Plant one-carbon metabolism and its engineering

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The metabolism of one-carbon (C1) units is vital to plants. It involves unique enzymes and takes place in four subcellular compartments. Plant C1 biochemistry has remained relatively unexplored, partly because of the low abundance or the lability of many of its enzymes and intermediates. Fortunately, DNA sequence databases now make it easier to characterize known C1 enzymes and to discover new ones, to identify pathways that might carry high C1 fluxes, and to use engineering to redirect C1 fluxes and to understand their control better.

One-carbon (C1) metabolism is essential to all organisms. In plants, it supplies the C1 units needed to synthesize proteins, nucleic acids, pantethenate and many methylated molecules1. Fluxes through C1 pathways are particularly high in plants that are rich in methylated compounds such as lignin, alkaloids and betaines because methyl groups make up several percent of their dry weight2. Transfers of C1 units are also central to the massive metabolism of plants because methyl moieties make up several percent of their dry weight. Betaines are rich in methylated compounds such as lignin, alkaloids and betaines because methyl moieties make up several percent of their dry weight. Betaines because methyl moieties make up several percent of their dry weight. Betaines because methyl moieties make up several percent of their dry weight. Betaines because methyl moieties make up several percent of their dry weight.

References

8. Hoecker, U. and Hans-Rose, W. (1996) Active repression mechanisms of plant one-carbon metabolism. In part this is because of the obstacles that C1 metabolism presents for classical biochemistry and genetics: its enzymes can be of low abundance and/or exist as several isoforms, mutants are lacking, and its key intermediates – C1 substituted folates – are labile and hard to quantify. Fortunately, classical approaches to C1 metabolism can now be complemented by genomics-driven approaches that exploit the fast-growing DNA sequence databases. Accordingly, this review has three aims: to illustrate how genomics-based approaches are advancing our knowledge of plant C1 biochemistry; to bring together biochemical and genomics-derived data to show which C1 pathways might operate in plants, and where they operate in the cell; to examine progress towards engineering C1 metabolism. Nucleotide sequence information – from genomes, cDNAs and ESTs – can be used to complement biochemical approaches in several ways. Because most enzymes of C1 metabolism are highly...
Conserved homology with bacterial, yeast or animal sequences can identify DNA sequences for known plant C1 enzymes. The proteins that these sequences encode can then be characterized by expression in heterologous systems. Homology searches can suggest the presence (or absence) of enzymes for which there is no biochemical information in plants. The differential expression of genes can be inferred from variation in the count of their cognate ESTs, cDNAs and genomic sequences. ESTs, cDNAs and genomic sequences can also give information about the organellar targeting of enzymes via their characteristic signal sequences, and about the sizes of gene families. Lastly, the availability of sequences from a wide range of plants can speed up the preparation of the cDNAs that are needed to make constructs for metabolic engineering.

Role of folates in one-carbon metabolism
Here we briefly introduce folate-mediated C1 metabolism (reviewed in Ref. 1) to provide background for the rest of the article. Many C1 transfers are mediated by the coenzyme tetrahydrofolate (THF). In essence, catabolism of serine, glycine and other molecules generates specific C1 derivatives of THF that are then interconverted between different oxidation states, ranging from 5,10-methenyl- and 5,10-methylene- to 5,10-methyl-THF (most oxidized) through 5,10-methylene- and 5,10-methylene- to 5-methyl-THF (most reduced) (Fig. 1). These interconversions of C1-substituted folates form the core of C1 metabolism, from which C1 units are withdrawn by anabolic reactions. The largest anabolic flux is the use of 5-methyl-THF to convert homocysteine to methionine, which is incorporated into proteins or converted to S-adenosylmethionine (AdoMet), the donor for methylations. In other anabolic reactions, 10-formyl-THF is used to synthesize purines and formylmethionyl-tRNA (for translation initiation in plastids and mitochondria), and 5,10-methylene-THF is used to produce thymidylate and pantethenate (Fig. 1).

Compartmentation of plant one-carbon metabolism
Folate-mediated and other C1 reactions in plants are highly compartmented (Fig. 2). The overall picture is like that for other eukaryotes inasmuch as many C1 enzymes occur in both the cytosol and the mitochondria. However, most of them are also found in plastids, and two enzymes are specific to peroxisomes. The data point to some surprising deductions about gaps in pathways and the inter-organellar traffic in C1-related metabolites that these gaps imply. Although some deductions presented here are robust inasmuch as they are based on concordance between DNA sequence data and biochemical findings, both types of information are subject to cavats. Thus, the algorithms available for predicting proteins from genomic DNA and organellar targeting from the protein sequence are fallible. Plant EST collections are far from a complete inventory of the transcriptome, and the Arabidopsis genome is only ~80% complete. Missing enzymes, especially organellar ones, might therefore yet be found. Also, clearcut results on enzyme compartmentation can be hard to achieve because of the difficulties in obtaining highly purified subcellular fractions. The following deductions should therefore be viewed as provisional and as stimuli for further investigation.

