TITLE: Fumaric acid: an overlooked form of fixed carbon in Arabidopsis and other plant species

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Abstract. Photoassimilates are used by plants for production of energy, as carbon skeletons and in transport of fixed carbon between different plant organs. Many studies have been devoted to characterizing the factors that regulate photoassimilate concentrations in different plant species. Most studies examining photoassimilate concentrations in C_3 plants have focused on analyzing starch and soluble sugars. However, work presented here demonstrates that a number of C_3 plants, including the popular model organism *Arabidopsis thaliana* (L.) Heynh., and agriculturally important plants, such as soybean [*Glycine max* (L.) Merr.], contain significant quantities of fumaric acid. In fact, fumaric acid can accumulate to levels of several mg per g fresh weight in Arabidopsis leaves, often exceeding starch and soluble sugar levels. Fumaric acid is a component of the tricarboxylic acid cycle and, like starch and soluble sugars, can be metabolized to yield energy and carbon skeletons for production of other compounds. Fumaric acid concentrations increase with plant age and light intensity in Arabidopsis leaves. Arabidopsis phloem exudates contain significant quantities of fumaric acid, raising the possibility that fumaric acid may function in carbon transport.

Introduction

Fixation of CO_2 through photosynthesis results in the production of a variety of photoassimilates. These photoassimilates are then used to generate energy and as carbon skeletons for formation of cellular components. As formation and metabolism of photoassimilates represents a key component of plant growth and metabolism, a large number of studies have been devoted to examining the factors regulating the accumulation of different photoassimilates (e.g. Stumpf and Burris 1981; Bieleski 1982; Caspar et al. 1985; Lin et al. 1988; Caspar et al. 1991; Poorter and Bergkotte 1992; Rawsthorne et al. 1992; Loescher et al. 1995; Pharr et al. 1995; Li et al. 1996; Poorter et al. 1997; Scheible et al. 1997). The abundance of different types of photoassimilates varies between plant species. Most studies on a particular species have focused on analyzing those photoassimilates believed to be most abundant in that

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species. For example, plants that carry out C_3 photosynthesis have typically been thought to accumulate predominantly starch and soluble sugars. As a result, most studies examining photoassimilate concentrations in C_3 plants have focused on analyzing starch and soluble sugars, such as glucose, sucrose and fructose. While a few studies have also characterized the factors regulating organic acid (Stumpf and Burris 1981; Poorter and Bergkotte 1992; Rawsthorne et al. 1992; Scheible et al. 1997) and sugar alcohol (Bieleski 1982; Stoop and Pharr 1993; Loescher et al. 1995; Pharr et al. 1995; Yamamoto et al. 1997) concentrations in C_3 plants, most studies on C_3 plants have not analyzed the concentrations of these types of compounds. In contrast, plants that carry out C_4 photosynthesis (reviewed in Edwards et al. 1985), C_3-C_4 intermediate photosynthesis (reviewed in Rawsthorne et al. 1992) or Crassulacean acid metabolism (reviewed in Ting 1985) have long been known to accumulate large quantities of organic acids, such as malic acid, in addition to soluble sugars and starch. As a result, many studies examining photoassimilate production in C_4 and Crassulacean acid metabolism plants have analyzed organic acid concentrations, in addition to soluble sugars and starch.

Photoassimilate concentrations are controlled by a number of factors, including light intensity and nutrient availability. For instance, starch levels increase with light intensity in *Arabidopsis thaliana* (Caspar et al. 1991). Exogenous nitrate decreases starch accumulation, while increasing organic acid concentrations, in tobacco (Scheible et al. 1997). In contrast, exogenous nitrate causes a decrease in malonate, an organic acid, in soybean (Stumpf and Burris 1981).

Besides acting as sources of energy and of carbon skeletons, photoassimilates are also involved in partitioning of fixed carbon between different plant parts. In most plant species, photoassimilate is believed to be transported through the phloem chiefly in the form of sucrose (Heineke et al. 1992; Riesmeier et al. 1994). However, exceptions have been found. For instance, some plant species, such as celery, transport some of their photoassimilate in the form of mannitol, a sugar alcohol (Loescher et al. 1995).

Materials and methods

Plant material and growth conditions. Wild-type *Arabidopsis thaliana* of the Columbia ecotype, *Arabidopsis griffithiana* and *Arabidopsis himalaica* and were obtained from the Arabidopsis Biological Resource Center at Ohio State University. The *pgm1* mutant (Caspar et al. 1985) of *Arabidopsis thaliana* was obtained from Dr. Tim Caspar, DuPont Company. Alyssum (*Lobularia martima* var Carpet of Snow), asparagus (*Asparagus officinalis* var Mary Washington), corn (*Zea mays* var Sweet Corn Jubilee Hybrid), godetia (*Clarkia amoena* var Dwarf Gem Mixed Colors), hollyhock (*Alcea rosea* var Chater's Double Mixed Colors), nasturtium (*Tropaeolum majus*), pansy (*Viola tricolor hortensis* var Swiss Giant Mixed Colors), poppy (*Eschscholtzia californica* var Orange), sunflower (*Helianthus annuus* var Autumn Beauty Mixed Colors), tomato (*Lycopersicon esculentum* var Homestead), turnip (*Brassica rapa* var Purple Top White Globe) and vinca (*Catharanthus roseus* var Tall Rosea Mixed Colors) were obtained from NK Lawn & Garden Company (Minneapolis, MN). Bean (*Phaseolus vulgaris* var Italian Pole Romano), beet (*Beta vulgaris* var Burpee's Red Ball), cabbage (*Brassica oleracea capitata* var Golden Acre), carrot (*Daucus carota sativa* var Danvers Half Long), cucumber (*Cucumis sativus* var Picklebush) and geranium (*Pelargonium x hortorum* var Paint Box Mixed) were obtained from W. Atlee Burpee & Co. (Warminster, PA). Cotton (*Gossypium hirsutum* var TAM 89E-51) was obtained from Dr. Wayne Smith, Texas A&M University. Rice (*Oryza sativa* var Lemont) was obtained from Dr. Anna McClung, USDA, Beaumont, TX. Rock cress (*Aubrieta deltoidea* var Pastel Shades) was obtained from Ferry-Morse Seed (Fulton, KY). Torch lily (*Kniphofia uvaria* var Mixed Colors) was obtained from Plantation Products, Inc. (South Easton, MA). Soybean (*Glycine max* var Jupiter-R) was obtained from Dude's Feed and Seed (Raymondville, TX).

