant defense responses (Dwurazna and Weintraub, 1969; Guest et al., 1989; Madrid et al.,

1999). Finally, the calculations were based on a transport rate which was collected 24h after induction in the light. Levels of import are likely to decrease at night (dark import rates are 20% lower than light-import rates, see **Fig.3.8A**, **Fig.3.10A**-1mM point). In addition, chloroplast abundance was decreased by JA (see **Fig.3.3D**), necessarily decreasing the abundance of PPT transporters per gram of leaf tissue. Thus, levels of imported PEP available for induced caffeoylputrescine accumulation were likely lower than 78pmol for the three day period following treatment.

**PPT and GPT do not Limit Enhanced Shikimate Pathway Activity**

Streatfield et al. (1999) found that Arabidopsis *ppt1-1* mutants lacking the *PPT* gene and thus PPT-mediated chloroplast PEP import have a phenotype with substantial decreases in aromatic amino acids, one of which (phenylalanine) is a substrate for phenylpropanoid reactions in the cytoplasm. These authors also found that levels of constitutive phenolics (hydroxycinnamic acids, flavonoids, simple phenolics, and anthocyanins) were decreased, but not completely abolished, in mutant plants. And while levels of anthocyanin accumulation were somewhat constrained in the mutants, there was still modest accumulation when plants were grown in high light. Thus, an alternative explanation of the results presented by Streatfield et al. (1999) is that the PPT is not solely responsible for supplying PEP to the chloroplast stromal compartment. This interpretation is consistent with our results, which implicate a source of PEP that does not involve transport across the chloroplast envelope. In fact, if chloroplasts were completely dependent on PPT activity in this capacity, then we would expect the *ppt1-1* mutation to be lethal because the SP synthesizes aromatic amino acids which are required additionally for protein synthesis. In a study with isolated chloroplasts, $^{14}$C-phenylalanine was synthesized from photoassimilated $^{14}$C-$CO_2$ at low rates (Schulze-Siebert and Schultz, 1989). In the same study, when PEP was added to the bathing chloroplast solution phenylalanine synthesis was significantly enhanced, indicating that shikimate pathway activity may depend on two pools of substrates, one from the stroma and the other imported from the cytoplasm.

Glucose-6P import is most active in amyloplasts from root cells, where its role in carbohydrate metabolism is well documented, as import of glucose-6P into amyloplasts has been linked to starch synthesis (Hill and Smith, 1991). The role of glucose-6P import via the GPT in green leaf chloroplast metabolism has not been characterized, and studies measuring glucose-6P import into isolated chloroplasts have generally demonstrated low transport rates (Batz et al., 1995; Quick et al., 1995). However, transport can be activated when plants are grown in heterotrophic conditions by supplying plants with an external carbon source (sucrose) in conjunction with shading (Quick et al., 1995). Because sink leaves display heterotrophic metabolism and can import carbon resources from source leaves, we expected that JA would stimulate GPT activity during phenolic induction responses, when young leaves may import carbon from distant sources (Arnold and Schultz, 2002). It is notable that the most inducible leaves (young leaves from unshaded plants) were the only ones that did not import glucose-6P, as chloroplasts from this group should have had the greatest need for additional substrates for the shikimate pathway. We necessarily employed a high concentration of glucose-6P (6.5mM) because previous studies showed that ambient transport rates were very low (Batz et al., 1995; Quick et al., 1995). The variable import responses we measured (including many 0 transport rates even outside of the young, unshaded leaf class) suggest that glucose-6P import into chloroplasts is not a primary source of substrates for carbon metabolism in green leaves.

**Alternative Sources of Stromal PEP and E4P during Induced Phenolic Synthesis**

Chloroplasts possess three additional mechanisms to acquire stromal PEP and E4P. First, the Calvin Cycle may serve as source of substrates for phenolic metabolism (Kleiner et al., 1999; Schulze-Siebert and Schultz, 1989). However, photosynthetic carbon assimilation is commonly decreased by enemies (Hermsmeier et al., 2001; Hibberd et al., 1996; Pennypacker et al., 1990; ), regeneration of ribulose-1,5-bisphophate, the substrate of Rubisco, is lessened during plant responses to enemies (Pennypacker et al., 1995), and incorporation of $^{14}$C-label from $^{14}C$-$CO_2$ can be diminished during phenolic induction responses (Zangerl et al., 1997). Thus, Calvin Cycle activity is unlikely to supply the additional substrates for induced phenolic synthesis. Second, supplementary phosphate

translocators in the chloroplast inner envelope may provide alternate means of importing substrates for induced phenolic synthesis. The triose phosphate:phosphate translocator (TPT) has high specificity for triose phosphates, which may be imported from the cytoplasm into chloroplasts (Flügge, 1999). For imported TPs to serve as a substrate for PEP synthesis would require the activity of phosphoglycerate mutase and enolase, but the presence and activity of these enzymes in chloroplasts is still a matter of debate (Hoppe et al., 1993; Ramachandra and Rama Das, 1995; Stitt and ap Rees, 1979; van der Straeten et al., 1991). The TPT is also capable of low levels of PEP transport (Fischer et al., 1997). However, the PEP transport we measured would have included that by both the TPT and PPT; and in either case JA decreased PEP import. The recently-discovered xylulose-5P:phosphate translocator (XPT) could provide an alternate means for chloroplasts to import intermediates of the OPP pathway (xylulose-5P or ribulose-5P) which could then be used to synthesize E4P (Eicks et al., 2002). Whether the XPT imports substrates in support of enhancing E4P supply to induced phenolic metabolism remains to be established. Finally, starch represents a significant pool of stored reserves in chloroplasts, and a link between starch and phenolics has been demonstrated in Arabidopsis (Mita et al., 1997). However, the ability of starch to provide PEP for induced phenolic metabolism would also require activity of phosphoglycerate mutase and enolase within the chloroplast. Thus, stromal PEP and E4P may be acquired from multiple trajectories in chloroplasts, affording these organelles with the ability to maintain continuous substrate supplies during phenolic induction responses.

**Summary and Conclusions**

During induced phenolic responses to simulated herbivory, plants must meet enhanced substrate demands imposed by the chloroplast shikimate pathway. We showed that chloroplasts do not meet these demands by increasing PEP and glucose-6P import, even under resource-limiting conditions imposed by low light and leaf development. Our inability to demonstrate enhanced PEP import was unexpected in light of current evidence showing that *PPT* mutants lacking the ability to import PEP via the PPT accumulated substantially fewer constitutive phenolics (Streatfield et al., 1999). Perhaps pentose phosphate import via the XPT plays a more significant role in chloroplast substrate import during phenolic

induction responses, especially in light of current evidence showing that the majority of OPP pathway enzymes are located in the chloroplast and not in the cytoplasm, as originally proposed (Eicks et al., 2002). Starch may provide an alternate source of both PEP and E4P substrates for the shikimate pathway, and this possibility could be investigated by exploring phenolic induction responses in starch mutants or plants engineered with deficiencies in starch metabolism. In conclusion, chloroplasts possess multiple avenues of acquiring stromal PEP and E4P which may allow them to maintain carbon resource allocation to induced phenolic metabolism under a variety of conditions that constrain carbon availability.

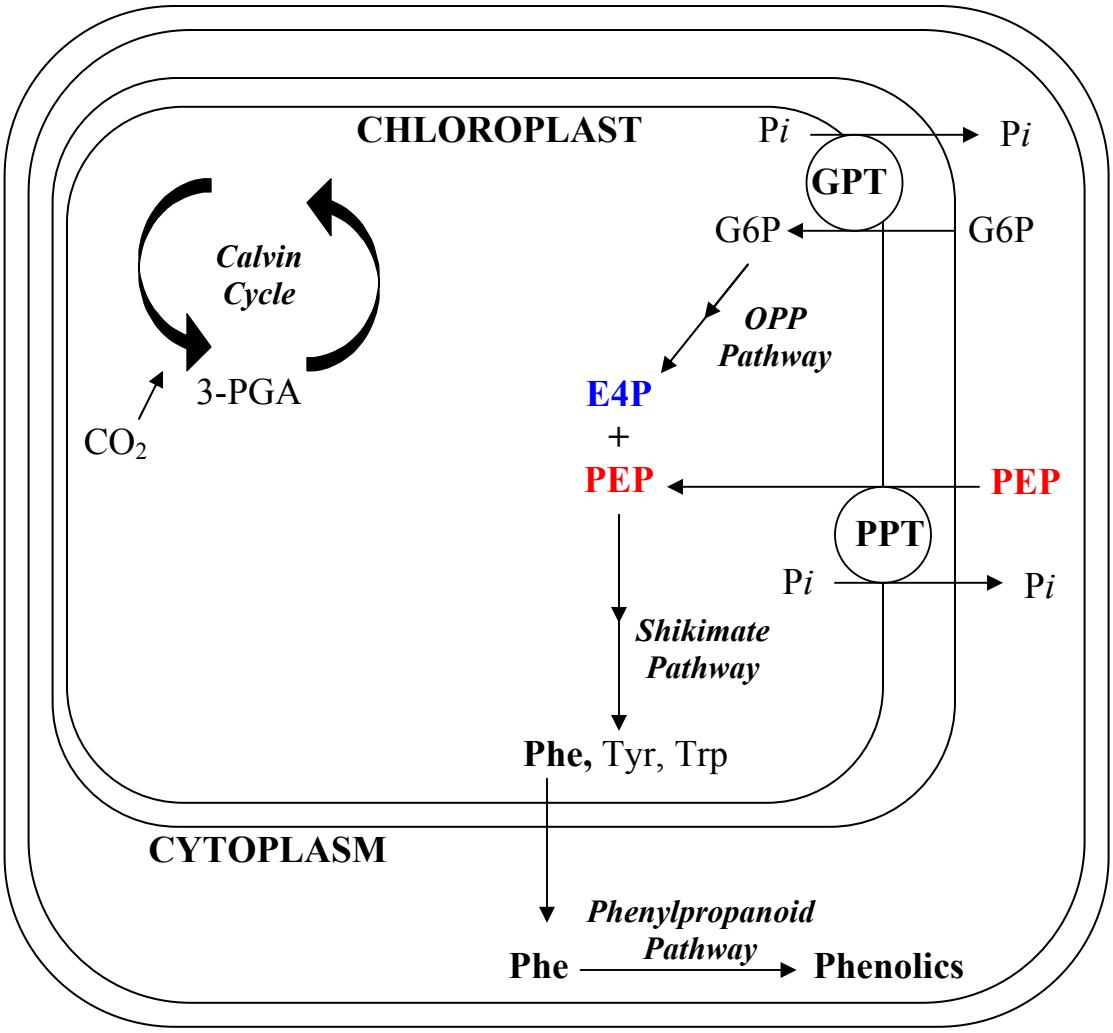


Figure 3.1. Possible routes of carbon resource supply to phenolic metabolism. E4P, erythrose-4-phosphate; G6T, glucose-6-phosphate; GPT, glucose-6P:P*i* translocator; OPP, oxidative pentose phosphate; PEP, phospho*enol*pyruvate; P*i*, inorganic phosphate; PPT, PEP:*Pi* translocator; Phe, 3-PGA, 3-phosphoglycerate; phenylalanine; Trp, tryptophan; Tyr, tyrosine.

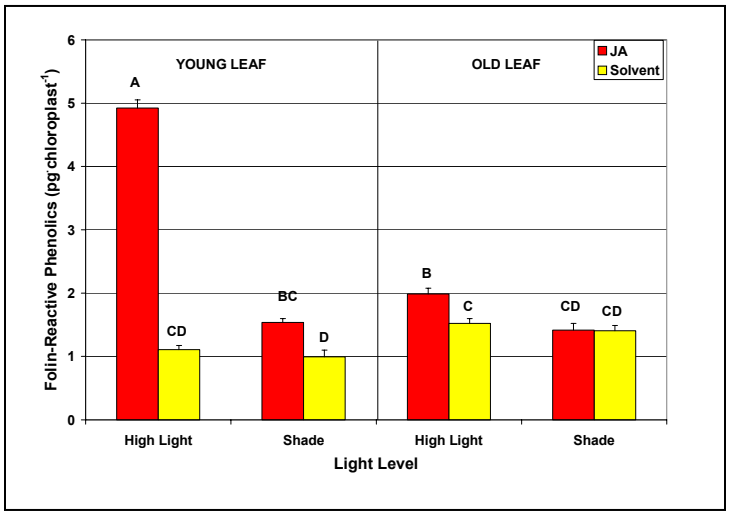


Figure 3.2. Folin reactive (total phenolic) compounds in young and old leaves of six-week old tobacco plants. Mean values ± standard error represent the levels of phenolics ($pg \cdot chloroplast^{-1}$) attained three days after treatment. Chloroplast numbers were assessed two days after treatment. Means with the same letter are not significantly different (p=0.05, Tukey test). Solvent, 3% (v/v) ethanol; JA, Jasmonic acid, 5mM dissolved solvent. High light, 204 $\mu mol \cdot m^{-2} \cdot s^{-1}$; shade, 26 $\mu mol \cdot m^{-2} \cdot s^{-1}$. N=three replicates per treatment

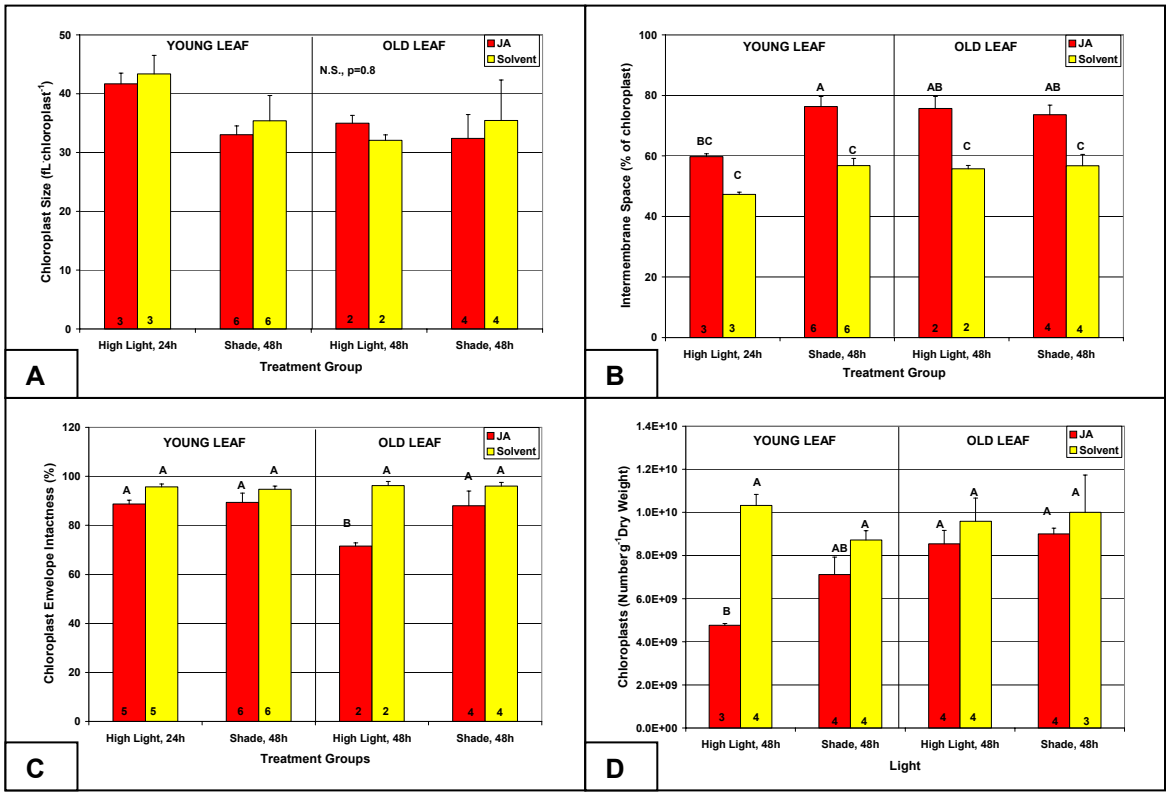


Figure 3.3. Chloroplast size (A), intermembrane space size (B), chloroplast envelope intactness (C) and chloroplast abundance (D) as affected by Jasmonic acid (JA) treatment in six-week old tobacco plants. Plants were sprayed with either JA (5mM in solvent) or solvent [3% (v/v) ethanol] and maintained in a day/night cycle with ambient light (204µmol·m$^{-2}$·s$^{-1}$) or shade (26µmol·m$^{-2}$·s$^{-1}$) for 24h or 48h prior to chloroplast isolation. Bars represent the mean ± standard error for N replicates (N is indicated on the bottom of each bar). Means with the same letter are not significantly different (p=0.05, Tukey test).