- The cytosol, mitochondria and plastids all contain the activities required to convert formate to 10-formyl-THF, to interconvert 10-formyl-, 5,10-methenyl- and 5,10-methylene-THF, and to generate 5,10-methylene-THF from serine. However,
5,10-methylene-THF reductase, which is required to convert 5,10-methylene-THF to 5-methyl-THF, is confined to the cytosol.

- The cytosol and mitochondria contain cobalamin-independent methionine synthase and can therefore form methionine from homocysteine and 5-methyl-THF. This suggests that mitochondria must import 5-methyl-THF, which is synthesized in plastids (Ref. 7) and is produced from S-adenosylhomocysteine (AdoHcy) only in the cytosol.

Although plastids carry out all steps in methionine synthesis up to homocysteine, and have a pool of 5-methyl-THF (in its monoglutamyl form, presumably imported), they do not contain cobalamin-independent methionine synthase. Plastids might therefore import methionine from the cytosol. Alternatively, plastids could have a methionine synthase of the cobalamin-dependent type found in bacteria and animals (Ref. 7). This possibility is attractive in light of evidence for the monoglutamyl form of 5-methyl-THF in plastids because the cobalamin-dependent enzyme can use this substrate. However, there are no published plant DNA sequences that encode this enzyme, which is highly conserved between bacteria and animals.

- Neither chloroplasts nor mitochondria have AdoMet synthetase, implying that they import AdoMet from the cytosol. A related deduction, albeit based only on sequence data (the absence of transit peptides), is that these organelles also lack...
AdoHcy hydrolase, and therefore must export AdoHcy to the cytosol for hydrolysis to homocysteine and adenosine. AdoHcy hydrolysis is crucial to the regulation of methylation reactions, because AdoHcy is a potent competitive inhibitor of AdoMet-dependent methyltransferases.

Other deductions from a combination of DNA data and biochemical results are (i) that the two 10-formyl-THF-dependent steps in purine synthesis occur in both mitochondria and plastids, and (ii) that plants have a bifunctional thymidylate synthase–dihydrofolate reductase, not two separate enzymes, and that this enzyme is probably present in both mitochondria and plastids.

Plants have two unique one-carbon reactions and two unusual ones

In terms of plant-specific enzymes, there are only a few major evolutionary novelties in plant C₁ metabolism. The deduced
Amino acid sequences of most plant C1 enzymes are strikingly similar to those of other organisms, even though the polypeptides specifying individual activities are sometimes fused together differently. Thus, although other eukaryotes have a trifunctional C1-THF synthase and plants have a 10-formyl-THF synthetase specifying individual activities are sometimes fused together differently. However, one enzyme is unique to plants, and there is some biochemical evidence for a second unique enzyme. A third enzyme is not known in other eukaryotes; a fourth occurs in anaerobic bacteria but has not been reported in other organisms. By creating a route from C1 to C2 compounds, such an enzyme, in conjunction with amino transferase and serine hydroxymethyltransferase activities, would enable the elaboration of C1 (and thence larger) structures solely from C1 units, as occurs in methylotrophs. Therefore, glyoxylate synthetase is potentially important. The direct reduction of CO2 to formate is also reported to occur in potato tuber chloroplasts, this reaction is known to occur in anaerobic bacteria but has not been reported in eukaryotes.

**Methionine S-methyltransferase (MMT)**

Methionine S-methyltransferase (MMT; Fig. 2, step 14) catalyzes the synthesis of S-methylmethionine (SMM) in the phloem. SMM can serve as a methyl donor for other methyltransferase activities, or other pathways. Therefore MMT is an evolutionary innovation, it is noteworthy that it exists as a homotetramer (uncommon among methyl transferases) and that it is regulated in an unusual way. These four enzymes are described below.