Unless otherwise specified, plants were grown under 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous cool white fluorescent light at approximately 21 °C. Potting soil consisted of a 2:1 (v/v) mixture of ProMix BX from Premier (Laguna Nigel, CA) and medium vermiculite. Pots were drenched with

1X minimal Arabidopsis media (Kranz and Kirchheim 1987) immediately prior to use and again each week, starting when plants were about 3 weeks old.

Fumaric acid assays and identification. Fumaric acid was extracted and derivatized to its corresponding dimethyl ester using a protocol designed for extraction and derivatization of fatty acids (Browse et al. 1986). In brief, tissue samples were incubated in 0.5 to 1.0 mL (depending on sample size) 1 N methanolic-HCl from Supelco (Bellefonte, PA) at 80 °C for 0.5 to 1 h. Equal volumes of 0.9% (w/v) NaCl and hexane were added to the samples, the samples were shaken vigorously for 1 to 2 min and spun at 2,000 *g* for 5 min at room temperature. For separation and quantitation of the dimethyl ester of fumaric acid, 1 to 5 µL aliquots of the hexane layer (containing the dimethyl ester of fumaric acid) were injected onto a 5890 Series II gas chromatograph from Hewlett Packard equipped with a 30 m SP2330 column (0.75 mm inner diameter) from Supelco. The oven containing the column was subjected to the following temperature program: 100 °C for 1 min, ramp to 160 °C at 25 °C•min^{-1}, ramp to 220 °C at 10 °C•min^{-1}, 220 °C for 4.6 min. The column was subjected to the following pressure program: 30 kPa for 9 min, ramp to 70 kPa at 40 kPa•min^{-1}, 70 kPa for 7 min. The carrier gas was helium. The injector temperature was 220 °C. The flame ionization detector temperature was 250 °C. Standards were prepared by derivatizing fumaric acid (Sigma), using the same protocol.

Gas chromatography - mass spectrometry was performed by the Mass Spectrometry Facility at Michigan State University. In brief, Arabidopsis leaf samples were extracted and derivatized as described above for fumaric acid quantification. These samples were then fractionated using gas chromatography and analyzed by mass spectrometry.

Starch and soluble sugar assays. Starch (Lin et al. 1988) and soluble sugar (Jones et al. 1977) levels were determined using established procedures, with slight modifications. In brief, tissue samples were weighed and then extracted several times with 80% ethanol at 80 °C to separate the soluble sugars (ethanol fraction) from starch (pellet). The combined ethanol fractions, containing

the soluble sugars, were dried down and the soluble sugars resuspended in water. The combined levels of sucrose, glucose and fructose were then determined by mixing aliquots of each sugar sample with 1 mL Glucose (HK) reagent (Sigma), 400 units invertase (Sigma) and 1 unit phophoglucose isomerase (Sigma). The reaction mixtures were incubated at 30 °C for 30 min and the A340 of each mixture was determined and compared with the readings from reaction mixtures prepared using a glucose standard curve. Starch levels were determined by first resuspending the ethanol insoluble pellets from the first step in 0.2 N potassium hydroxide at 98 °C. Acetic acid was then added to each starch sample, to a final pH of 5.5. Each starch sample was then digested with 10 units alpha-amylase (Sigma) for 30 min at 37 °C, followed by digestion with 7 units of amyloglucosidase (Sigma) for 1 h at 55 °C, to convert the starch to glucose. The glucose derived from the digested starch was assayed using the Glucose (HK) reagent (Sigma), according to the manufacturer's directions.

Analysis of phloem exudates. Phloem exudates were collected using a modified version of an established procedure (Costello et al. 1982). Leaf petioles were cut with a razor. The leaves were then placed under water and cut again to remove another small segment of the petiole. The leaves were weighed and the cut ends of the petioles placed in tubes with 4 mL of 20 mM EDTA. Two leaves were placed in each tube. The tubes were loosely capped and placed in the dark. At different times after the leaves were placed in the tubes, 150 µL aliquots of the EDTA plus phloem exudate were removed from each tube and assayed for fumaric acid and sugar (a combination of glucose, sucrose and fructose) content, as described above. The total amounts of fumaric acid or sugar exuded into a tube over a given period of time were then divided by the weight of the leaves placed in that tube.

Results

Arabidopsis contains high concentrations of fumaric acid. Plants that carry out C_3 photosynthesis, such as *Arabidopsis thaliana*, are generally believed to channel most of their photosynthate into soluble sugars (such as sucrose, glucose and fructose) and starch. Interestingly, Arabidopsis also accumulates high levels of fumaric acid. This determination was made during the course of experiments designed to analyze fatty acid composition. Arabidopsis leaves were heated in methanolic-HCl to convert fatty acids and other compounds to their corresponding methyl esters. These methyl esters were then separated and quantified using a gas chromatograph. These gas chromatographs revealed the presence of a large peak with a retention time that is distinct from that of the methyl ester of any common Arabidopsis fatty acid. Gas chromatography followed by mass spectrometry revealed that the peak is composed of the dimethyl ester of fumaric acid (data not shown). The size of the peak indicates that Arabidopsis leaves contain substantial amounts of fumaric acid.