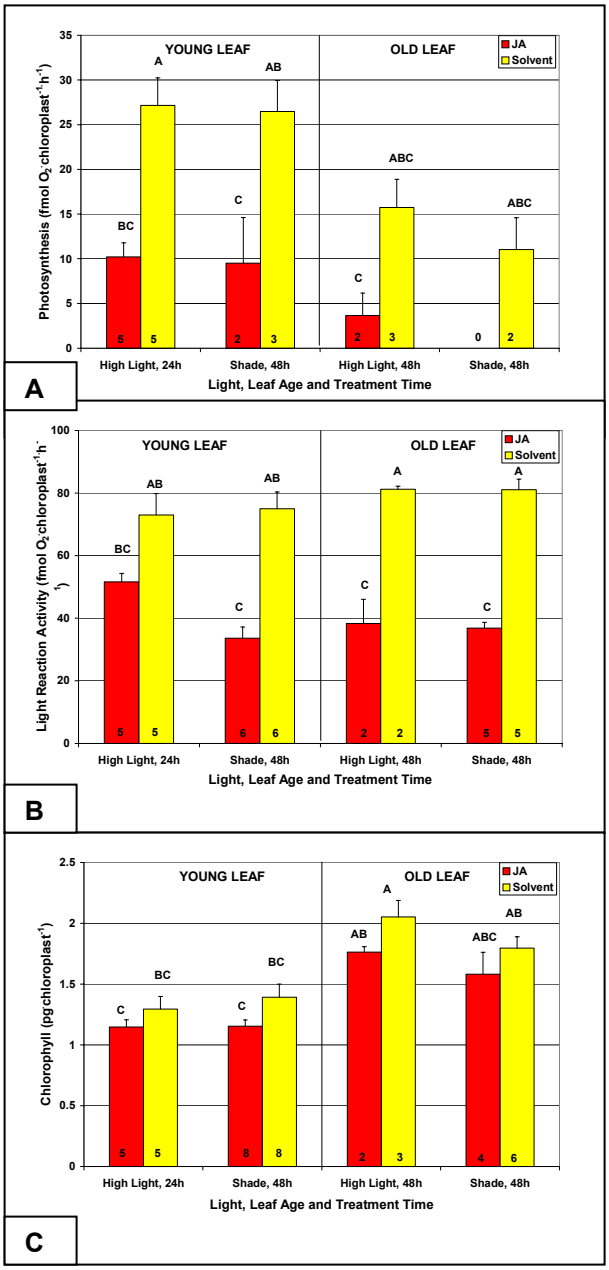


Figure 3.4. Photosynthetic oxygen evolution (A), electron transport (B), and chlorophyll levels (C) as affected by Jasmonic acid (JA) treatment in chloroplasts isolated from six-week old tobacco plants. Plants were sprayed with either JA (5mM in solvent) or solvent [3% (v/v) ethanol] and maintained in a day/night cycle with ambient light (204µmol·m$^{-2}$·s$^{-1}$) or shade (26µmol·m$^{-2}$·s$^{-1}$) for 24h or 48h prior to chloroplast isolation. Oxygen evolution was measured in a Hansatech oxygen electrode at 20ºC with 200 µmol·m$^{-2}$·s$^{-1}$ red light. Ferricyanide-dependent oxygen evolution in ruptured chloroplasts (B) is representative of photosynthetic electron transport. Bars represent the mean ± standard error for N replicates (N is indicated on the bottom of each bar). Means with the same letter are not significantly different (p=0.05, Tukey test).

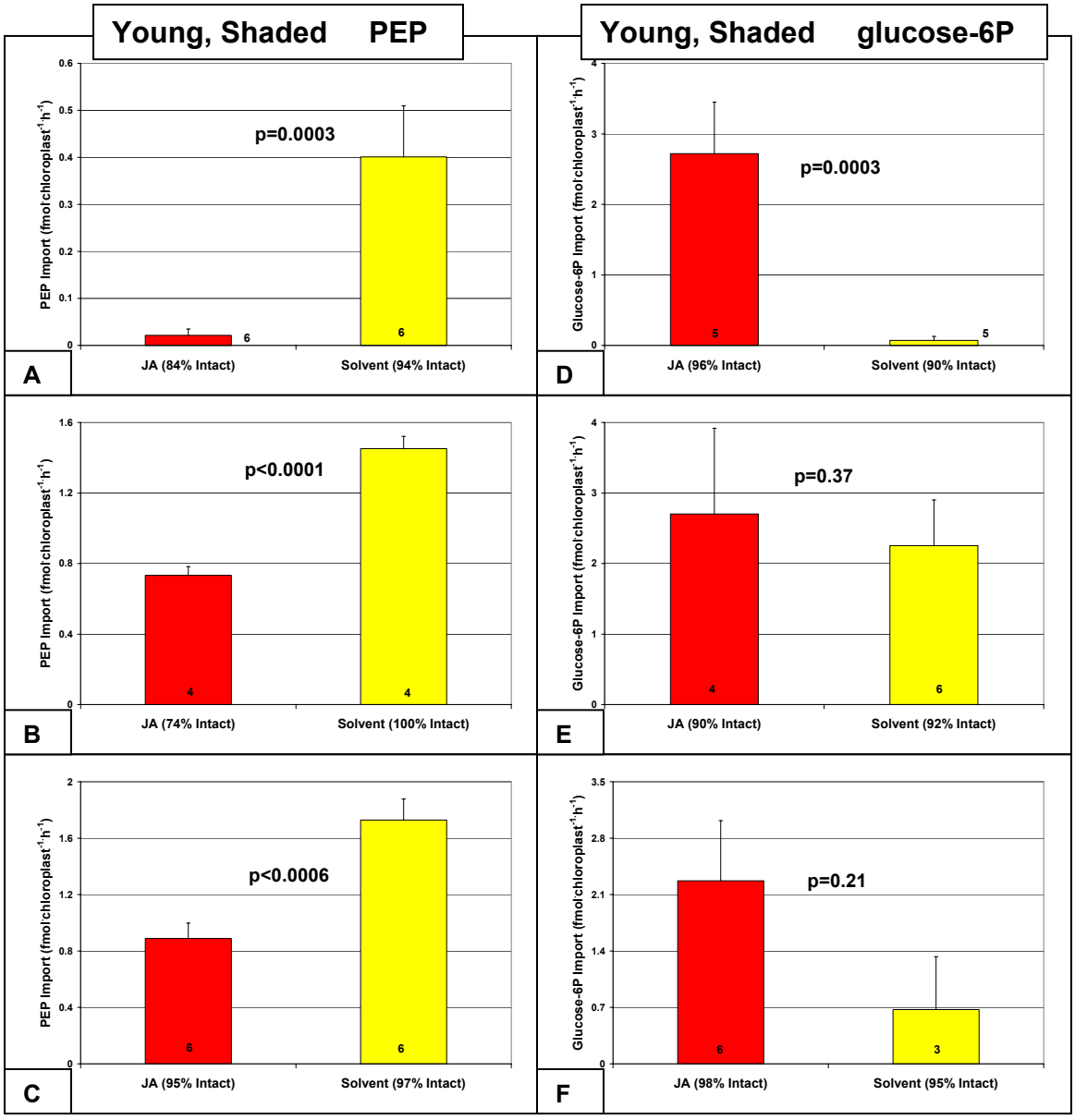


Figure 3.5. Transport rates of 1mM $^{14}$C-PEP (A, B, C) or 6.5mM $^{14}$C-glucose-6P (D, E, F) into chloroplasts isolated from young, shaded tobacco leaves. Data from three replicate experiments are presented. Plants were sprayed with either solvent [3% (v/v) ethanol] or JA (5mM in solvent) and maintained in a day/night cycle with shade (26µmol·m$^{-2}$·s$^{-1}$) for 48h prior to chloroplast isolation. Metabolite uptake experiments were conducted in the dark. Bars represent the mean ± standard error for N replicates (N is indicated on the bottom of each bar). Statistical differences estimated with 1-tailed Student's t-test.

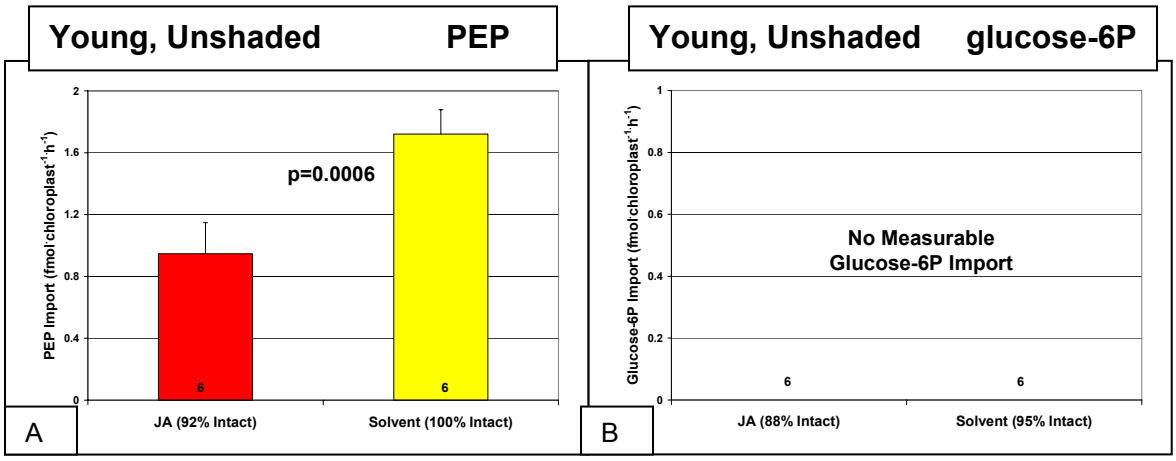


Figure 3.6. Transport rates of 1mM $^{14}$C-PEP (A) and 6.5mM $^{14}$C-glucose-6P (B) into chloroplasts isolated from young, unshaded tobacco leaves. Plants were sprayed with either solvent [3% (v/v) ethanol] or JA (5mM in solvent) and maintained in a day/night cycle with high light (204 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 24h prior to chloroplast isolation. Metabolite uptake experiments were conducted in the dark. Bars represent the mean ± standard error for N replicates (N is indicated on the bottom of each bar). Statistical differences estimated with 1-tailed Student's t-test.

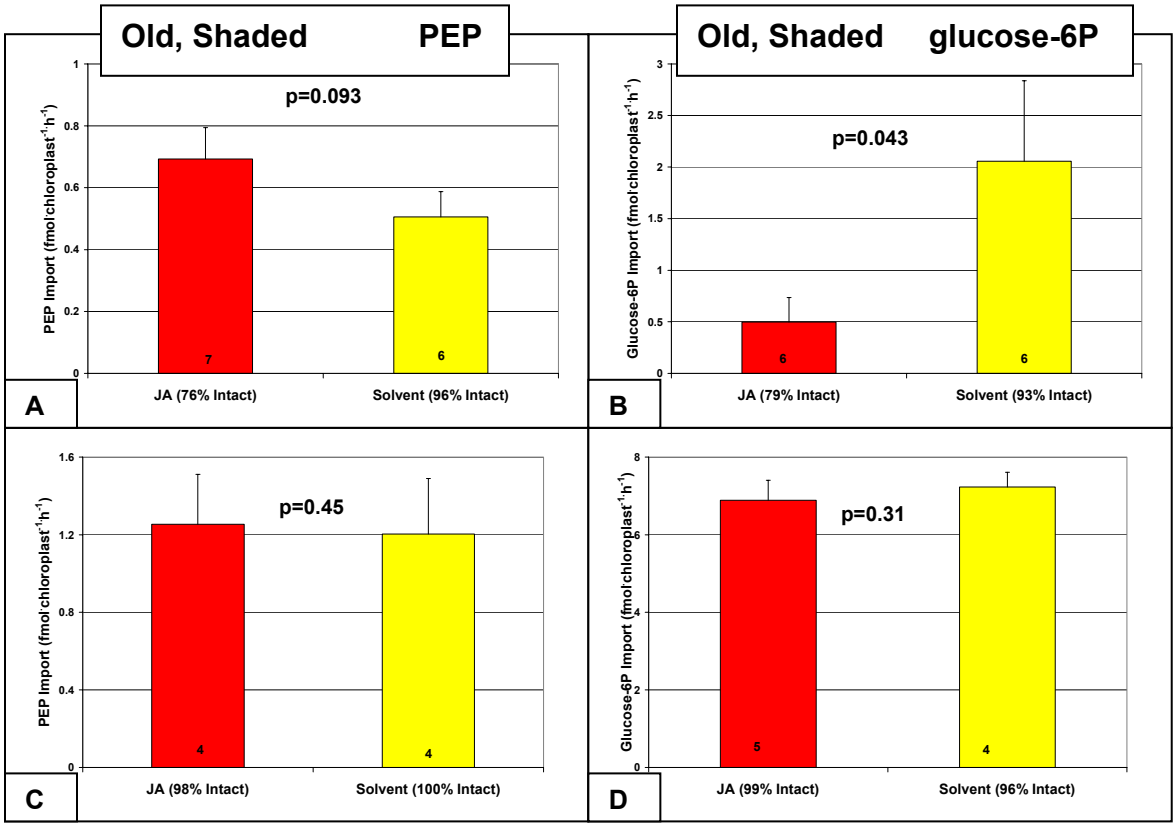


Figure 3.7. Transport rates of 1mM $^{14}$C-PEP (A, B) and 6.5mM $^{14}$C-glucose-6P (C, D) into chloroplasts isolated from old, shaded tobacco leaves. Results from two replicate experiments for each metabolite are presented. Plants were sprayed with either solvent [3% (v/v) ethanol] or JA (5mM in solvent) and maintained in a day/night cycle with shade ($26\mu mol \cdot m^{-2} \cdot s^{-1}$) for 48h prior to chloroplast isolation. Metabolite uptake experiments were conducted in the dark. Bars represent the mean ± standard error for N replicates (N is indicated on the bottom of each bar). Statistical differences estimated with 1-tailed Student's t-test.