**Glyoxylate synthetase**

Glyoxylate synthetase (Fig. 2, step 21) has only been reported in potato tuber chloroplasts and has not been cloned. It appears to mediate a THF-dependent condensation of two formate molecules to give glyoxylate, a reaction with no precedent in other organisms. By creating a route from C1 to C2 compounds, such an enzyme, in conjunction with amino transferase and serine hydroxymethyltransferase activities, would enable the elaboration of C1 (and thence larger) structures solely from C1 units, as occurs in methylotrophs. Therefore, glyoxylate synthetase is potentially important. The direct reduction of CO2 to formate is also reported to occur in potato tuber chloroplasts; this reaction is known to occur in anaerobic bacteria but has not been reported in eukaryotes.

**NADH-dependent, AdoMet-insensitive 5,10-methylene-THF reductase**

The 5,10-methylene-THF reductases (MTHFRs; Fig. 2, step 9) from Arabidopsis and maize were recently cloned by genomics-based methods, and characterized by expression in yeast. Both enzymes were found to be NADH-dependent and AdoMet-insensitive, unlike the mammalian and yeast enzymes, which use NADPH and are allosterically inhibited by AdoMet – the AdoMet-binding site being in a C-terminal, regulatory domain. The pyridine nucleotide specificity is crucial because the NADPH-dependent MTHFR reaction is physiologically irreversible, because of the large free energy change involved in the reduction of 5,10-methylene-THF and the high cytosolic NADPH:NADP+ ratio. An NADPH-dependent reaction thus commits C1 units to methyl group synthesis, and potentially can deplete the 5,10-methylene-THF pool; feedback inhibition by AdoMet checks such depletion. By contrast, because the cytosolic NADH:NAD+ ratio in plants is low, the NADH-dependent MTHFR reaction might well be reversible; this would obviate the need for feedback-inhibition by AdoMet. The NADH-dependence and AdoMet-insensitivity of plant MTHFRs have important implications for the control of C1 fluxes in plants and their engineering. MTHFRs provide cautionary examples of how proteins sharing high homology can have critically different properties that cannot be predicted from their primary sequences.
Mitochondrial formate dehydrogenase

Formate dehydrogenase (FDH, Fig. 2, step 23) occurs in fungi as a cytosolic enzyme, but both biochemical and DNA sequence data show it to be a mitochondrial matrix enzyme in plants15. Moreover, FDH is one of the most abundant soluble proteins in mitochondria from non-green tissues (e.g. up to 9% of the total protein content in potato tuber mitochondria17) although it is a minor protein in mitochondria from illuminated, unstressed leaves18. FDH is remarkable for being strongly induced in leaves by darkness, by C1-related compounds, by environmental stresses16, and in roots by hypoxia and by Fe deficiency17. These patterns of developmental and environmental regulation strongly suggest that formate is a major metabolite in certain tissues and conditions.

Genomic evidence points to unsuspected one-carbon reactions

There is evidence that formate can be formed in the leaves of illuminated C3 plants as a result of a chemical reaction between glyoxylate and H2O2 (Refs 18,19) (perhaps facilitated by the peroxidatic activity of catalase20). However, it is not at all clear how formate is produced in the dark, in non-photosynthetic organs, or by C4 species. Until now, the most plausible route was considered to be from serine or glycine via 5,10-methylene-THF and 5,10-methenyl-THF to 10-formyl-THF, followed by reversal of the 10-formyl-THF synthetase reaction (Fig. 1). Therefore, it is significant that DNA sequence data point to three additional possibilities for the origin of formate that have received little or no attention.

1. Hydrolysis of 10-formyl-THF: the Arabidopsis genome contains two homologs of the E. coli purU gene, encoding 10-formyl-THF deformylase. Both the putative PurU proteins appear to be mitochondrial (Fig. 2, step 2). Cognate ESTs indicate that these genes are expressed in Arabidopsis and other plants at a modest level (Fig. 4). The 10-formyl-THF deformylase, which in E. coli is activated by methionine and inhibited by glycine21, releases formate from 10-formyl-THF in an essentially irreversible reaction. Therefore, if 10-formyl-THF deformylase is active in plants, it could drive flux out of C1-substituted folate pools into formate. It is noteworthy that if the C4 folates were derived via serine and the 3-phosphoglycerate from glycolysis, a flux from C1 folates to formate (and ultimately CO2) could operate as an energy-yielding dissimilatory route, bypassing the Krebs cycle.