Fumaric acid, or *trans*-butenedioic acid, is a component of the tricarboxylic acid cycle. As such, fumaric acid, like soluble sugars and starch, can be metabolized to yield energy or used to form other compounds. The size of the fumaric acid peak seen on gas chromatographs suggested that fumaric acid is present in high concentrations in Arabidopsis leaves, and so might represent a significant fraction of the fixed carbon in that tissue. Fumaric acid, sugar and starch assays were performed to determine the relative abundance of those compounds in Arabidopsis rosette leaves. As shown in Table 1, Arabidopsis rosette leaves typically contain higher concentrations of fumaric acid than of soluble sugars (the combined levels of sucrose, glucose and fructose were measured in these assays). In the experiment shown in Table 1, leaves from plants growing in continuous light also contained more fumaric acid than starch. Leaves from plants growing in a 12-h photoperiod contained more fumaric acid than starch at the beginning of the light period, whereas starch was more abundant in leaves collected at the end of the light period. The results

Table 1

of this experiment indicate that fumaric acid represents a very abundant form of fixed carbon in Arabidopsis rosette leaves.

Table 2

Fumaric acid levels were also measured in other Arabidopsis tissues. As shown in Table 2, the results of these experiments indicate that fumaric acid is abundant in leaves, stems, flowers and siliques, but is largely lacking from roots and mature seeds. These results suggest that fumaric acid accumulates predominately in photosynthetically active tissues.

Fumaric acid accumulation in other plant species. The finding that *Arabidopsis thaliana* contains high levels of fumaric acid raised the possibility that other plants that carry out C_3 photosynthesis might also accumulate high levels of fumaric acid. To determine whether this is the case, fumaric acid levels were measured in leaves from a diverse selection of flowering plant species. Species that are from the same genus or family as *Arabidopsis thaliana*, as well as more distantly related species, were chosen for this analysis. In addition, while most of the species chosen carry out C_3 photosynthesis, representative C_4 plants (e.g. corn) were also analyzed. As

Table 3

shown in Table 3, some, but not all, of the species tested accumulate high levels of fumaric acid in their leaves. Particularly high levels of fumaric acid are seen in *Arabidopsis griffithiana*, *Arabidopsis himalaica*, hollyhock and rock cress, while moderately high levels are present in common bean, carrot, soybean, sunflower and vinca. These experiments indicate that species that accumulate high levels of fumaric acid can not be predicted on purely taxonomic grounds. For instance, some species, such as cabbage, that are in the same family as *Arabidopsis thaliana* contain very low levels of fumaric acid while some relatively distantly related species, such as hollyhock, contain very high levels of fumaric acid. Also, while hollyhock and cotton both belong to the family Malvaceae (Zomlefer 1994), hollyhock has very high levels of fumaric acid while cotton has very low levels of fumaric acid. These experiments also indicate that fumaric acid likely constitutes a significant fraction of the fixed carbon present in a number of plant species, in addition to *Arabidopsis thaliana.*

Fig. 1 *Growth conditions and plant age affect fumaric acid levels in Arabidopsis.* As shown in Fig. 1, Arabidopsis grown in a 12-h photoperiod have significantly higher fumaric acid levels at the end

Fig. 2 of the light period than at the end of the dark period. In addition, as shown in Fig. 2, fumaric acid levels decrease rapidly in plants shifted from continuous light to darkness. These experiments suggest that fumaric acid may be turned over in amounts that are at least equal to the fluctuation in fumaric acid levels seen during a 24-h cycle. Alternatively, fumaric acid may be exported from

Fig. 3 leaves during growth in the dark. The finding, described in Fig. 3, that starch biosynthesis (*pgm1*) mutants (Caspar et al. 1985) accumulate higher levels of fumaric acid than wild-type plants when grown in a 12-h photoperiod suggests that some of the photosynthate that would normally be used to produce starch is instead being used to make fumaric acid in the mutant plants.

Arabidopsis starch levels have been shown to increase with increasing light intensity (Caspar et al. 1991). To determine whether light intensity also affects fumaric acid levels, fumaric acid was assayed in leaves from Arabidopsis grown under a range of light intensities. As

Fig. 4 shown in Fig. 4, fumaric acid levels increase at higher light intensities, particularly in plants grown under continuous light and in plants grown under a 12-h photoperiod and sampled at the end of the light period. These results suggest that increases in photosynthate production lead to increases in fumaric acid accumulation. Alternatively, the high fumaric acid levels could be the result of light-induced increases in phospho*enol*pyruvate carboxylase activity (see Discussion section).

Fumaric acid, sugar and starch levels were quantified in Arabidopsis plants at different

Fig. 5 developmental stages. As shown in Fig. 5, these experiments indicate that fumaric acid levels increase as plants get older. In contrast, starch levels were highest in younger plants and declined with age. Plant age had no clear effect on soluble sugar (glucose, sucrose and fructose) levels. These results indicate that, as Arabidopsis ages, fumaric acid represents an increasingly greater percentage of the fixed carbon.

Phloem exudates contain significant quantities of fumaric acid. Most plant species transport the bulk of their fixed carbon in the form of sucrose (Heineke et al. 1992; Riesmeier et al. 1994). To investigate whether fumaric acid also plays a role in transport of fixed carbon, phloem exudates were collected by placing the cut ends of Arabidopsis petioles, with the leaves attached, in a dilute EDTA solution. Samples of the EDTA plus phloem exudate were collected at different time points and analyzed for fumaric acid and soluble sugar (glucose, sucrose and fructose) contents. As shown in Fig. 6, phloem exudates contain significant quantities of fumaric acid. For instance, phloem exudates collected from leaves over a 6-h period contain 22% as much fumaric acid as soluble sugar. These results suggest that fumaric acid may play a role in transport of fixed carbon. Alternatively, the primary role of phloem-localized fumaric acid may be in nitrate transport. Other organic acids, particularly malate, have previously been postulated to function in transport of fixed carbon (Hamilton and Davies 1988) or nitrate (Ben Zioni et al. 1971; Touraine et al. 1988; Touraine et al. 1992).