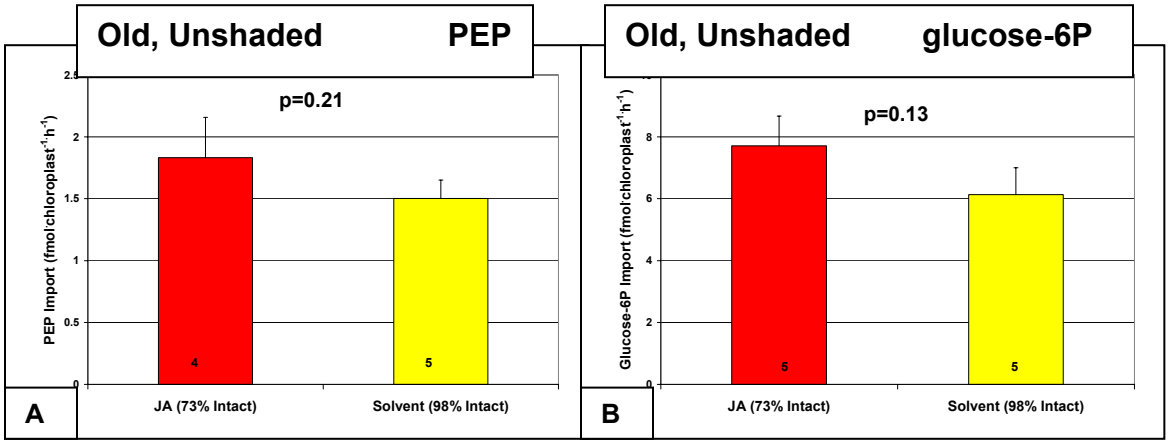


Figure 3.8. Transport rates of 1mM $^{14}$C-PEP (A) and 6.5mM $^{14}$C-glucose-6P (B) into chloroplasts isolated from old, unshaded tobacco leaves. Plants were sprayed with either solvent [3% (v/v) ethanol] or JA (5mM in solvent) and maintained in a day/night cycle with high light (204 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 48h prior to chloroplast isolation. Metabolite uptake experiments were conducted in the dark. Bars represent the mean ± standard error for N replicates (N is indicated on the bottom of each bar). Statistical differences estimated with 1-tailed Student's t-test.

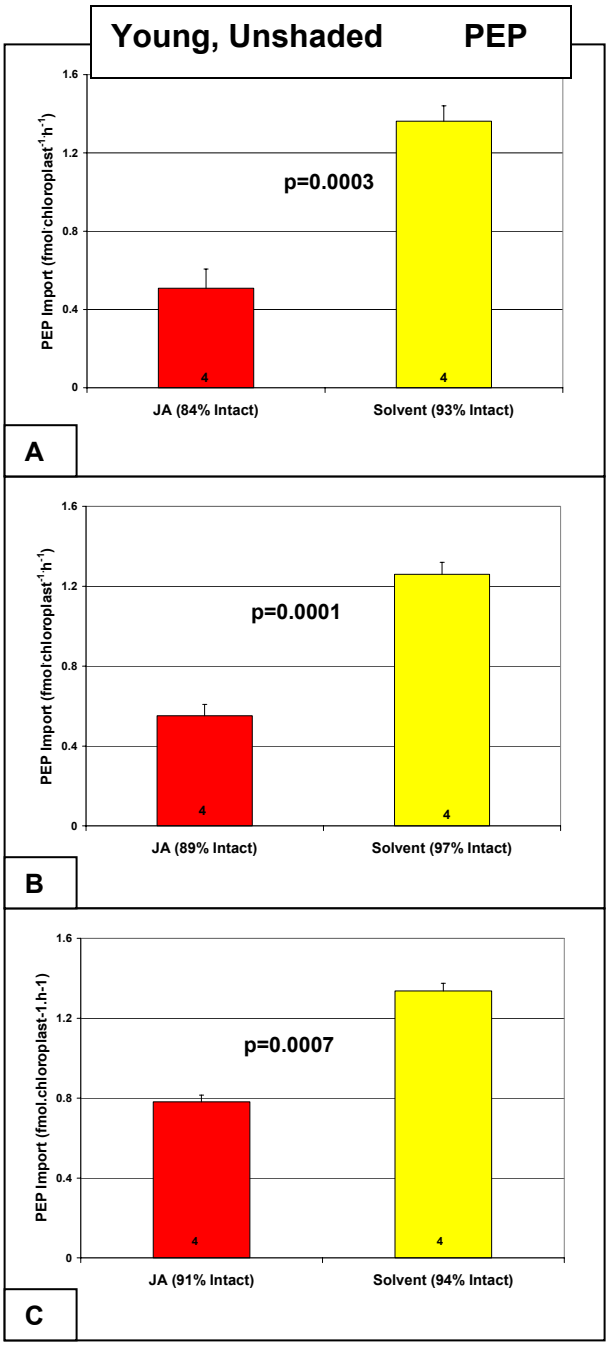


Figure 3.9. Transport rates of 0.5mM $^{14}$C-PEP into chloroplasts isolated from young, unshaded tobacco leaves. Data from three experimental replicates are presented. Plants were sprayed with either solvent [3% (v/v) ethanol] or JA (5mM in solvent) and maintained in a day/night cycle with high light (204 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 24h prior to chloroplast isolation. Metabolite uptake experiments were conducted in the light. Bars represent the mean ± standard error for N replicates (N is indicated on the bottom of each bar). Statistical differences estimated with 1-tailed Student's t-test.

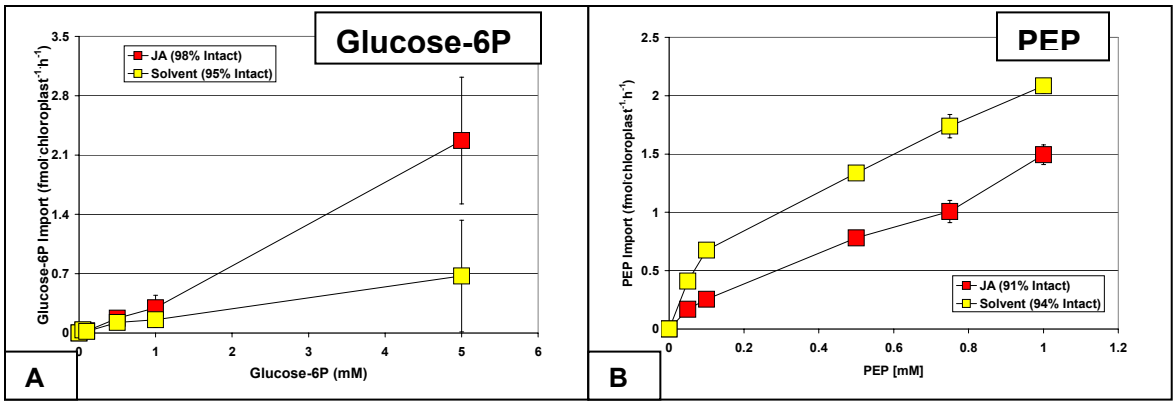


Figure 3.10. Substrate saturation of $^{14}$C-glucose-P import into chloroplasts isolated from young, unshaded leaves (A) and $^{14}$C-PEP import into chloroplasts isolated from young, shaded leaves from six-week old tobacco plants. Plants were sprayed with either solvent [3% (v/v) ethanol] or JA (5mM in solvent) and maintained in a day/night cycle with high light (A, 200 µmol·m$^{-2}$·s$^{-1}$) or shade (B, 26 µmol·m$^{-2}$·s$^{-1}$) for 24 or 48h, respectively. Means ± standard error, N= three to four replicates per data point.

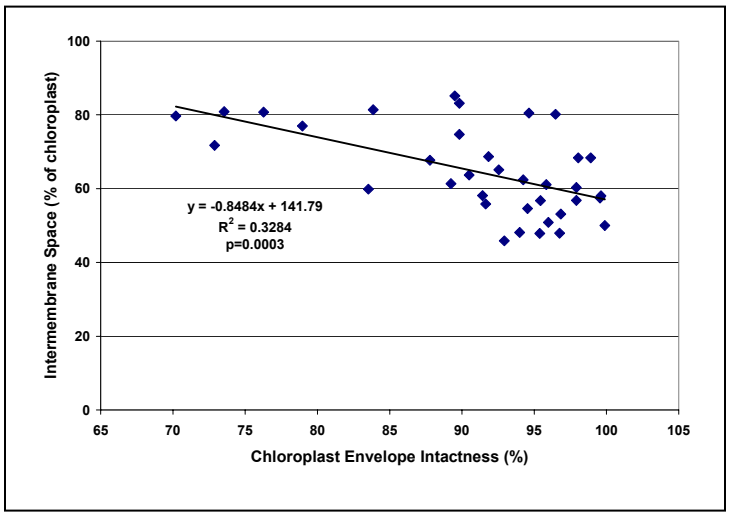


Figure 3.11. Regression of chloroplast envelope intactness versus intermembrane space size in chloroplasts isolated from six-week old tobacco plants. Plants were sprayed with either solvent [3% (v/v) ethanol] or JA (5mM in solvent) and maintained in a day/night cycle with high light (204 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or shade (26 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 24h or 48h prior to chloroplast isolation. Data points were combined for all treatments.

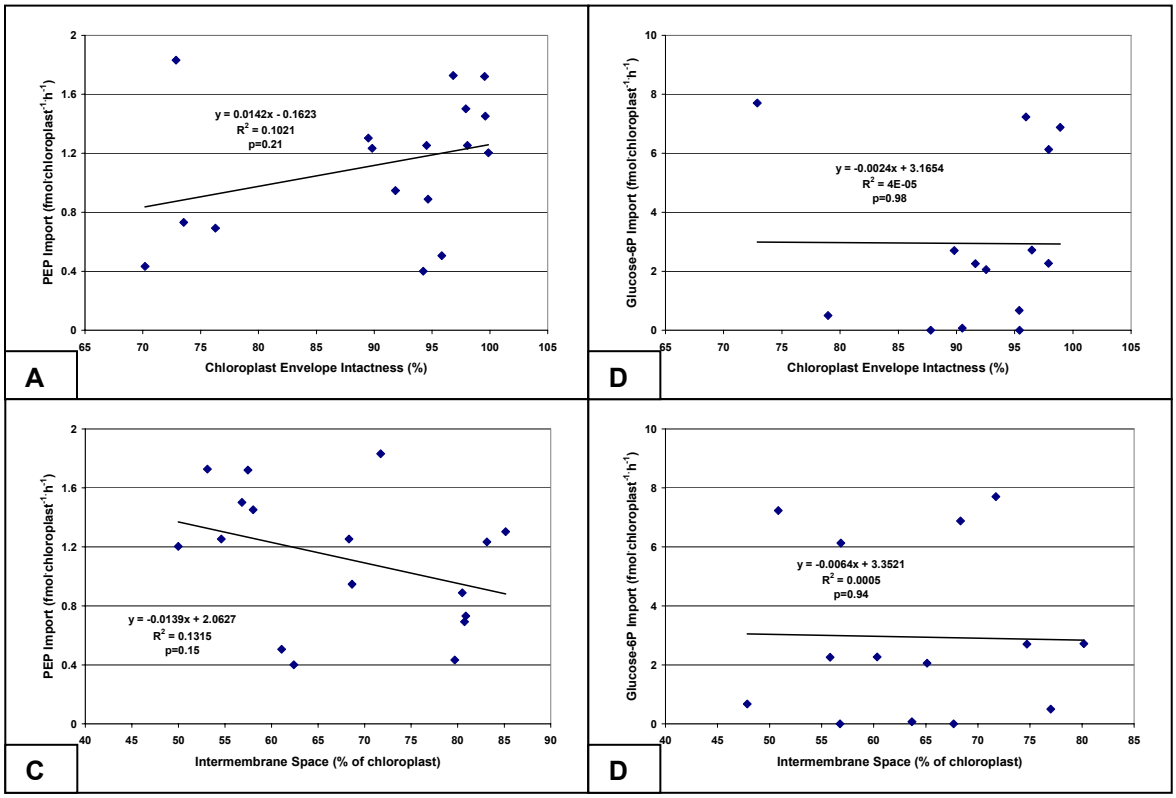


Figure 3.12. Regressions of chloroplast envelope intactness versus PEP import (A), chloroplast envelope intactness versus glucose-6P import (B) intermembrane space versus PEP import (C), and intermembrane space versus glucose-6P import (D). Chloroplasts were isolated from six-week old tobacco plants sprayed with either solvent [3% (v/v) ethanol] or JA (5mM in solvent) and maintained in a day/night cycle with high light (204 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or shade (26$\mu mol \cdot m^{-2} \cdot s^{-1}$) for 24h or 48h prior to chloroplast isolation. Data points were combined for all treatments.