2. Oxidation of formate by 10-formylglutathione hydrolase (SFGH, also known as class III alcohol dehydrogenase), coupled with the presence of an Arabidopsis gene and cognate ESTs that putatively specify S-formylglutathione hydrolase (SGFH, also known as esterase D) (Fig. 2, steps 7 and 8) suggest another possible origin of formate. FADH converts the glutathione adduct of formaldehyde (which forms spontaneously) to S-formylglutathione, from which formate is released by SGFH. FADH and SGFH are not highly specific enzymes and so might in principle be playing roles unrelated to each other or to formaldehyde oxidation. However, at least for bacteria, this appears not to be the case because FADH and SGFH are members of a preserved operon in many species. There are various possible sources of formaldehyde; these
include spontaneous dissociation of 5,10-methylene-THF formed from glycine or serine14, and catalase-mediated oxidation of methanol derived from pectin hydrolysis15,16.

(3) Oxidation of sarcosine or other N-methylamino acids. The Arabidopsis genome includes a homolog of bacterial and mammalian/mimicronic sarcosine oxidases, and there are cognate ESTs from Arabidopsis and other plants (Fig. 4). Sarcosine oxidase (Fig. 2, step 20) converts sarcosine to glycine and formaldehyde, which can be oxidized to formate. Caution is necessary with this genomic evidence because (a) mammalian sarcosine oxidases also attack piperolic acid and proline (without release of formaldehyde), (b) a sarcosine oxidase-like enzyme in E. coli acts on N-methylpyrothepin, and (c) it is not clear if there are major sources of sarcosine or other N-methylated amino acids in plants. It is therefore significant that sarcosine is a strong inducer of FDI in potato leaves17, because this suggests that sarcosine is indeed metabolized to formate.

Towards engineering of one-carbon metabolic fluxes

Apart from the photosynthetic flux through glycine and serine2, little is known about C1 fluxes in plants. Therefore a major reason to engineer plant C1 metabolism is to modify the fluxes through the main pathways (Fig. 2), to understand how they are controlled and to learn about their possible functions. Although such studies have yet to be made using engineered plants, the utility of 13C NMR techniques in plant C1 metabolism has been demonstrated with wild-type plants21,22. Future engineering challenges will be to attack folate-mediated C1 metabolism, for instance by preventing C1 units derived from glycine or serine from entering the C1 folate pool and by stopping a catabolic route producing methanethiol was induced29. This shows that methionine itself exerts little control over its own synthesis in glycine-accumulating mutants of barley and tobacco was lowered by antisense RNA expression, the plants showed that methionine itself exerts little control over its own synthesis30. In another approach to lowering AdoMet levels, the plants were engineered to produce excess methylthioadenosine. The transformants produced less ethylene from glycine or serine3, and catalase-mediated oxidation of methylthioadenosine (Acetobacter glutaminus) cell cultures30. This work established, inter alia, that:

- The glycine decarboxylase complex and the mitochondrial serine hydroxymethyltransferase are tightly coupled via a pool of 5,10-methylene-THF that does not equilibrate with the overall pool31.
- S3-methylcysteine is probably limited by the THF supply32,33.
- Plants convert supplied S-formyl-THF to other C3 substituted folates34.

Engineered modification of C3 fluxes in plants has begun in the area of AdoMet metabolism. Based on the observation that reductive AdoMet formation in tobacco, leaf tissue accumulated high levels of free methionine (400-fold that of the wild type) and that the catabolic route producing methanethiol was induced29. This shows that methionine itself exerts little control over its own synthesis, and supports an earlier finding that plants have an inducible methionine-γ-lyase that can liberate excess methylthio groups35. In another approach to lowering AdoMet levels, the bacterial phage T3 enzyme AdoMet hydrolase was expressed in tomato36 (AdoMet hydrolase converts AdoMet to homoserine and methylthioadenosine). The transformants produced less ethylene (a metabolite of AdoMet), which suggests that AdoMet pools were depleted. Lastly, when AdoHcy hydrolase activity in tobacco was lowered by antitox genes, RNA expression, the plants were morphologically abnormal and showed hypomethylation of DNA (Ref. 31). The most remarkable aspect of these C3 engineering experiments is that the engineered plants were viable, implying that plants can somehow maintain essential C3 fluxes in spite of major perturbations.

Future engineering challenges will be to attack folate-mediated C3 metabolism, for instance by preventing C3 units derived from formate and serine entering the C3 folate pool and by stopping methyl groups from leaving it. In addition, the activity of the SMN cycle could be reduced, and the demand for methyl groups increased or decreased by adding or subtracting secondary pathways. Such engineering research, in conjunction with studies of C4 gene expression patterns change in the engineered plants, will provide insight into how C4 fluxes are controlled in plants. To date, we know in a general way that C1 metabolism is highly regulated at both metabolic and gene levels, and that its capacity can vary greatly as a function of development and environment. However, little is known about either the mechanisms or the extent of this plasticity.

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