Fig- 6

Discussion

Although organic acids are known to represent important forms of fixed carbon in plants carrying out C_4 photosynthesis and Crassulacean acid metabolism, the contribution of organic acids to fixed carbon levels in C_3 photosynthetic plants has usually been overlooked. Results presented here indicate that fumaric acid is an important form of fixed carbon in at least some C_3 plants. In fact, fumaric acid levels can exceed starch and soluble sugar (sucrose, glucose and fructose) levels in *Arabidopsis thaliana* (Table 1). Fumaric acid is a component of the tricarboxylic acid cycle. Fumaric acid is synthesized from succinic acid in the mitochondria, through the action of succinate dehydrogenase. Fumaric acid is also made from malate through the action of fumarate hydratase. The malate used in this reaction is formed from oxaloacetate produced from phospho*enol*pyruvate by phospho*enol*pyruvate carboxylase (Goodwin and Mercer 1983). The reason that some plants accumulate large quantities of fumaric acid remains

to be determined. Possibly there are no significant advantages associated with accumulating a particular organic acid. Different C_3-C_4 intermediate plant species accumulate different organic acids (Rawsthorne et al. 1992), suggesting there is no overwhelming advantage associated with accumulating a particular organic acid in these species. The intra-cellular location of fumaric acid storage remains to be determined. However, given the high concentrations of fumaric acid found in a number of plant species and the fact that vacuoles occupy a large percentage of the volume of many plant cells, the vacuole is the most likely site of fumaric acid accumulation.

Fumaric acid likely has several functions in plants, like Arabidopsis, where it accumulates to high levels. First, fumaric acid may function to help maintain cellular pH and turgor pressure. In addition, results presented here suggest that fumaric acid may be metabolically accessible and, like starch and soluble sugar, may function as a transient storage form of fixed carbon. For instance, *pgm1* mutants that can't make starch (Caspar et al. 1985) accumulate higher levels of fumaric acid than wild-type plants (Fig. 3), suggesting that some of the photosynthate that would normally be used to make starch is instead being used to form fumaric acid in the mutant plants. The fact that Arabidopsis phloem exudates contain significant quantities of fumaric acid (Fig. 6) suggests that fumaric acid may also be involved in transport of fixed carbon from one part of the plant to another. Alternatively, the primary function of fumaric acid present in phloem exudates could be in nitrate transport. Phloem-localized malate has been proposed to function in carbon transport (Hamilton and Davies 1988) and in transport of nitrate from roots to shoots (Ben Zioni et al. 1971; Touraine et al. 1988; Touraine et al. 1992).

Fumaric acid levels increase in response to several of the same factors that lead to increases in starch accumulation. When Arabidopsis is grown in a 12-h photoperiod, fumaric acid levels increase during the light period and decrease during the dark period (Fig. 1). Similar results have been reported for starch accumulation in a wide variety of plant species, including Arabidopsis (Caspar et al. 1985). Both fumaric acid (Fig. 4) and starch (Caspar et al. 1991) levels also increase at higher light intensities. High light intensities may increase fumaric acid levels by increasing photosynthetic rates. Increased fumaric acid levels at high light intensities

may also be the result of increases in phospho*enol*pyruvate carboxylase activity. Light has been shown to activate phospho*enol*pyruvate carboxylase kinase leading, in turn, to higher levels of phospho*enol*pyruvate carboxylase activity (Li et al. 1996). Fumaric acid (Table 2) and starch (Caspar and Pickard 1989; Caspar et al. 1991) also show similar distribution patterns, as both accumulate to high levels in leaves, stems and developing siliques of Arabidopsis, but are low in mature seeds and in roots. However, fumaric acid and starch levels do not always fluctuate in parallel. In Arabidopsis, fumaric acid levels rise with plant age (Fig. 5A), while starch levels decrease (Fig. 5C). These results indicate that the relative importance of starch and fumaric acid varies with plant age. Interestingly, fumaric acid levels decrease with increasing plant age in soybean (Stumpf and Burris 1981). These results indicate that, not only do fumaric acid levels vary tremendously among even closely related species, but the factors that regulate fumaric acid levels can also be species specific.

Conclusions. The results presented here indicate that fumaric acid can represent a major form of fixed carbon, even in plant species that carry out C_3 photosynthesis. In Arabidopsis, fumaric acid is found predominantly in photosynthetically-active tissues, increases with plant age and light intensity and is present in significant amounts in phloem exudates. Future experiments will be aimed at characterizing the effects of plant age, tissue type and growth conditions on the accumulation of other organic acids in C_3-photosynthetic plants.

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References

Ben Zioni A, Vaadia Y, Lips SH (1971) Nitrate uptake by roots as regulated by nitrate reduction products of the shoot. Physiol Plant 24: 288-290

Bieleski RL (1982) Sugar alcohols. In: Loewus FA, Tanner W (eds) Plant Carbohydrates I. Intercellular Carbohydrates. Encyclopedia of Plant Physiology, New Series, Vol 13A. Springer-Verlag, New York, pp 158-192

Browse J, McCourt PJ, Somerville CR (1986) Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. Anal Biochem 152: 141-145

Caspar T, Huber SC, Somerville C (1985) Alterations in growth, photosynthesis, and respiration in a starchless mutant of Arabidopsis thaliana (L.) deficient in chloroplast phosphoglucomutase activity. Plant Physiol 79: 11-17

Caspar T, Lin TP, Kakefuda G, Benbow L, Preiss J, Somerville C (1991) Mutants of Arabidopsis with altered regulation of starch degradation. Plant Physiol 95: 1181-1188