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# CHAPTER 4

# EFFECTS OF PHOSPHOENOLPYRUVATE/PHOSPHATE TRANSLOCATOR MUTATION ON INDUCED DEFENSES AND HERBIVORE FITNESS

## INTRODUCTION

Plants are frequently induced to accumulate defensive phenolic compounds in response to attacking insect herbivores and plant pathogens (Karban and Baldwin 1997). During such defense responses, it is common to measure enhanced gene expression and activities of enzymes in the shikimate and phenylpropanoid pathways which constitute the core reactions of phenolic metabolism (See Chapter 1 for references). Because induced shikimate pathway reactions occur within the chloroplast, we would expect enhanced levels of the substrates erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) to be available within the chloroplast during phenolic induction responses (**Fig. 4.1**). Since Calvin Cycle intermediates can replenish stromal PEP and E4P (Schulze-Siebert and Shultz, 1989), we expected photosynthesis to be enhanced in plants undergoing phenolic induction responses. However, Calvin Cycle activity is often repressed under these conditions (Chapter 2 and references therein) and allocation of products of the Calvin Cycle to phenolic synthesis may actually decrease when plants are chewed by insects (Zangerl et al. 1997). Thus, the Calvin Cycle is unlikely to be the primary source of substrates for the shikimate pathway during phenolic induction responses.

An alternate source of substrates for shikimate metabolism in the chloroplast may exist in the cytoplasm, as the chloroplast inner envelope contains several phosphate transporters that mediate exchange of metabolites between the cytoplasm and chloroplast stroma (Flügge, 1999). Furthermore, activity of one of these transporters, the PEP:phosphate translocator (PPT), has recently been linked to phenolic synthesis (Streatfield et al., 1999); the Arabidopsis *ppt1-1* mutant lacks *PPT* gene expression and is unable to import PEP into chloroplasts via activity of this transporter. Using the *ppt1-1* mutant, these authors demonstrated that levels of constitutive phenolics were substantially lower in mutant plants

when compared to the wild-type. These results indicate that cytoplasmic PEP provides an additional source of substrates for the chloroplast shikimate pathway, leading to phenolic synthesis. Whether phenolic induction responses elicited by enemies are also constrained by PPT activity remains to be tested.

The following hypothesis was developed to investigate the role of the PPT in providing PEP substrates to the chloroplast during phenolic induction responses: induced phenolic accumulation that decreases insect fitness requires PEP:phosphate translocator activity. We exploited the availability of two previously characterized Arabidopsis *PPT* mutants, *cue1-6* in Columbia and *ppt1-1* from the Bensheim ecotype (Li et al., 1995; Streatfield et al., 1999). Phenolic metabolism was stimulated by treating rosettes with Jasmonic acid (JA), a signal which elicits phenolic induction responses in plant leaves much as does wounding or herbivory (Creelman and Mullet, 1997; Parthier, 1991; Rickauer et al., 1992; Thaler et al., 1996) and measured changes in foliar phenolics following treatment. If PEP imported via the PPT is a significant source of stromal PEP for the synthesis of induced phenolics, we would expect to observe little or no phenolic induction in *ppt1-1* and *cue1-6* mutants following JA treatment. To investigate the ecological significance of this mutation, we measured fitness responses of larvae of the cabbage butterfly (*Pieris rapae*) to previously treated plants. We predicted that the *ppt1-1* and *cue1-6* mutants would support higher growth rates if the *PPT* mutation limited phenolic accumulation or induction responses.

## METHODS

### Seed Source and Plant Growth Conditions

We obtained wild type plants and the corresponding *PPT* mutants from two Arabidopsis ecotypes, Columbia and Bensheim. Columbia wild-type (Co-O, CS1092) and its mutant (*cue1-6*, CS3168) and the Bensheim mutant plants (*ppt1-1*, CS8858) were provided by the Arabidopsis Biological Resource Center (Columbus, OH). Bensheim wild type seeds (pOCA108, described in Li et al. 1995) were a gift from Hsou-min Li (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). Seeds were vernalized for two to three days at

4ºC in 0.15% (w/v) agar prior to rinses in 95% (v/v) ethanol, 10% (v/v) bleach, and autoclaved water and subsequently transferred to Petri dishes containing growth medium [Murashige Skoog Salt Mixture, Gamborg's vitamin mixture, 0.05% (w/v) MES, and 0.8% (w/v) phytagel, pH 5.7] supplemented with 1% (w/v) sucrose (Li et al., 1995). Plants were reared in a growth chamber with 100-150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent and incandescent light, 12h photoperiod, 24ºC day, 22ºC night (Environmental Growth Chambers, Chagrin Falls, OH). Two weeks after sowing seeds, we transferred seedlings to 5.5cm x 6cm pots and allowed the plants to become acclimated to chamber humidity levels over a one-week period. Plants were fertilized with Miracle Grow and randomly repositioned within the growth chamber semi-weekly.

**Egg Source and Insect Growth Conditions**

We purchased cabbage butterfly (*Pieris rapae*) eggs from Carolina Biological Supply Company (Burlington, NC) and subsequently maintained insect colonies through adulthood on five-week old cabbage plants (Chinese cabbage, Burpee Co.) contained in sealed growth cages. Pupae were placed into separate growth cages and following emergence, adults were provided with 10% sucrose solutions containing yellow dye as previously described (Grant-Petersson and Renwick, 1996).

**Phenolic Induction Experiment**

Plants were four to five weeks old at the beginning of the phenolic induction experiments. We conducted experiments at this time to allow mutant plants to produce enough foliage to support insect feeding for two days. Plants were randomly assigned to one of three treatments: water, solvent [1.47% (v/v) ethanol and 0.125% (v/v) Triton] or Jasmonic acid (JA, 2.5mM dissolved solvent). Treatments were applied as a fine mist to the adaxial side of all rosette leaves on the first day of the experiment and again two additional times during a one-week period. The induction experiment was terminated seven days following the first treatment, after which plants were either set aside for the insect bioassay or rosette leaves were frozen in liquid nitrogen and stored at -20ºC. Plant growth was

assessed by measuring rosette diameters on the first and last days of the phenolic induction experiment and calculating specific growth rates ($mm \cdot mm^{-1}$rosette diameter$\cdot d^{-1}$).

**Insect Bioassay Experiment**

Prior to the experiment, early first or second instar larvae were transferred from cabbage leaves to Petri dishes containing moistened filter paper and maintained in the dark at 4ºC for up to three days. We prepared plants for the bioassay by attaching a frass collector (a circle prepared from soft plastic) beneath each rosette. Insects were removed from cold storage, weighed, and placed onto randomly assigned plants. Plants were returned to the growth chamber and the bioassay was allowed to proceed until nearly all of the JA-treated mutants were eaten (*two days*: Bensheim bioassays with first and second instars and Columbia bioassay with second instars; *four days*: Columbia bioassay with first instars). At this time, insects were removed from rosettes, weighed, and dried at 75ºC. Frass pellets were collected from each plant and oven dried. Larval specific growth rates were calculated as $mg \cdot mg^{-1}$fresh weight$\cdot d^{-1}$. Total insect growth per amount of consumed leaf was measured as mg larval dry weight$\cdot mg^{-1}$ dry weight frass equivalents$\cdot d^{-1}$ with the exception of second instars on Bensheim plants, where we used larval fresh weights since dry weights were not calculated for this experiment.

**Leaf Chemistry**

Frozen leaves were freeze-dried and subsequently ground to a fine powder with liquid nitrogen using a mortar and pestle. Mutant plants were often too small to grind individually, so two to six mutant rosettes were randomly combined for each data point. Leaf protein content was estimated using BSA as the standard (Jones et al., 1989). Anthocyanins were extracted in acidified methanol, chlorophyll was removed with chloroform, and absorbance measured at 530nm, correcting for chlorophyll ($A_{530}$-$A_{652}$) (Martin et al., 2002). For protein and anthocyanin assays, absorption readings for each sample were collected in triplicate using a SPECTRAMax Plus microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). Flavonoid glycosides and aglycones were extracted from leaf samples and

measured using HPLC procedures as described by Pelletier et al. (1999). We used a Waters chromatography system consisting of a model 515 dual-pump system, a model 486 single wavelength detector, and a model 717 autosampler set to 4ºC (Waters, Bedford, MA). Samples were detected (255nm) after fractionation at room temperature on a reversed phase column (Nova-Pak 60 Å, 4µm, 3.9mm x 150mm) at a flow rate of 2.5 mL/min.

**Statistical Analyses**

We used the general linear model procedure in SAS (SAS Institute, Cary, NC) to assess the effects of JA on leaf chemistry and insect growth parameters. Ecotypes were considered separately in all analyses. When investigating leaf chemistry responses, genotype and treatment effects were included as factors, but insect growth statistics were considered separately for mutant and wild type plants within each ecotype since pleiotropic effects of the *PPT* mutation may have influenced feeing behaviors. When the general linear model produced a significant result ($p<0.05$), we used the Tukey lines procedure to assess statistical differences between treatment means.

## RESULTS

**Changes in Foliar Chemistry Induced by Jasmonic Acid**

*Anthocyanins*

Constitutive anthocyanin levels were not influenced by the *PPT* mutation, as levels were similar between wild type and mutant plants (**Fig. 4**). JA strongly induced anthocyanins in both ecotypes (**Fig. 4.2A**, $F=180.9$, $p<0.0001$; **Fig. 4.2B**, $F=92.4$, $p<0.0001$; **Fig. 4.2C**, $F=55.7$, $p<0.0001$; **Fig. 4.2D**, $F=38.4$, $p<0.0001$). Anthocyanins were similarly induced 8.5-9.5 times over control levels in both wild type and *cue1-6* rosettes, but the final levels attained were 65-84% greater in wild type when compared to *cue1-6* rosettes (**Fig. 4.2A**, $F=15.5$, $p=0.0009$; **Fig. 4.2B**, $F=5.02$, $p=0.0283$). In the Bensheim ecotype, anthocyanins were strongly induced in the wild type (13-fold), but *ppt1-1* mutants were

induced in only one of two replicate experiments (**Fig. 4.2C**, 6-fold; **Fig. 4.2D**, 4-fold increase, but not significantly higher than controls). Final levels induced by JA were 2.4-3.7 times higher in wild-type rosettes compared to *ppt1-1* mutants (**Fig. 4.2C**, F=11.1, p=0.0049; **Fig. 4.2D**, F=15.7, p=0.0012).

*Protein*

Eight to ten percent of the rosette dry weight in both genotypes within each Arabidopsis ecotype was comprised of protein, and levels of protein were unaffected by JA treatment in all replicates of this experiment (**Fig. 4.3A**, F=1.3, p=0.34; **Fig. 4.3B**, F=0.91, p=0.51; **Fig. 4.3C**, F=1.36, p=0.33; **Fig. 4.3D**, F=1.74, p=0.22).

*Flavonoid glycoside HPLC technique*

HPLC was used to examine flavonoid glycosides from leaf tissues (Pelletier et al., 1999). Representative HPLC chromatograms are shown in **Fig. 4.4**. After hydrolyzing flavonoid glycoside samples, we performed chromatography for aglycones using the method described by Pelletier et al. (1999). Hydrolyzed extracts yielded no quercetin and very little, if any, kaempferol from our plants (data not shown), indicating low levels of flavonoid glycosides in mature *Arabidopsis* leaves. The induced peak in **Fig. 4.5** was identified as an indole-derivative. 3-Indoleacetonitrile was confirmed using LC/MS analysis on a Quattro II mass spectrometer (Micromass, Beverly, MA). Analyses were performed using atmospheric pressure chemical ionization (APCI) in positive ion mode. The following ions were observed: m/z 157 ($[M^+H]^+$) and 130 ($[M^+H\text{-}HCN]^+$). Concentrations of this compound were increased by JA treatment in both ecotypes (**Fig. 4.5 A**, F=195.8, p<0.001; **Fig. 4.5B**, F=55.4, p<0.0001; **Fig. 4.5C**, F=100.8, p<0.0001; **Fig. 4.5D**, F=27.7, p=0.0001) and final induced levels were two times higher in *cue1-6* rosettes compared to Columbia wild types and 5-8 times greater in *ppt1-1* compared to Bensheim wild type rosettes (**Fig. 4.5 A**, F=18.9, p=0.0004; **Fig. 4.5B**, F=4.75, p=0.033; **Fig. 4.5C**, F=61.6, p<0.0001; **Fig. 4.5D**, F=8.8, p=0.0076).

### *Arabidopsis thaliana* Growth Responses to Jasmonic Acid

The *PPT* mutation severely restricted rosette growth in both ecotypes. On the first day of the experiment, wild type rosettes were three to five times larger when compared to mutants (**Fig. 4.6A**, F=2239, p<0.0001; **Fig. 4.6B**, F=1954, p<0.0001; **Fig. 4.6C**, F=2815, p<0.0001; **Fig. 4.6D**, F=559, p<0.0001). During the one-week treatment period, wild type and *cue1-6* rosettes in the Columbia ecotype grew at the same rate (**Fig. 4.7A**, F=0, p=0.95; **Fig. 4.7B**, F=3.74, p=0.055); whereas, *ppt1-1* mutants grew at about half the rate of Bensheim wild type rosettes (**Fig. 4.7C**, 114.7, p<0.0001; **Fig. 4.7D**, 69.7, p<0.0001). Growth rates of both wild type and mutant rosettes were strongly decreased by JA treatment in each ecotype (60-75%, **Fig. 4.7A**, F=239, p<0.0001; **Fig. 4.7B**, F=250, p<0.0001; **Fig. 4.7C**, F=167, p<0.0001; **Fig. 4.7D**, F=77, p<0.0001).

### *Pieris rapae* growth

On the first day of the bioassay, first instar larvae weighed 100-120 μg (fresh weight), while second instars were 7-8 times larger. First and second instars of *P. rapae* grew similarly on wild type rosettes treated with JA or control solutions in all cases but one. In this instance, JA-treated rosettes decreased first instar growth rates 35% on wild type Columbia rosettes (**Fig. 4.8A**, F=20.45, p<0.0001). In contrast, JA-treated *cue1-6* rosettes caused significant reductions in first and second instar growth rates (45% and 40%, respectively, **Fig. 4.9A**, F=31.8, p<0.0002, **Fig. 4.9B**, F=6.6, p=0.0035), while only first instar growth was inhibited on JA-treated *ppt1-1* rosettes (20%, **Fig. 4.9C**, F=6.21, p=0.0049). Second instar growth did not differ between JA treated *ppt1-1* rosettes and controls (**Fig. 4.9D**, F=1.1, p=0.35).

Larval growth was calculated as a function of the dry weight of frass produced to estimate growth efficiency per unit leaf consumed. JA-treated rosettes revealed general inhibitory effects on larval growth when data were expressed in this manner. With the exception of first instars feeding on Columbia wild type rosettes (**Fig. 4.10A**), larvae in all JA treatment groups exhibited decreased growth per amount of leaf consumed (25%

inhibition of second instars on Columbia, **Fig. 4.10B**, F=10.06, p=0.0003; 25% inhibition of first instars on Bensheim, **Fig. 4.10C**, F=9.6, p=0.011; 30% inhibition of second instars on Bensheim, **Fig. 4.10D**, F=3.8, p=0.0346). On mutant plants, three treatment groups displayed decreased larval growth per amount of leaf consumed (25% inhibition of first instars on *cue1*-6, **Fig. 4.11A**, F=5.99, p=0.0056; 43% inhibition of first instars on *ppt1-1* rosettes, **Fig. 4.11C**, F=4.33, p=0.026; 30% inhibition of second instars on *ppt1-1*, **Fig. 4.11D**, F=4.97, p=0.0142) but second instars feeding on *cue1-6* rosettes were unaffected by JA (**Fig. 4.11B**).