Caspar T, Pickard BG (1989) Gravitropism in a starchless mutant of Arabidopsis: implications for the starch-statolith theory of gravity sensing. Planta 177: 185-197

Costello LR, Bassham JA, Calvin M (1982) Enhancement of exudation from *Fraxinus uhdei* Wenz. (evergreen ash) using ethylenediaminetetraacetic acid. Plant Physiol 69: 77-82

Edwards GE, Nakamoto H, Burnell JN, Hatch MD (1985) Pyruvate, Pi dikinase and NADP-malate dehydrogenase in C4 photosynthesis: properties and mechanism of light/dark regulation. Annu Rev Plant Physiol 36: 255-286

Goodwin TW, Mercer EI (1983) Introduction to Plant Biochemistry. Pergamon Press, Oxford, U.K., pp 1-677

Hamilton DA, Davies PJ (1988) Sucrose and malic acid as the compounds exported to the apical bud of pea following $^{14}CO2$ labeling of the fruit. No evidence for a senescence factor. Plant Physiol 88: 466-472

Heineke D, Sonnewald U, Bussis D, Gunter G, Leidreiter K, Wilke I, Raschke K, Willmitzer L, Heldt H (1992) Apoplastic expression of yeast-derived invertase in potato: effects on photosynthesis, leaf solute composition, water relations, and tuber composition. Plant Physiol 100: 301-308

Jones MGK, Outlaw WH, Lowry OH (1977) Enzymic assays of 10^{-7} to 10^{-14} moles of sucrose in plant tissues. Plant Physiol 60: 379-383

Kranz AR, Kirchheim B (1987) Arabidopsis Inf Serv 24: 4.2.1

Li B, Zhang X-Q, Chollet R (1996) Phospho*enol*pyruvate carboxylase kinase in tobacco leaves is activated by light in a similar but not identical way as maize. Plant Physiol 111: 497-505

Lin TP, Caspar T, Somerville C, Preiss J (1988) Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADP glucose pyrophosphorylase activity. Plant Physiol 86: 1131-1135

Loescher WH, Everard JD, Cantini C, Grumet R (1995) Sugar alcohol metabolism in source leaves. In: Madore MA, Lucas WJ (eds) Carbon Partitioning and Source-Sink Interactions in Plants. Current Topics in Plant Physiology: An American Society of Plant Physiologists Series, Vol 13. American Society of Plant Physiologists, Rockville, MD, pp 170-179

Pharr DM, Stoop JMH, Williamson JD, Feusi MES, Massel MO, Conkling MA (1995) The dual role of mannitol as osmoprotectant and photoassimilate in celery. J Hort Sci 30: 1182-1188

Poorter H, Bergkotte M (1992) Chemical composition of 24 wild species differing in relative growth rate. Plant Cell Environ 15: 221-229

Poorter H, van Berkel Y, Baxter R, den Hertog J, Dijkstra P, Gifford R, Griffin K, Roumet C, Roy J, Wong S (1997) The effect of elevated CO2 on the chemical composition and construction costs of leaves of 27 C3 species. Plant Cell Environ 20: 472-482

Rawsthorne S, von Caemmerer S, Brooks A, Leegood RC (1992) Metabolic interactions in leaves of C3-C4 intermediate plants. In: Tobin AK (eds) Plant Organelles. Society for Experimental Biology Seminar Series, Vol 50. Cambridge University Press, Cambridge, pp 113-139

Riesmeier J, Willmitzer L, Frommer W (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. EMBO J 13: 1-7

Scheible WR, Gonzalez-Fontes A, Lauerer M, Muller- Rober B, Caboche M, Stitt M (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Plant Cell 9: 783-798

Stoop JMH, Pharr DM (1993) Effect of different carbon sources on relative growth rate, internal carbohydrates, and mannitol 1-oxidoreductase activity in celery suspension cultures. Plant Physiol 103: 1001-1008

Stumpf DK, Burris RH (1981) Organic acid contents of soybean: age and source of nitrogen. Plant Physiol 68: 989-991

Ting IP (1985) Crassulacean acid metabolism. Annu Rev Plant Physiol 36: 595-622

Touraine B, Grignon N, Grignon C (1988) Charge balance in NO3--fed soybean. Estimation of K+ and carboxylate recirculation. Plant Physiol 88: 605-612

Touraine B, Muller B, Grignon C (1992) Effect of phloem-translocated malate on NO3(-) uptake by roots of intact soybean plants. Plant Physiol 99: 1118-1123

Yamamoto YT, Zamski E, Williamson JD, Conkling MA, Pharr DM (1997) Subcellular localization of celery mannitol dehydrogenase: a cytosolic metabolic enzyme in nuclei. Plant Physiol 115: 1397-1403

Zomlefer WB (1994) Guide to Flowering Plant Families. University of North Carolina Press, Chapel Hill, pp 1-430

Table 1. Fumaric acid, starch and soluble sugar levels in rosette leaves of 5 week old wild-type Arabidopsis. Plants were grown under 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light for 4 weeks. Half of the plants were then transferred to a 12-h photoperiod with 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light and all plants were grown for an additional week prior to sampling. Results are means ± SD (n = 8-10)

Plants Grown In	Samples Collected	mg/ g fresh weight		
		Fumaric Acid	Soluble Sugar	Starch
12-h photoperiod	beginning of light period	0.44 ± 0.11	0.34 ± 0.18	0.00 ± 0.00
12-h photoperiod	end of light period	1.00 ± 0.29	0.28 ± 0.22	2.23 ± 1.28
continuous light	-	2.10 ± 0.56	0.90 ± 0.25	1.35 ± 0.69

Table 2. Fumaric acid levels in different Arabidopsis organs. Plants were grown under 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light. Results are means ± SD (n = 4-10)