## DISCUSSION

### Accumulation of Constitutive and Induced Phenolics is not Prevented in *PPT* Mutants

A link between PPT activity and phenolic synthesis was originally demonstrated by Streatfield et al. (1999) as reduced constitutive phenolic synthesis in *ppt1-1* mutants. Those authors reported 80% lower anthocyanin concentrations in *ppt1-1* plants grown under 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for seven days following transfer from low light (35 $\mu mol \cdot m^{-2} \cdot s^{-1}$) than in comparably-treated wild types. We were unable to demonstrate such dramatic decreases in constitutive levels in either *cue1-6* or *ppt1-1* mutants. Concentrations of constitutive phenolics were decreased by only 55% in *cue1-6* mutants but unaffected in *ppt1-1*, the same genotype used by Streatfield et al. (1999). The Streatfield et al. study differed from ours in three significant ways. First, those authors expressed anthocyanin concentration on a fresh weight basis, which could artificially decrease anthocyanin levels if water content were elevated in mutant tissue. Second, plants in the Streatfield et al. study were 2.5 weeks younger and were grown in tissue culture medium supplemented with 1% sucrose, while our plants were removed from culture medium after the first two weeks of growth. Hexoses can decrease photosynthesis and stimulate phenolic metabolism (Ehness et al., 1997) and increase glucose-6P import into chloroplasts (Quick et al., 1995), while phenolic metabolism is sensitive to leaf development (Cosio and McClure, 1984), all differences which may have influenced anthocyanin levels independently of the *PPT* mutation in the earlier study, thus making it incomparable with our results. Streatfield et al. (1999) also reported decreases in

other phenolics (57-80%, expressed as a proportion of leaf dry weight of cultured, 26-day old plants) in *ppt1-1* mutants but neither presented specific results nor methods used to identify these putative phenolics.

Our results indicate that phenolic induction responses can occur in the absence of PPT-mediated PEP import into the chloroplast, the site of induced shikimate metabolism and thus we reject the hypothesis that induced phenolic accumulation requires PPT activity. We found that concentrations of induced anthocyanins in *ppt1-1* and *cue1-6* rosettes, which lack *PPT* gene expression (Streatfield et al., 1999), were somewhat lower than corresponding Columbia and Bensheim wild type plants. But we also found that JA-elicited increases in anthocyanins were only slightly constrained in *ppt1-1* mutants and were unaffected by the *PPT* mutation in *cue1-6* plants. These results indicate that chloroplasts must have alternate sources of PEP for induced phenolic metabolism. This is not surprising, given the central role of the shikimate pathway in plant metabolism. Shikimate pathway intermediates are substrates for the synthesis of a variety of compounds with important roles in plant growth and development, including proteins, signal molecules (salicylic acid), electron carriers (plastoquinone) and hormones (auxin).

The strong pleiotropic effects of the *PPT* mutation illustrate the central role that this translocator plays in plant development. Mutant plants require sucrose to germinate, exhibit stunted root and shoot growth, and show altered leaf morphology (fewer mesophyll cells which also contain smaller chloroplasts) (Li et al., 1995; Streatfield et al., 1999). In addition, chlorophyll levels and photosynthetic gene expression are strongly decreased in *PPT* mutants (e.g. *CAB*, *rbcL*, *RBCS*, *psbA*) (Li et al., 1995). If PEP import via the PPT were the only source of stromal PEP for shikimate metabolism, the mutation would be lethal and phenolic induction by JA would not have occurred. Constraints on chloroplast PEP import were suggested by Streatfield et al.'s (1999) results, but the authors did not isolate chloroplasts from *ppt1-1* plants to confirm that PEP import was prevented by the mutation.

There are several potential alternative sources of PEP for the chloroplast. Stromal PEP could be supplied to the shikimate pathway via other transporters. While the triose

phosphate:phosphate translocator (TPT) has specific preference for triose phosphates (dihydroxyacetone phosphate and glyceraldehyde-3P) and 3-phosphoglycerate, it also transports PEP, although it has lower affinity for this substrate compared to triose phosphates (Fischer et al. 1997). TPT activity can be enhanced to meet changing carbohydrate demands within the plant (Knight and Gray, 1994; Schulz et al., 1993), but its role in providing stromal PEP during defense responses is unstudied. Calvin Cycle intermediates are unlikely sources of additional PEP for induced phenolic synthesis, since enemies and wound signals commonly repress photosynthetic gene expression and carbon assimilation (Chapter 2 and references therein). In fact, carbon resource allocation to phenolic synthesis actually decreases in wild parsnip plants when phenolic synthesis is elicited (Zangerl et al., 1997). The substantial pool of stored carbon reserves in plastidic starch may provide an alternate source of substrates for phenolic synthesis (Mita et al., 1997). However, the ability of starch to provide PEP for induced phenolic metabolism would require activity of phosphoglycerate mutase and enolase within the chloroplast, but the presence and activity of these enzymes in chloroplasts is still a matter of debate (Hoppe et al., 1993; Ramachandra and Rama Das, 1995; Stitt and ap Rees, 1979; van der Straeten et al., 1991).

**Insect Growth is Decreased on JA-Treated *PPT* Mutants**

We expected *PPT* mutants to be less sensitive to JA treatment, yielding a higher quality food source for *P. rapae* larvae when compared to treated wild types. What we found was the opposite. Mutant plants treated with JA were a poorer food source for larvae than were similarly treated wild types. Compared to controls, treated *cue1-6* rosettes caused the greatest decreases in specific growth rate of both first and second instars, followed by *ppt1-1*, Columbia and finally Bensheim wild types, the latter having no effect on insect growth. Even in the absence of JA, wild-type rosettes in general supported higher specific growth rates of *P. rapae* larvae. When we expressed insect growth in proportion to frass produced, growth efficiency was reduced on both wild type and *PPT* mutant tissues when treated with JA compared to controls. These data indicate that mutant plants lacking the ability to import PEP into the chloroplast via the PPT are not constrained in their ability to deter an insect herbivore.

However, increases in phenolics do not explain the decreased larval growth responses to JA-treated *PPT* mutants. Since insect growth was worst on treated mutant rosettes, we expected to detect the highest levels of induced phenolics in these tissues if phenolic induction was responsible for repressed insect growth. But final anthocyanin concentrations in induced *cue1-6* and *ppt1-1* rosettes were lower than in the wild types, indicating that induced anthocyanin responses were not the primary foliar change contributing to reduced larval growth. This may not be unexpected, as evidence that anthocyanins act as chemical defenses is equivocal (Steyn et al., 2002). We also anticipated flavonoid glycoside and aglycone increases in JA-treated plants since these phenolics are routinely detected in Arabidopsis (Pelletier et al., 1999), are induced by UV treatment (Martin et al., 2002) and have a demonstrated toxicity toward *P. rapae* (Grant-Petersson and Renwick, 1996). But mature rosettes contained very low levels of kaempferol and we detected no quercetin glycosides or aglycones. Our inability to detect these compounds may be related to the ecotypes we used in this study (Bensheim and Columbia). These compounds are more commonly measured in the Landsberg ecotype (Pelletier et al., 1999). These results point to the fact that such metabolites decrease with age in Arabidopsis, as they do in other plants (Kerckhoffs et al., 1992), a phenomenon which can be attributed to decreased activity of flavonoid metabolism in mature leaves (Cosio and McClure, 1984).

Foliar nitrogen in the form of protein and amino acids is also a major factor influencing leaf quality as food for insects (Mattson, 1980). A previous study showed that *ppt1-1* mutants had higher levels of free amino acids than did wild type plants (Streatfield et al., 1999). In that study, levels of aromatic amino acids (shikimate pathway products: phenylalanine, tyrosine and tryptophan) were proportionately lower than non-aromatic amino acids in this mutant compared to the wild type. In addition, both *RBCS* and *rbcL* mRNA levels were decreased in *ppt1-1* mutants (Li et al., 1995), and since Rubisco is the most abundant protein in plant leaves, we anticipated finding lower constitutive protein levels in *PPT* mutants. However, total protein levels were similar in wild type and mutant plants, and protein was unaffected by JA treatment. Mutants must synthesize a different complement of foliar proteins since total protein levels are comparable to the wild type. Thus, larval growth

repressions caused by *PPT* mutants or JA-treated rosettes are most likely not caused by protein deficiencies.

Foliar trichomes may have contributed to the decreased larval growth responses on *PPT* mutants. We often noticed larvae walking on trichomes of *PPT* rosettes, the leaves of which presented a fuzzy appearance and may have contained elevated trichome density per unit leaf area compared to wild type rosettes (personal observation). These defenses present physical barriers that have been shown to deter insect herbivores on crucifer plants (Lamb, 1980). Trichomes can be additionally induced in crucifers by JA and herbivory (Agrawal, 1999), and *P. rapae* growth can be decreased by crucifers exhibiting induced trichome defenses, but in a species-dependent manner (Agrawal, 1999; Traw and Dawson, 2002). We did not measure trichome densities in JA-treated plants, so are unable to relate growth decreases to trichome induction. However, it is unlikely that trichome induction was involved in induced defenses in this study, since induced trichomes occur on newly-formed leaves (Agrawal, 1999; Traw and Dawson, 2002). Mutant plants did not appear to initiate new leaf growth during the one-week treatment period and larval feeding did not appear to be related to leaf age in wild-type plants (personal observation).

Variation in growth rates of *P. rapae* larvae on JA-treated plants was closely correlated with concentrations of the indole compound we found in HPLC analyses. Specific larval growth rates were lowest on treated *cue1-6* rosettes, followed by *ppt1-1*, Columbia wild type and Bensheim wild type. This pattern is the inverse of indole concentrations among plants and treatments. This variation probably reflects indole glucosinolate (IGS) induction in JA-treated treated plants. While we did not measure IGSs directly, they are commonly induced by herbivory (Koritsas et al., 1991; Bodnyark, 1992; Doughty et al., 1995; Bartlet et al., 1999), and their induction by JA has recently been demonstrated in Arabidopsis (Brader et al., 1999). While *P. rapae* is considered to be adapted to glucosinolate-containing diets (Agrawal and Sherriffs, 2001), our results suggest that the concentrations we found in our experimental system can influence feeding and growth of very young (first instar) larvae (see Agrawal, 2000).

It is remarkable that *PPT* mutants were more inducible than wild type plants given that tryptophan, an intermediate of the shikimate pathway, is a biosynthetic precursor to IGS synthesis (Brader et al., 2001). Our indole accumulation data provide further evidence that the *PPT* mutation does not constrain shikimate-derived inducible defense responses in *Arabidopsis*. This leads us to reject the hypothesis that induced shikimate-based defenses that decrease insect fitness require PPT activity.

**Summary and Conclusions**

Our results suggest that chloroplasts possess multiple means of acquiring PEP substrates for elicited increases in shikimate metabolism. In *cue1-6* and *ppt1-1* mutants, chloroplasts are unable to import PEP via the PPT. We anticipated that the inability to import PEP via this transporter would also hinder JA-elicited increases in chemicals synthesized from shikimate pathway products, phenylalanine, tyrosine, and tryptophan. However, induced increases in anthocyanins were not suppressed in *cue1-6* mutants and were only modestly reduced in *ppt1-1* mutants compared to wild types. At the same time, indole-containing compounds increased more in JA-treated mutant plants than in wild type plants. Because tryptophan is a precursor of indole synthesis while phenylalanine is required for phenylpropanoid synthesis, these results suggest a trade-off between these two defense classes based on competition for substrates derived from the shikimate pathway. Protein synthesis, another sink for products of the shikimate pathway was unaffected by the *PPT* mutation in either ecotype. We did not assess possible changes in protein composition in these plants. The pleiotropic effects of the *PPT* mutation on plant metabolism and insect fitness highlight the inherent complexities in studying effects of single gene removal on plant metabolism, which is inherently plastic in a changing environment.

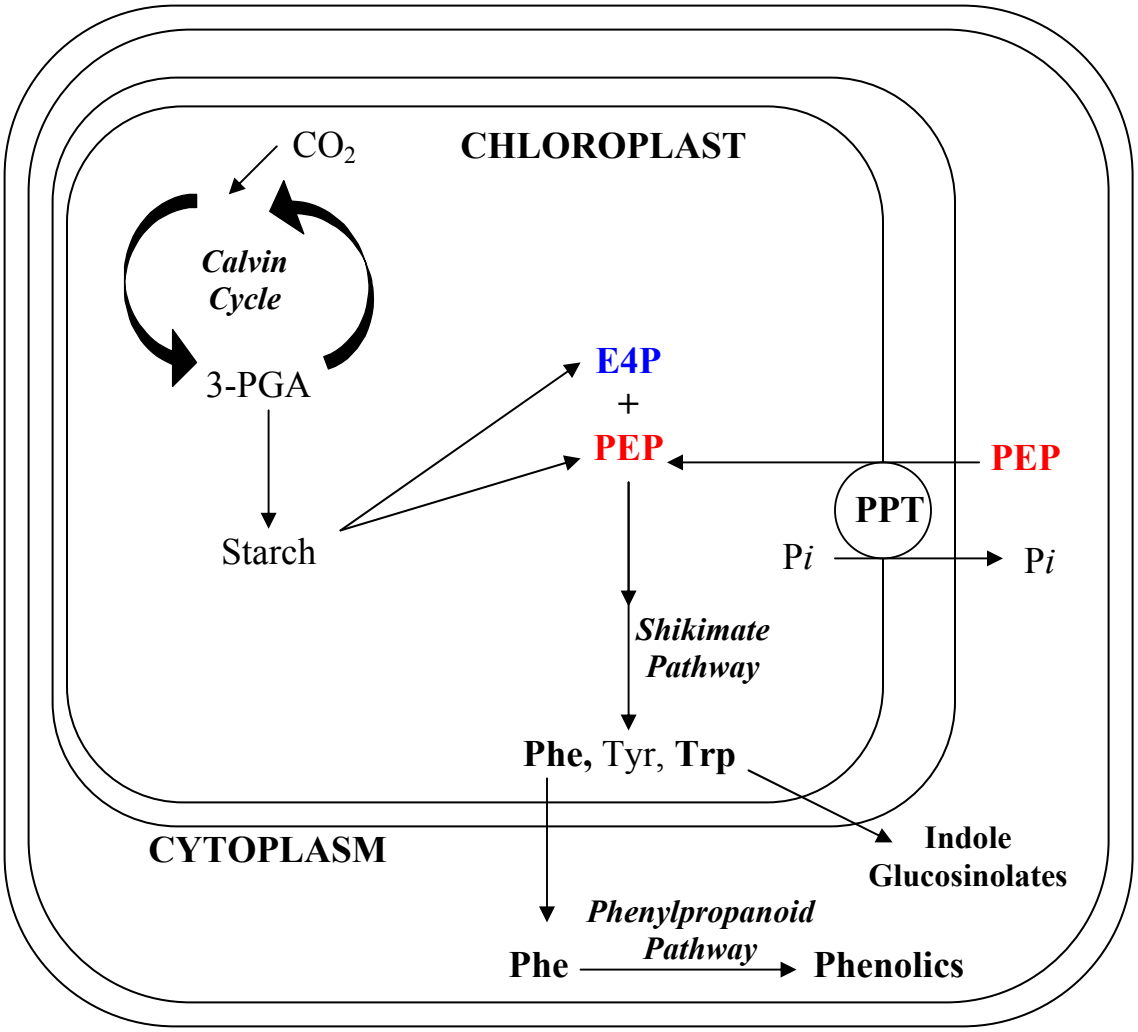


Figure 4.1. Possible routes of carbon resource supply to phenolic metabolism. E4P, erythrose-4-phosphate; ENO, enolase; OPP, oxidative pentose phosphate; PEP, phosphoenolpyruvate; PGM, phosphoglycerate mutase; P*i*, inorganic phosphate; PPT, PEP:P*i* translocator; Phe, 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; phenylalanine; Trp, tryptophan; Tyr, tyrosine.

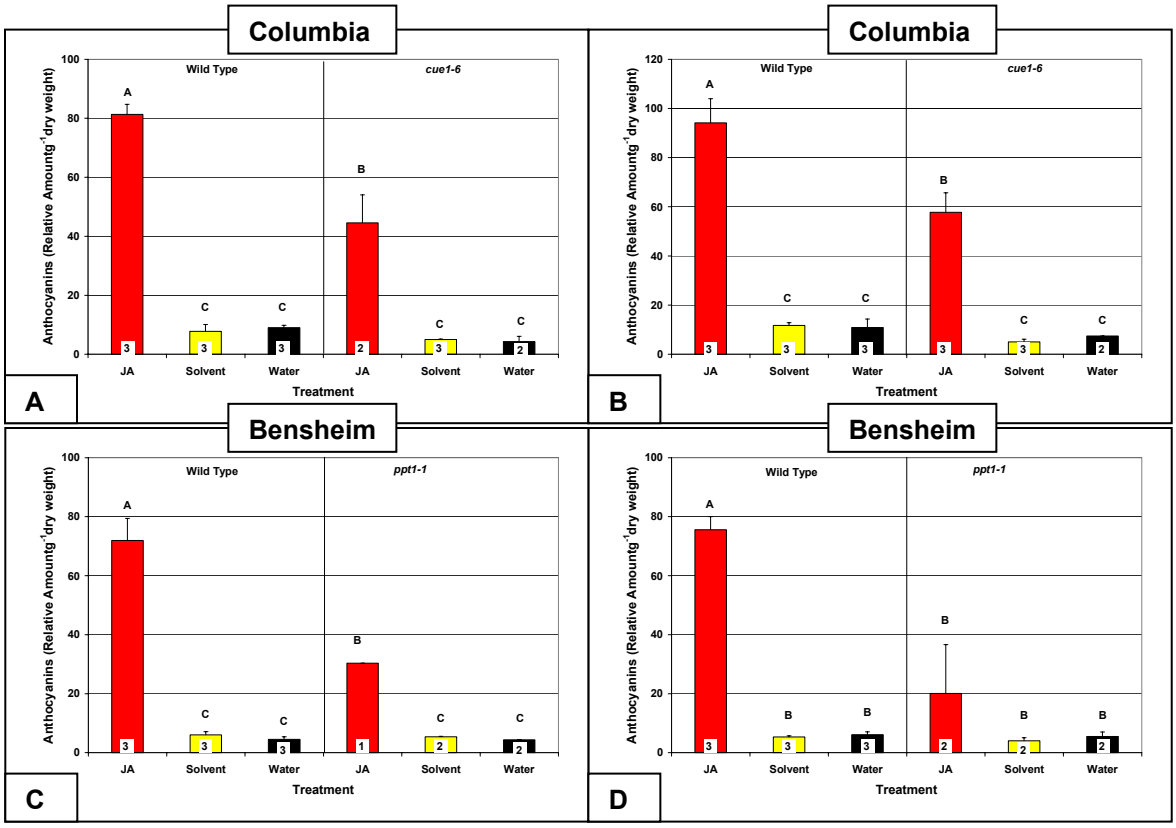


Figure 4.2. Anthocyanin content of Arabidopsis wild type and PEP:phosphate translocator mutants in Columbia (A, B) and Bensheim (C, D) ecotypes. Mean values ± standard error represent relative amounts of anthocyanins in response to treatment. Treatments were applied three times during one week. Solvent [1.9% (v/v) ethanol and 0.125% (v/v) Triton], Jasmonic acid (JA, 2.5mM dissolved in solvent). Sample sizes are indicated on bars. Means with the same letter are not significantly different (p=0.05, Tukey test).

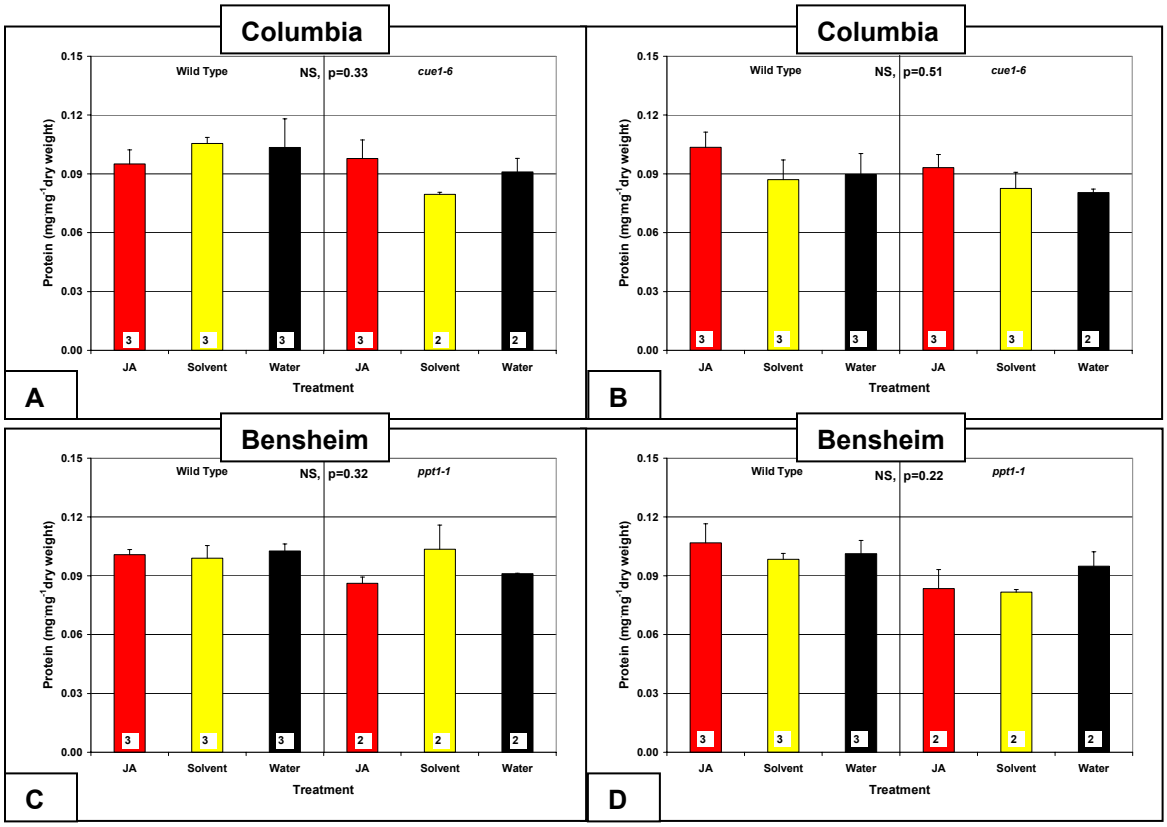


Figure 4.3. Protein content of Arabidopsis wild type and PEP:phosphate translocator mutants in Columbia (A, B) and Bensheim (C, D) ecotypes. Mean values ± standard error represent relative amounts of protein in response to treatment. Treatments were applied three times during one week. Solvent [1.9% (v/v) ethanol and 0.125% (v/v) Triton], Jasmonic acid (JA, 2.5mM dissolved in solvent). Sample sizes are indicated on bars. Means with the same letter are not significantly different (p=0.05, Tukey test).

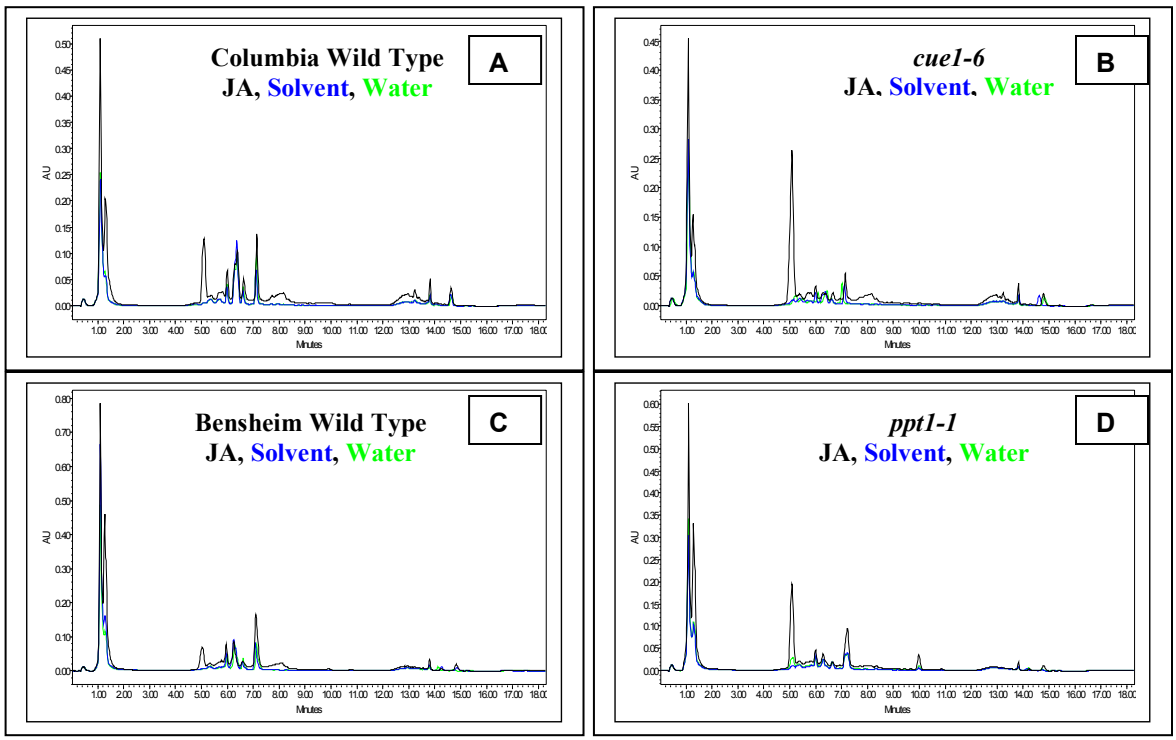


Figure 4.4. HPLC chromatograms from Columbia wild type and *cue1-6* mutant (A, B) and Bensheim wild type and *ppt1-1* mutant (C, D). Treatments were applied three times during one week. Values are absorbance units (AU) at 255nm. Solvent [1.9% (v/v) ethanol and 0.125% (v/v) Triton], Jasmonic acid (JA, 2.5mM dissolved in solvent). Results are presented for representative plants from bioassay experiments with first instar *Pieris rapae* larvae.

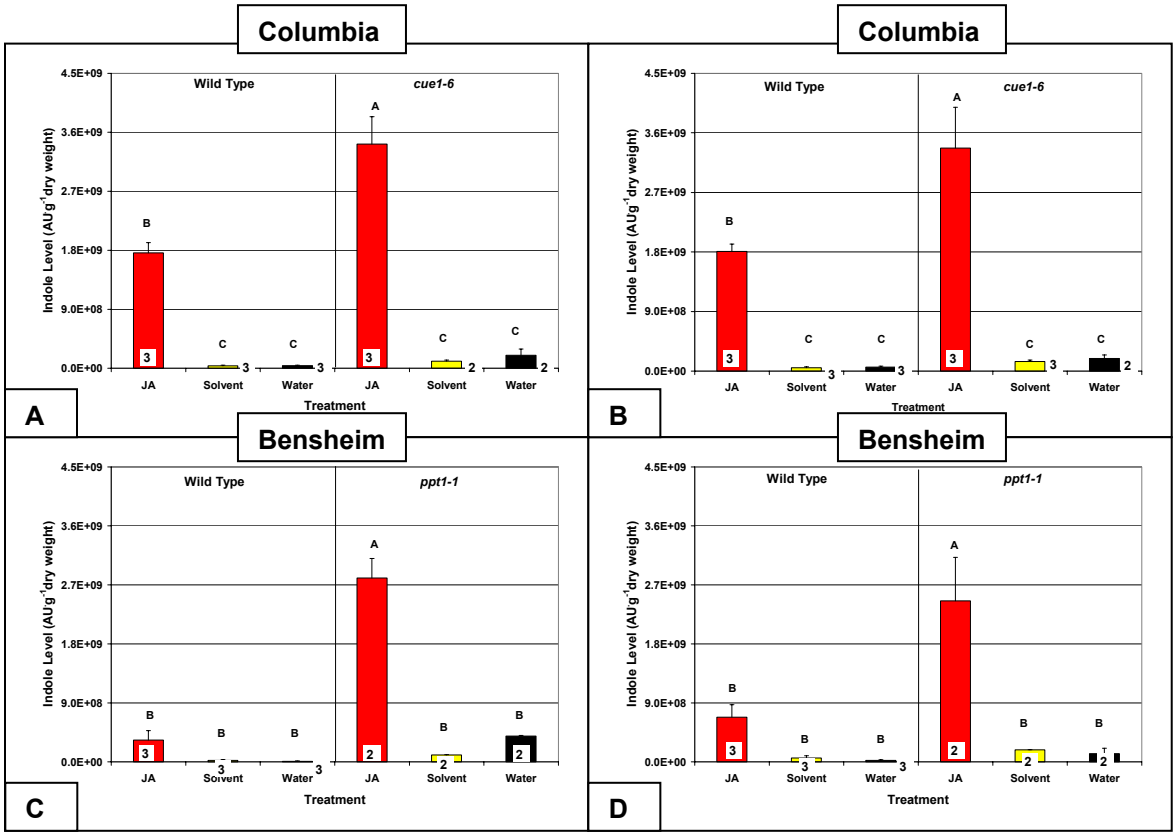


Figure 4.5. Content of putative indole compound in Arabidopsis wild type and PEP:phosphate translocator mutants in Columbia (A, B) and Bensheim (C, D) ecotypes. Mean values ± standard error represent relative amounts of indole in response to treatment. Treatments were applied three times during one week. Solvent [1.9% (v/v) ethanol and 0.125% (v/v) Triton], Jasmonic acid (JA, 2.5mM dissolved in solvent). Sample sizes are indicated on bars. Means with the same letter are not significantly different (p=0.05, Tukey test).