Organ (age of plant in d)	Fumaric Acid (mg/g FW)
Rosette leaves (29)	2.1 ± 0.4
Cauline leaves (29)	1.4 ± 0.7
Stem tissue (29)	1.5 ± 0.7
Roots (37)	0.01 ± 0.007
Flowers (29)	1.8 ± 0.3
Small, immature siliques (29)	2.7 ± 0.7
Large, immature siliques (37)	1.8 ± 0.4
Seeds (57)	0.09 ± 0.04

Table 3. Fumaric acid accumulation in different plant species. Fumaric acid levels are expressed as mg fumaric acid/g fresh weight of leaf material. The values presented are the means ± SD (n = 5 for experiment 1; n = 8 for experiment 2). nd = not detected. ns = not sampled. Age = Age of plants in d at the time the samples were collected. Plants were sampled at two ages for experiment 1 and at one age for experiment 2

	Experiment 1:				Experiment 2:	
Plant:	Fumaric acid:	Age:	Fumaric Acid:	Age:	Fumaric Acid:	Age:
alyssum	0.008±0.004	28	0.021±0.007	44	nd	14
Arabidopsis griffithiana	1.8±0.4	28	5.2±1.7	41	ns	
Arabidopsis himalaica	1.3±0.2	41	3.8±1.1	56	ns	
asparagus	0.04±0.03	28	0.07±0.09	44	0.014±0.01	39
bean	0.34±0.04	16	0.38±0.09	30	0.38±0.11	12
beet	0.014±0.009	16	0.015±0.005	44	0.023±0.005	12
cabbage	0.08±0.09	16	0.07±0.04	30	0.03±0.02	14
carrot	0.2±0.1	28	0.16±0.05	51	0.42±0.11	14
corn	0.04±0.08	16	0.003±0.001	30	nd	14
cotton	ns		ns		0.027±0.013	32
cucumber	0.018±0.004	16	0.024±0.006	44	0.025±0.017	12
geranium	0.017±0.013	26	0.031±0.015	42	0.005±0.001	14
godetia	0.002±0.001	28	0.035±0.014	44	0.056±0.024	14
hollyhock	3.9±0.7	16	9.4±1.3	44	6.4±1.9	12
nasturtium	0.07±0.02	16	0.066±0.006	30	0.10±0.03	32
pansy	0.097±0.04	28	0.06±0.03	44	ns	
poppy	0.05±0.05	44	ns		0.06±0.04	12
rice	ns		ns		0.007±0.001	39
rock cress	1.5±0.8	28	4.7±2.7	44	8.4±2.2	39
soybean	ns		ns		0.50±0.11	12
sunflower	0.37±0.18	16	0.20±0.15	51	0.45±0.16	14
tomato	0.02±0.01	28	0.026±0.009	44	0.077±0.018	12
torch lily	0.03±0.03	44	0.04±0.01	59	ns	
turnip	0.05±0.03	16	0.011±0.008	51	0.020±0.014	14
vinca	0.20±0.09	28	0.24±0.17	44	0.22±0.15	39

Figure Legends

Fig. 1. Diurnal changes in fumaric acid concentration. Wild-type *Arabidopsis thaliana* were grown for 28 d under ~ 110 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light. Plants were then shifted to a 12-h photoperiod, at a light intensity of ~ 110 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and grown for an additional 7 d. Rosette leaves were collected at 4-h intervals throughout a 24-h diurnal cycle and assayed for fumaric acid content. The zero-h time point corresponds to the beginning of the light period, lights were turned off at 12 h and back on at 24 h. Results are means ± SD ($n = 5$)

Fig. 2. Fumaric acid levels in Arabidopsis shifted to extended darkness. *Arabidopsis thaliana* were grown in 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light for 26 d. Plants were shifted to total darkness, rosette leaves collected at 12-h intervals and assayed for fumaric acid content. Results are means of 8 to 10 independent assays. Bars indicate standard deviation (only the top halves of the bars are shown, for clarity)

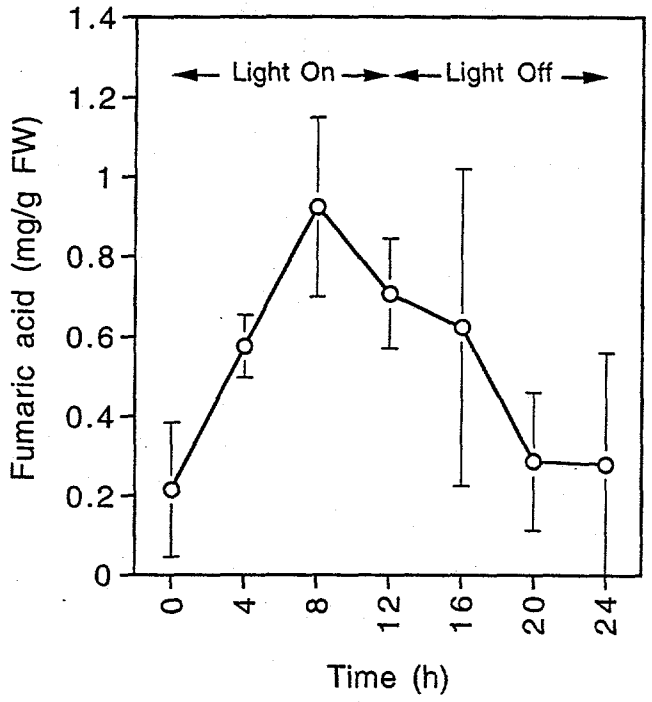
Fig. 3. Fumaric acid levels in wild-type and starchless (*pgm1*) plants. Wild-type (▨) and *pgm1* (■) *Arabidopsis thaliana* were grown under 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light for 3 weeks. Half of the plants were then transferred to a 12-h photoperiod with 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light and all the plants were grown for an additional week prior to sampling. Beginning of light period = plants grown in a 12-h photoperiod and sampled at the beginning of the light period. End of light period = plants grown in a 12-h photoperiod and sampled at the end of the light period. Continuous light = plants grown in continuous light. Results are means ± SD ($n = 8$-10)