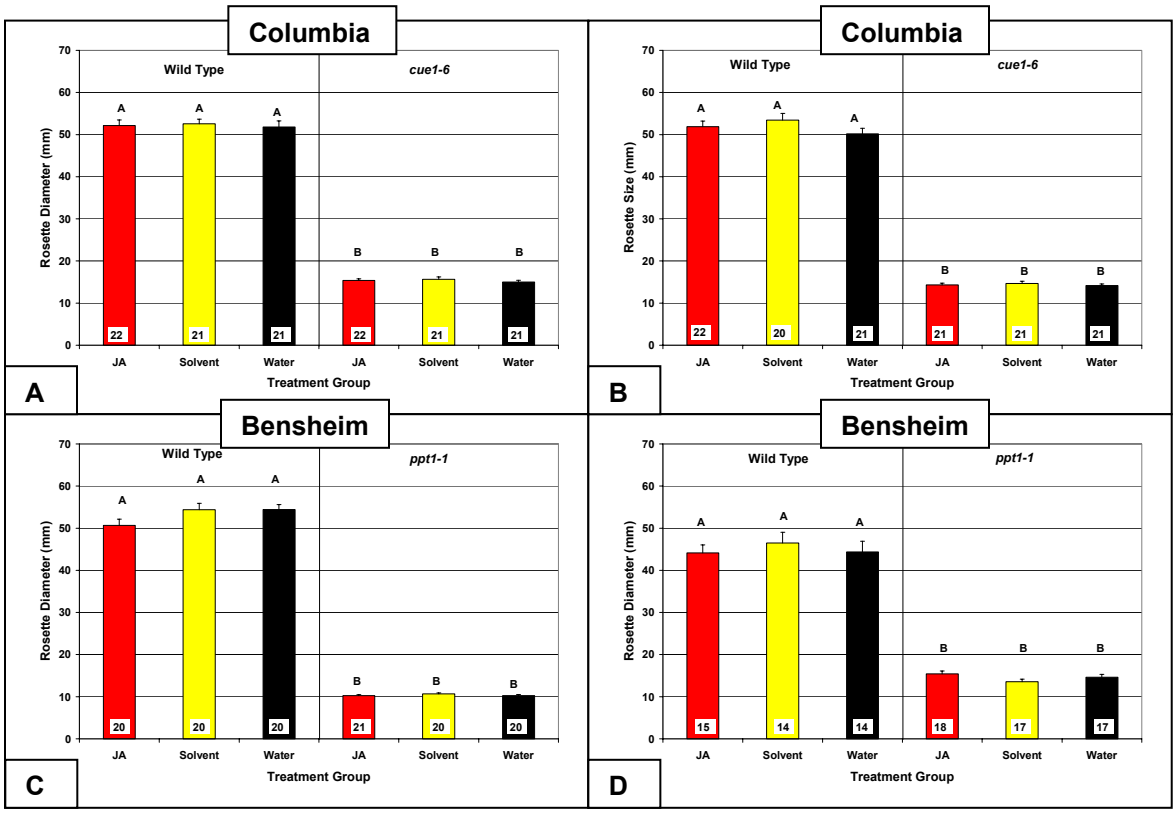


Figure 4.6. Sizes of Arabidopsis wild-type and PEP:phosphate translocator mutants in Columbia (A, B) and Bensheim ecotypes before Jasmonic acid treatment (C, D). Mean values ± standard error represent rosette sizes (diameter, mm) 28 (A, B), 31 (C), or 33 (D) days after planting. Sample sizes are indicated on bars. Means with the same letter are not significantly different (p=0.05, Tukey test).

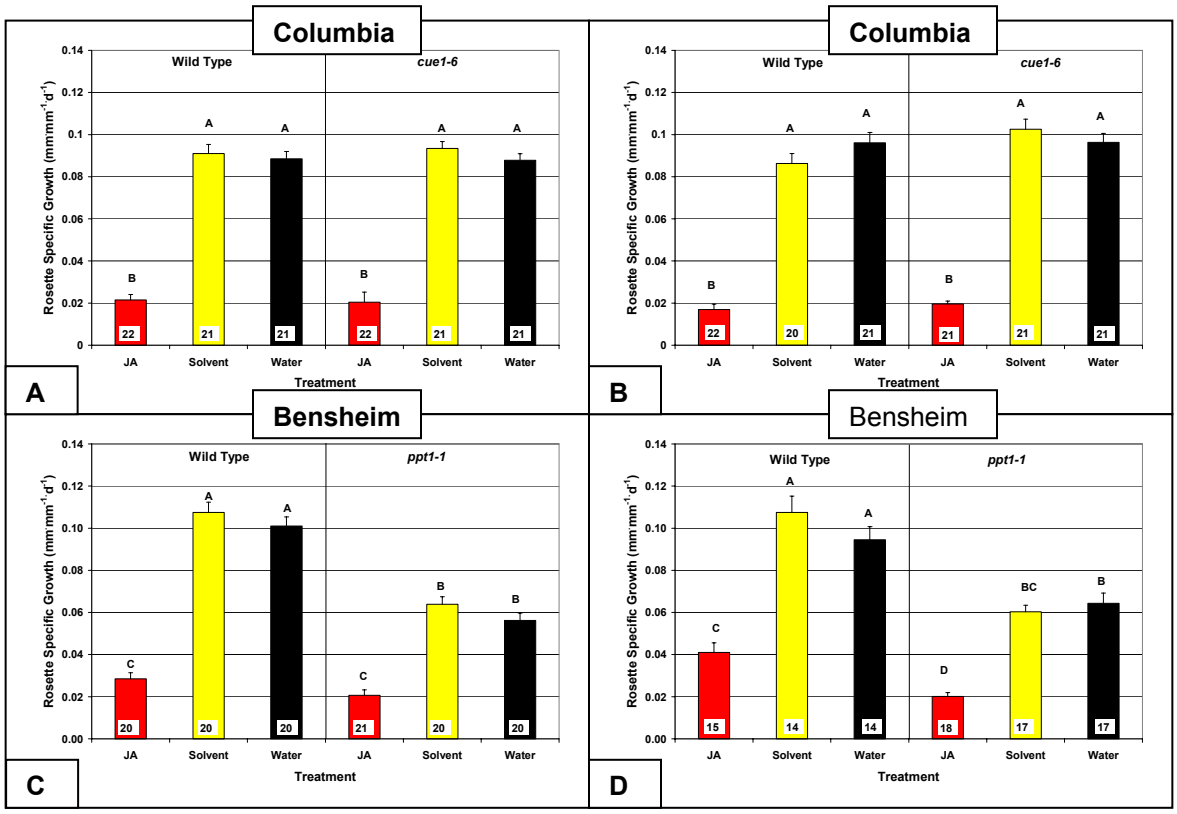


Figure 4.7. Specific growth rates of Arabidopsis wild-type and PEP:phosphate translocator mutants in Columbia (A, B) and Bensheim (C, D) ecotypes. Mean values ± standard error represent rosette specific growth rates in response to treatment. Treatments were applied three times during one week. Solvent [1.9% (v/v) ethanol and 0.125% (v/v) Triton], Jasmonic acid (JA, 2.5mM dissolved in solvent). Sample sizes are indicated on bars. Means with the same letter are not significantly different (p=0.05, Tukey test).

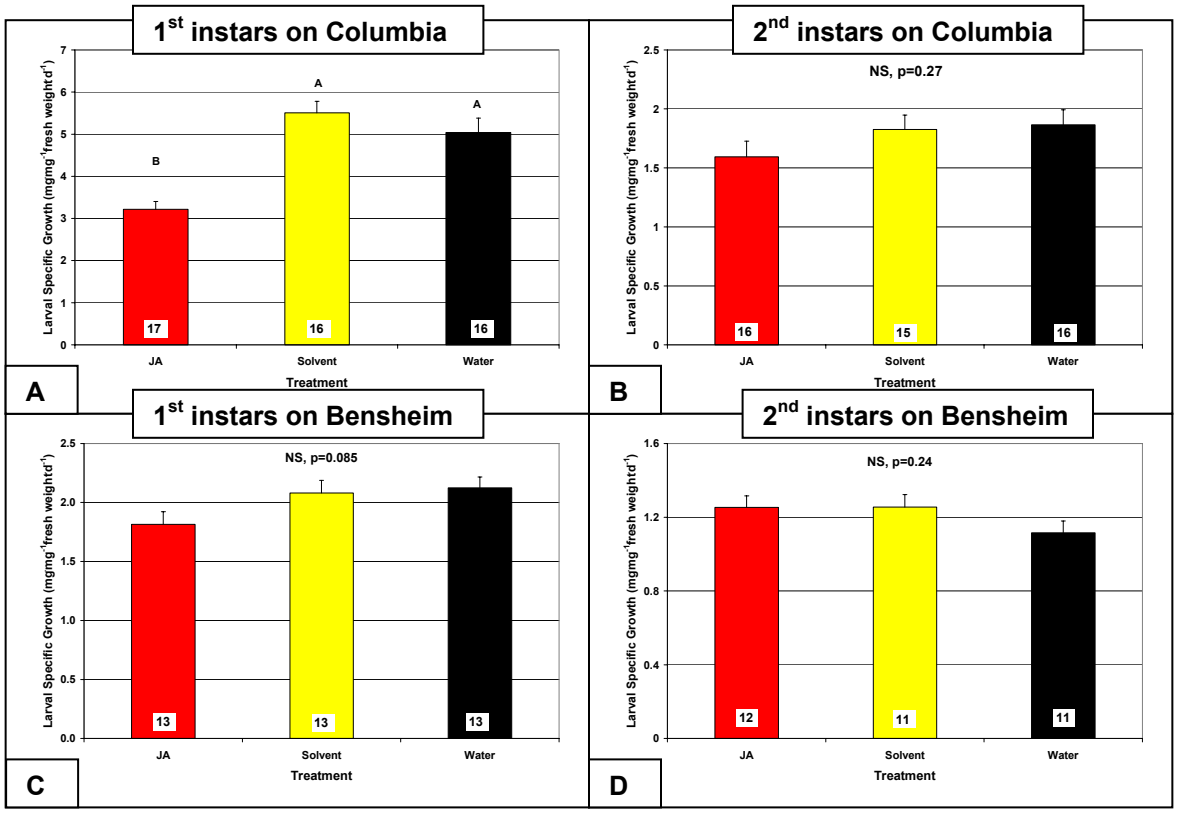


Figure 4.8. Specific growth rates (fresh weight) of *Pieris rapae* larvae feeding on JA-treated Arabidopsis wild type plants from Columbia (A, B) and Bensheim (C, D) ecotypes. Mean values ± standard error represent specific growth rates of first instars (A, C) or second instars (B, D). Solvent [1.9% (v/v) ethanol and 0.125% (v/v) Triton], Jasmonic acid (JA, 2.5mM dissolved in solvent). Sample sizes are indicated on bars. Means with the same letter are not significantly different (p=0.05, Tukey test).

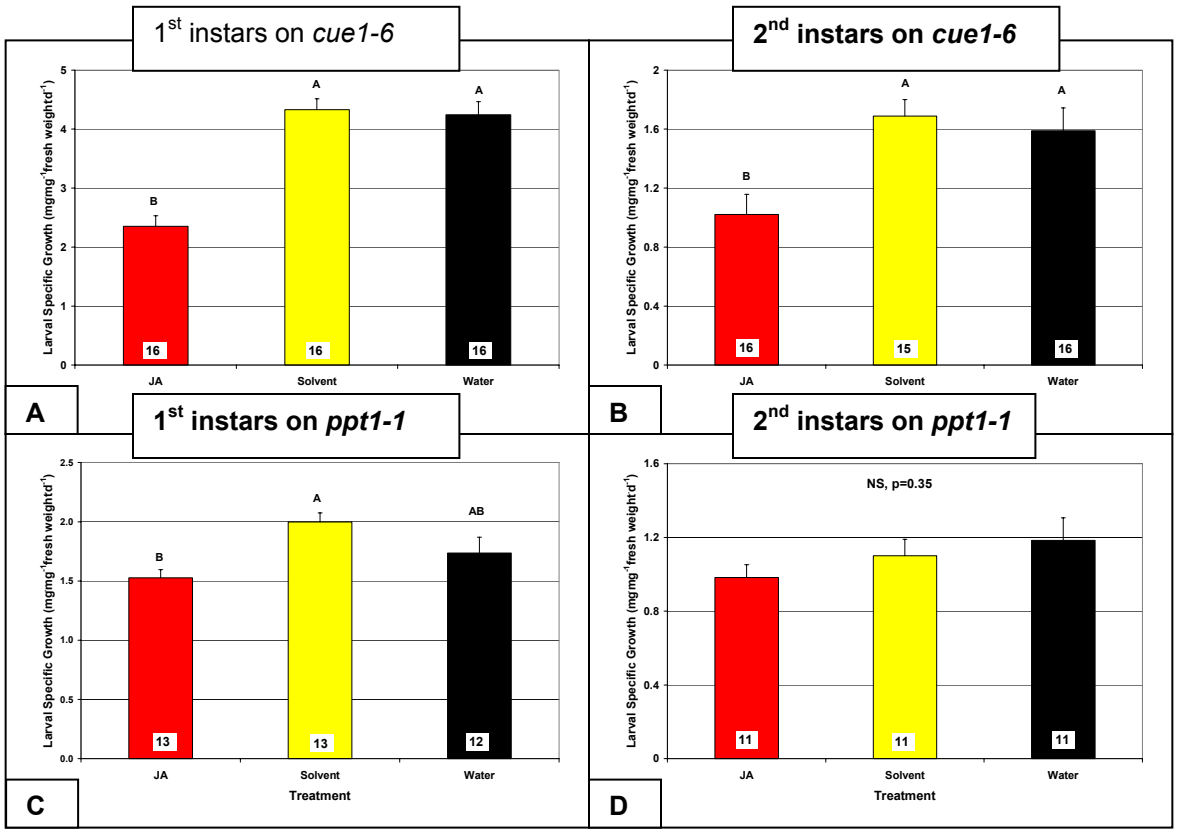


Figure 4.9. Specific growth rates (fresh weight) of *Pieris rapae* larvae feeding on JA-treated Arabidopsis PEP:phosphate translocator mutants from Columbia (A, B) and Bensheim (C, D) ecotypes. Mean values ± standard error represent specific growth rates of first instars (A, C) or second instars (B, D). Solvent [1.9% (v/v) ethanol and 0.125% (v/v) Triton], Jasmonic acid (JA, 2.5mM dissolved in solvent). Sample sizes are indicated on bars. Means with the same letter are not significantly different (p=0.05, Tukey test).

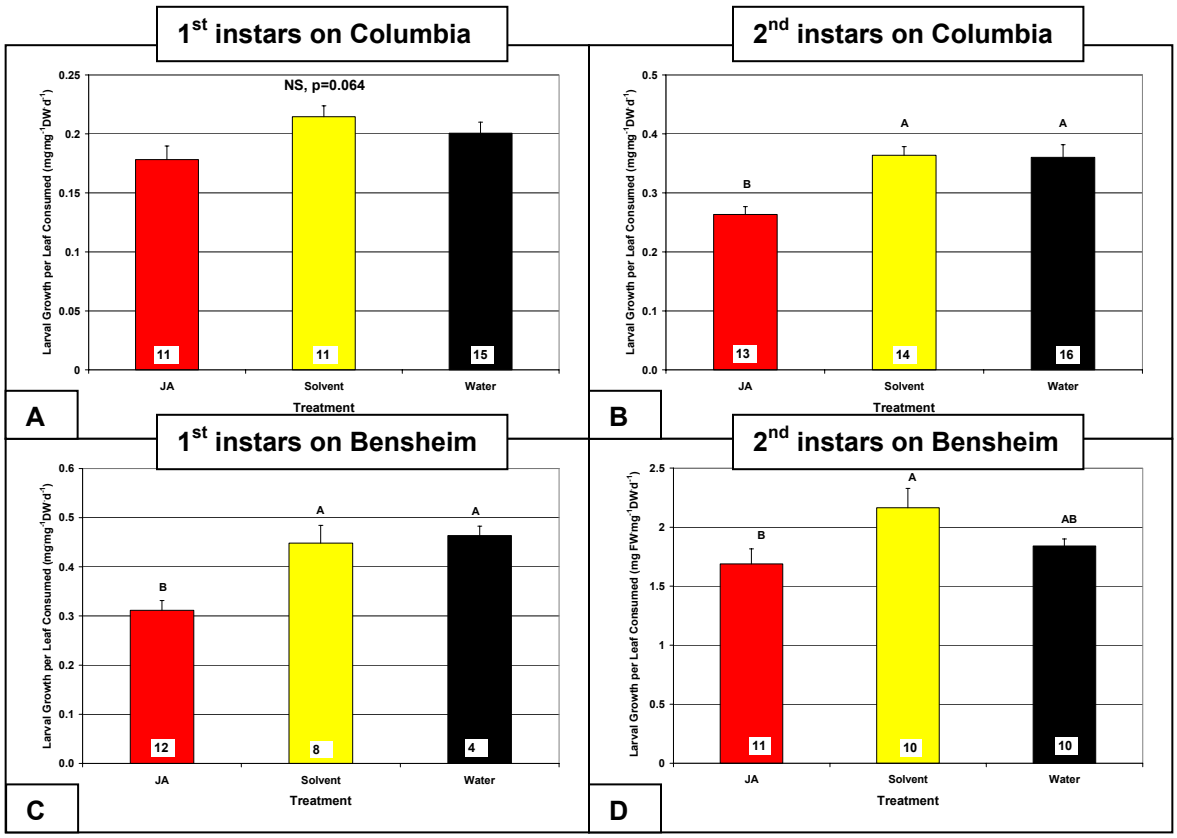


Figure 4.10. Larval growth per leaf consumption by *Pieris rapae* feeding on JA-treated Arabidopsis wild type plants from Columbia (A, B) and Bensheim (C, D) ecotypes. Mean values ± standard error represent the total larval growth per amount eaten (frass equivalents) by first instars (A, C) or second instars (B, D) during the bioassay. Solvent [1.9% (v/v) ethanol and 0.125% (v/v) Triton], Jasmonic acid (JA, 2.5mM dissolved in solvent). Sample sizes are indicated on bars. Means with the same letter are not significantly different (p=0.05, Tukey test).

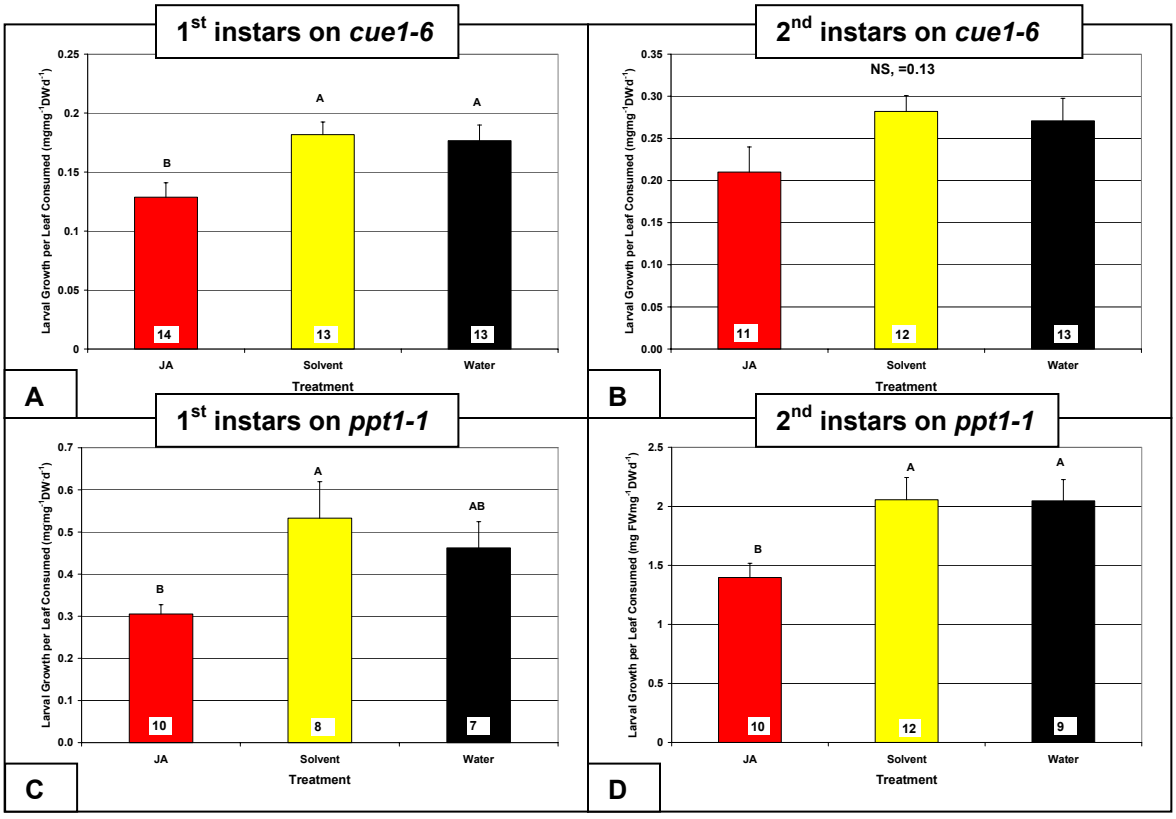


Figure 4.11. Larval growth per leaf consumption by *Pieris rapae* feeding on JA-treated Arabidopsis PEP:phosphate translocator mutants from Columbia (A,B) and Bensheim (C, D) ecotypes. Mean values ± standard error represent the total larval growth per amount eaten (frass equivalents) by first instars (A, C) or second instars (B, D) during the bioassay. Solvent [1.9% (v/v) ethanol and 0.125% (v/v) Triton], Jasmonic acid (JA, 2.5mM dissolved in solvent). Sample sizes are indicated on bars. Means with the same letter are not significantly different (p=0.05, Tukey test).

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# CHAPTER 5

## SUMMARY AND FUTURE DIRECTIONS

The previous three chapters presented the results of a series of experiments aimed at elucidating the role of the chloroplast in modulating phenolic induction responses. Shading experiments, metabolite uptake experiments with isolated chloroplasts, and experiments with plants containing specific chloroplast transporter mutations allowed me to investigate how plants obtain carbon resource substrates for induced phenolics. The biochemical basis of how resources are allocated to phenolic metabolism within the chloroplast has not been examined prior to these studies, and a number of novel results emerged from this research:

1. Phenolic compounds cannot be induced in darkened tobacco plants, but plants can perceive night-time JA application and respond with phenolic induction during the day.

2. During the day, low light and leaf development constrain phenolic induction.

3. The Calvin Cycle is not the only source of substrates for induced phenolic synthesis.

4. Chloroplast metabolite import is not enhanced during phenolic induction responses. Imported glucose-6P is not a significant carbon resource for chloroplasts. Imported PEP, even when decreased by JA treatment, may be a sufficient carbon resource for induced phenolic synthesis.

5. PPT-mediated import of PEP into the chloroplast stroma is not required for plants to induce strong shikimate-based defense responses (anthocyanins and possibly indole glucosinolates) that deter an insect herbivore.

Studies using the Folin Denis assay to estimate carbon resource investment in total phenolic defenses may inaccurately portray quantitative changes by not considering

qualitative phenolic responses elicited by enemies. In Chapter 2, I showed that caffeoylputrescine was induced while caffeoylquinic acids were either unresponsive or decreased by JA treatment. In those leaves experiencing concurrent increases and decreases in different phenolic compounds, the Folin Denis assay may detect no net change in phenolics. I was unable to reproduce total phenolic induction responses in old leaves using HPLC techniques. This inconsistency may be attributed to using a native standard for colorimetric analyses which was prepared from a mixture of young and old leaves. Appel et al. (2001) demonstrated potential inaccuracies in total phenolic assays when non-native standards were employed and also recommended that standards be prepared for each leaf age class and treatment group. To accurately measure resource investment in phenolic defenses, future studies should optimally use HPLC but at the least, native-standards from each age and treatment group must be used in Folin Denis assays of total phenolics.

Light and leaf age constrain phenolic induction responses in tobacco plants, but the influence of these constraints on ecological interactions between plants and their enemies remains largely unexplored. Fitness of insects may be enhanced by their mobility, which provides them the potential to escape plant defenses in space and time. In contrast, pathogens may be more sensitive to changes in plant defenses because of their comparably lower mobility on plant surfaces (Kessler and Baldwin, 2002). It would be interesting to investigate whether different species of plants evolved either constitutive or inducible defense strategies in response to insects or pathogens, respectively. For example, plants employing a constitutive defense strategy may be better defended against insects, which could otherwise escape inducible phenolic defenses by feeding on younger leaves at night. In contrast, inducible defenses may be more efficient at deterring pathogens, which are not as mobile and cannot escape plant defenses of younger leaves by moving to alternate tissues.

In developing leaves, neither the Calvin Cycle nor the cytoplasm independently supplies all of the additional substrates for induced phenolic synthesis. Results from Chapter 3 indicated that PEP transport rates in chloroplasts from JA-treated tobacco plants may have been sufficient to supply stromal PEP resources for phenolic induction, but the mechanism of import remains a mystery since PPT-mediated PEP import is not required for strong phenolic

induction responses in Arabidopsis (Chapter 4). Assuming that measured rates of PEP import into chloroplasts from unshaded, developing leaves are sufficient to supply PEP for induced caffeoylputrescince synthesis, the source of E4P is still unresolved. Carbon assimilated by the Calvin Cycle was estimated to provide between 1-6% of the total carbon resources for caffeoylputrescine induction. However, if the Calvin Cycle were to provide only E4P (but not PEP) resources for shikimate metabolism, measured PCA rates could supply 14% of the E4P carbon resources for induced phenolic synthesis. There still would be an 86% shortfall of E4P required for caffeoylputrescine synthesis.

Starch may serve as an additional source of substrates for induced phenolic synthesis, and this possibility remains unexplored. Starch levels increase in high light (Bertin et al., 1999) and throughout the day (Fondy and Geiger, 1987) and it is possible that resource constraints on phenolic induction responses are caused by limited availability of starch reserves in shaded plants (Mita et al., 1997). Glucose-6P produced from starch catabolism may supply substrates for the OPP pathway, which is enhanced in plants responding to enemies. Since E4P is an intermediate of the OPP pathway, enhanced substrate allocation to this pathway could increase stromal E4P concentrations. The path from starch catabolism to PEP synthesis is not clearly defined. Phosphoglycerate mutase and enolase convert 3-phosphoglycerate to PEP, but whether activities of these enzymes in chloroplasts are sufficient to synthesize PEP substrates for induced phenolic synthesis is uncertain. To explore the possibility that starch supplies substrates for shikimate-based defenses, genetic mutants of starch biosynthesis could be employed. The *pgm* mutant in Arabidopsis is deficient in the phosphoglucomutase gene and thus cannot synthesize starch but the phenotype otherwise appears similar to the wild type (Sicher and Kremer, 1992). If starch is a significant source of stromal E4P for the shikimate pathway, then *pgm* mutants would be expected to show constrained induction of anthocyanin and indole glucosinolates compared to wild type plants.

The results presented in this thesis lead me to conclude that mechanisms of resource allocation to induced phenolics are much more complex than originally envisioned. I was unable to disprove the chloroplast control hypothesis, that phenolic induction responses are

constrained by levels of carbon resources in the stroma, because my results implicated an additional source of substrates for phenolic synthesis, possibly starch. Thus, chloroplasts may possess at least three routes of acquiring substrates for induced phenolic synthesis. It is possible that multiple avenues of resource supply exist as a redundancy to ensure sufficient substrates for the shikimate pathway, which plays a central role in plant metabolism. Pathway regulation is an alternate means of controlling phenolic induction responses, and since phenolic enzymes are activated by light (Henstrand et al., 1992; Homeyer and Schultz, 1988; Koch et al., 1990; Logemann et al., 2000), it is possible that fine control of phenolic metabolism contributes to some of the light-constraints on phenolic induction responses. The degree to which substrate supply (via three possible sources) or pathway activation controls phenolic induction responses under different light conditions and in response to leaf age can only be determined by conducting a comprehensive study of both factors in one plant system simultaneously.

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