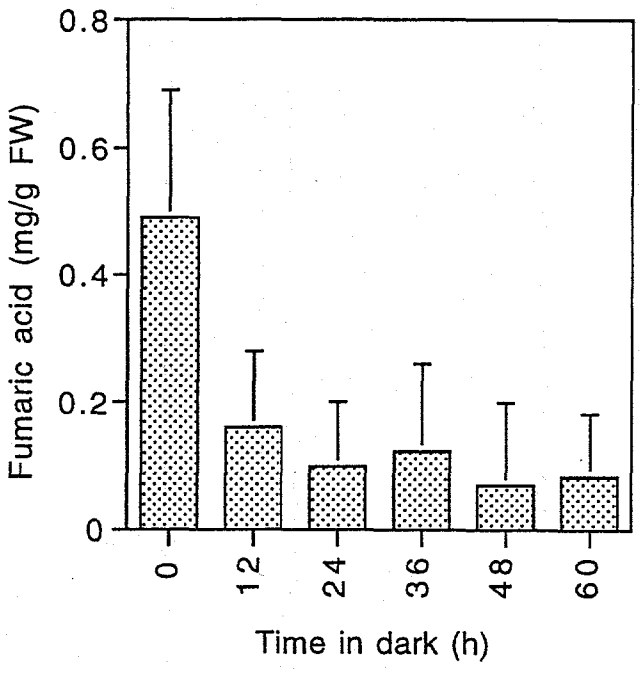
Fig. 4. Effect of light intensity on fumaric acid levels. Wild-type *Arabidopsis thaliana* were grown under ~110 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light for 31 d. Plants were then shifted to the indicated light intensities and grown under continuous light (▩) or a 12-h photoperiod for an

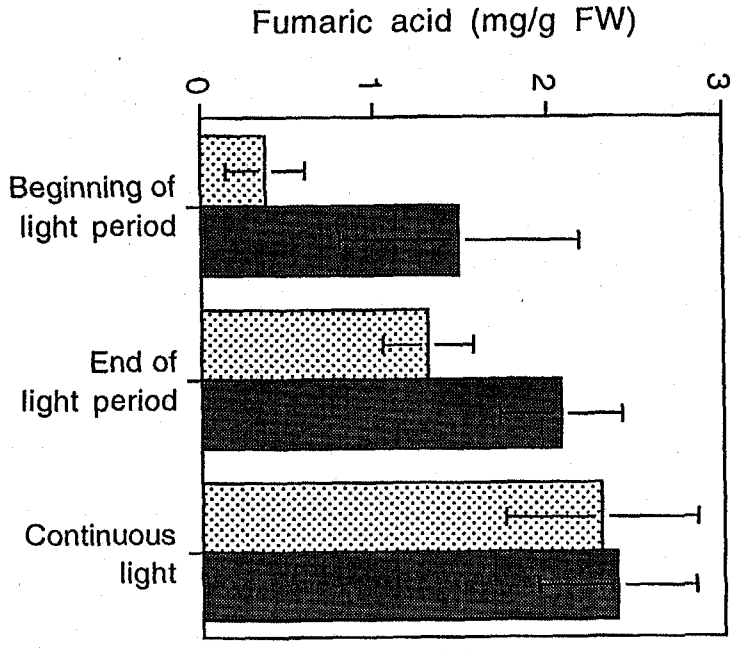
additional 7 d prior to sampling. Plants growing in a 12-h photoperiod were sampled at the end of the dark period (■) and at the end of the light period (□). Results are means ± SD (n = 5)

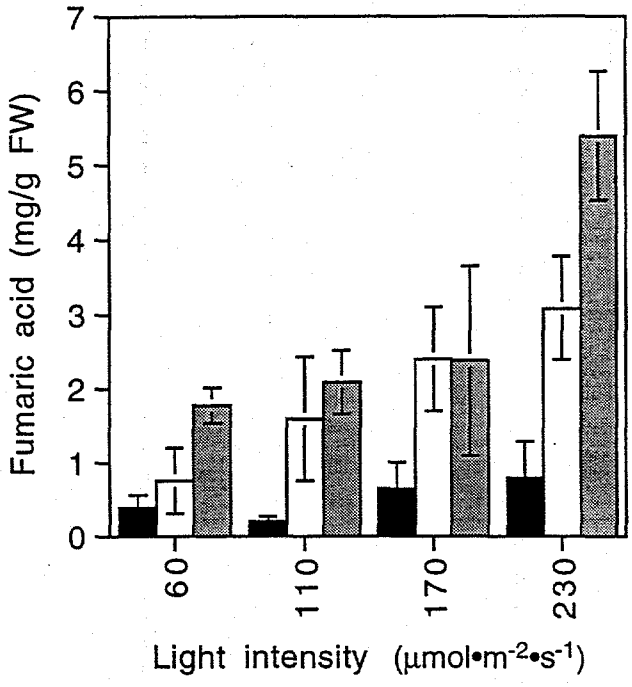
Fig. 5A-C. Effect of developmental stage on fumaric acid, starch and sugar levels. *Arabidopsis thaliana* were grown under 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light and then shifted to a 12-h photoperiod with 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for one week prior to sampling, or were kept under continuous light (▩). Plants growing in a 12-h photoperiod were sampled at the end of the dark period (■) and at the end of the light period (□). **A** mg fumaric acid per g fresh weight of rosette leaf. **B** mg soluble sugar (the combined amounts of glucose, sucrose and fructose were measured in these assays) per g fresh weight of rosette leaf. **C** mg starch per g fresh weight of rosette leaf. Results are means ± SD (n = 8-10)

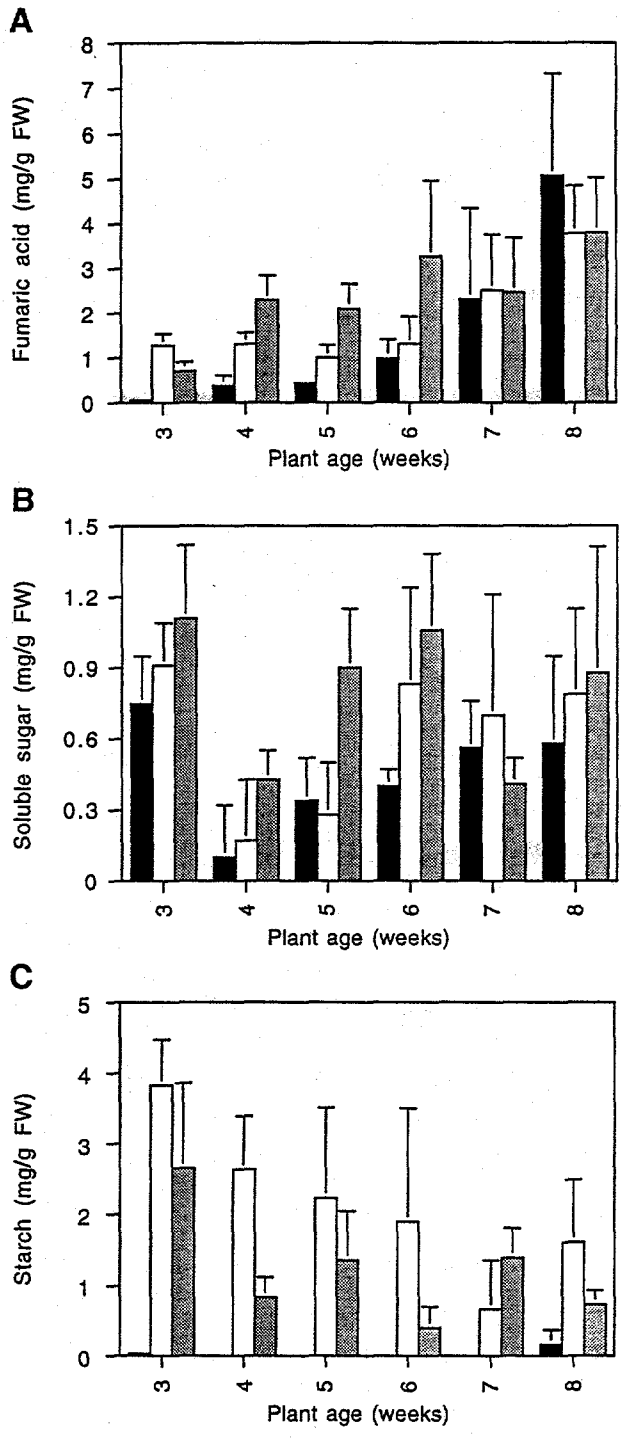
Fig. 6. Fumaric acid and sugar levels in phloem exudates. Wild-type *Arabidopsis thaliana* were grown for 35 d under 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light. Phloem exudates were collected from pairs of leaves for different lengths of time. The samples were assayed to determine the amount of fumaric acid (—o—) and soluble sugar (—▲—) exuded during that time period. The amount of fumaric acid or sugar in each sample was divided by the fresh weight of the leaves from which that sample was collected. Results are means ± SD (n = 8)

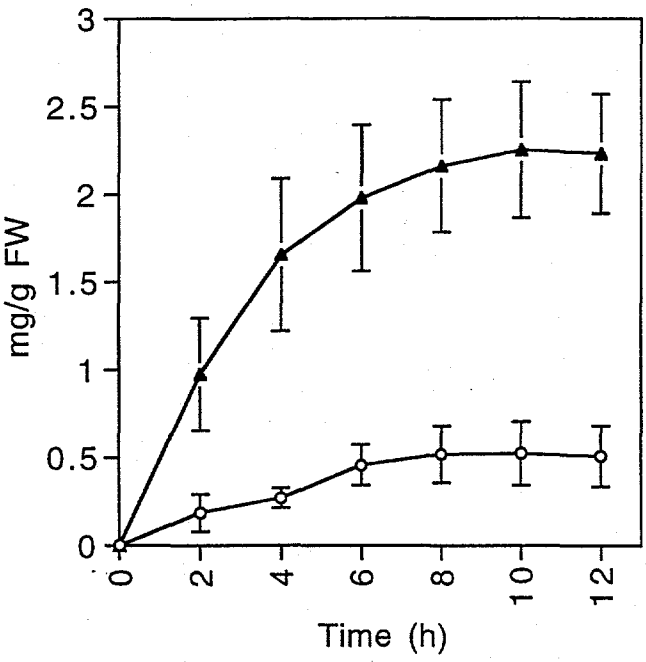

Light On
Light Off
Fumaric acid (mg/g FW)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
4
8
12
16
20
24
Time (h)


0.8
0.6
0.4
0.2
0
Fumaric acid (mg/g FW)
0
12
24
36
48
60
Time in dark (h)


Fumaric acid (mg/g FW)
0
1
2
3
Beginning of light period
End of light period
Continuous light


Fumaric acid (mg/g FW)
0
1
2
3
4
5
6
7
60
110
170
230
Light intensity (μmol•m-2•s-1)


A
Fumaric acid (mg/g FW)
0
1
2
3
4
5
6
7
8
3
4
5
6
7
8
Plant age (weeks)
B
Soluble sugar (mg/g FW)
0
0.3
0.6
0.9
1.2
1.5
3
4
5
6
7
8
Plant age (weeks)
C
Starch (mg/g FW)
0
1
2
3
4
5
3
4
5
6
7
8
Plant age (weeks)


3
2.5
2
1.5
1
0.5
0
mg/g FW
0
2
4
6
8
10
12
Time (h)