Lipid Utilization, Gluconeogenesis, and Seedling Growth in Arabidopsis Mutants Lacking the Glyoxylate Cycle Enzyme Malate Synthase*

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The aim of this research was to test the role of the glyoxylate cycle enzyme malate synthase (MLS) in lipid utilization, gluconeogenesis, and seedling growth in Arabidopsis. We hypothesized that in the absence of MLS, succinate produced by isocitrate lyase (ICL) could still feed into the tricarboxylic acid cycle, whereas glyoxylate could be converted to sugars using enzymes of the photosynthetic pathway. To test this hypothesis we isolated knock-out mls mutants and studied their growth and metabolism in comparison to wild type and icl mutant seedlings. In contrast to icl seedlings, which grow slowly and are unable to convert lipid into sugars (Eastmond, P. J., Germain, V., Lange, P. R., Bryce, J. H., Smith, S. M. & Graham, I. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5669–5674), mls seedlings grow faster, use their lipid more rapidly, and are better able to establish as plantlets. Transcriptome and metabolome analyses show that icl seedlings exhibit many features characteristic of carbohydrate starvation, whereas mls seedlings differ relatively little from wild type. In the light mls seedlings generate more sugars than icl seedlings, and when fed with 14C-labeled acetate, 14C-labeling of sugars is three times greater than in icl mutants and more than half that in wild type seedlings. The mls seedlings also accumulate more glycine and serine than icl or wild type seedlings, consistent with a diversion of glyoxylate into these intermediates of the photorespiratory pathway. We conclude that, in contrast to bacteria and fungi in which MLS is essential for gluconeogenesis from acetate or fatty acids, MLS is partially dispensable for lipid utilization and gluconeogenesis in Arabidopsis seedlings.

The glyoxylate cycle catalyzes the conversion of two acetate molecules into succinate, providing the means for microorganisms to grow on ethanol, acetate, or fatty acids (1). This function has been confirmed through the analysis of mutants lacking isocitrate lyase (ICL); EC 4.1.3.1) and malate synthase (MLS; EC 4.1.3.2), key enzymes of the glyoxylate cycle. Bacterial and fungal mutants do not grow on acetate, ethanol, or fatty acids (2–5). Loss of ICL or MLS also leads to avirulence in bacterial and fungal pathogens of plants and mammals (2–5). For example Mycobacterium tuberculosis lacking ICL cannot grow on fatty acids or acetate and is unable to persist in macrophages, where lipid is the primary carbon source available (2). Similarly Candida albicans and Saccharomyces cerevisiae lacking ICL cannot grow on acetate, and the Candida mutant is less infectious in mice where macrophage lipid is also the primary carbon source (3). ICL and MLS have therefore been identified as targets for therapeutic drugs to treat some bacterial and fungal infections since the glyoxylate cycle is absent from vertebrates.

In germinating oilseeds the glyoxylate cycle also enables acetate from lipid breakdown to be converted to four-carbon gluconegenic substrates to support seedling growth (6). In Arabidopsis, seedlings of icl mutants grow poorly because they are unable to convert acetate from fatty acid β-oxidation into sugars (7). Instead, the seedlings slowly respire their fatty acids by transferring either acetate or citrate from the peroxisome to the mitochondrion (8, 9). Without ICL, the tricarboxylic acid cycle cannot be supplied with succinate or with malate produced from glyoxylate by MLS. Thus, oxaloacetate cannot be generated for gluconeogenesis. We considered the possibility that if instead of blocking the glyoxylate cycle at ICL, it is blocked downstream at MLS, the succinate would still feed into the tricarboxylic acid cycle to regenerate oxaloacetate, and the glyoxylate cycle could potentially feed into the photorespiratory pathway for conversion to sugar (Fig. 1). Although seed germination (radicle emergence) is fueled by a limited reserve of sugars in Arabidopsis, the subsequent growth of the seedling is fueled largely by oil breakdown, which occurs concurrently with the acquisition of photosynthetic competence. It has been clearly established by three independent studies that glyoxylate cycle and photosynthetic enzymes co-exist in the same peroxisomes during seedling growth in oilseed species (10–12). Thus, mls mutants, glyoxylate produced by ICL could serve as a substrate for serine-glyoxylate aminotransferase, leading to glycine and subsequently to serine, hydroxypropyruvate, glycinate, and ultimately sugars (Fig. 1). We, therefore, hypothe-

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¶ The abbreviations used are: ICL, isocitrate lyase; MLS, malate synthase; T-DNA, transfer-DNA; TAG, triacylglycerol; RT, reverse transcription; Suc, sucrose.

42916 This paper is available on line at http://www.jbc.org
sized that mls mutant seedlings would be capable of gluconeogenesis from lipid and would grow better than icl seedlings. To test this hypothesis we isolated two independent mls mutants and studied their growth and metabolism relative to icl mutants. Consistent with our hypothesis, mls mutant seedlings grow much better, break down their lipid more rapidly, and accumulate more sugars than icl mutant seedlings. In addition they are capable of gluconeogenesis from acetate, unlike icl seedlings (7).

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Seeds of Arabidopsis were surface-sterilized, stratified, and germinated as described by Sherson et al. (13). Except where stated, seedlings were grown for 48 h, corresponding to principal growth stage 0.6 as defined by Boyes et al. (14) in continuous light (100 µmol photons m⁻² s⁻¹) or in the absence of 1% (w/v) sucrose.

Screening of T-DNA Insertion Lines and Isolation of mls Knock-out Mutants—PCR screening was used to identify T-DNA insertions of the Arabidopsis MLS gene (At5g03860). The gene-specific primers MS50 (5’-ATG GAG CTC GAG ACC TCA GTT TAT C-3’) and MS30 (5’-GCT GCT TCC GGT TCT C-3’), MS30 (5’- GAC GCT TGA GAC ATT GAT AGG GTA G-3’), and MS31 (5’-ACA AGT ACG GAT GAG AAG ATC AGA G-3’) were used in combination with T-DNA left border primers (13, 15, 16). This screening identified an insertion in a plant in the Wassilewskija ecotype from the Versailles collection (15, 16). DNA sequence analysis revealed two copies of the T-DNA in the first intron of the MLS gene (mls-1; Fig. 2A). A second mutant was identified in the SALK SIGnAL T-DNA collection, which was generated in the Col-0 ecotype (17), containing a T-DNA insertion in the third intron of the MLS gene (mls-2, SALK_006987; Fig. 2A). In both knock-out lines kanamycin resistance conferred by the T-DNA co-segregated with the interrupted MLS gene (data not shown). A wild type segregant was isolated concurrently with each mutant and named MLS-1 and MLS-2 to indicate the mutant to which each corresponds.

RT-PCR Analysis—RNA was isolated from 2-day-old seedlings using the Qiagen RNAeasy kit and used to generate cDNA with the Qiagen Omniscript RT-PCR kit according to manufacturers’ instructions. PCR was carried out with gene-specific primers MS51 and MS30 for MLS and IC51 and ICL30 for ICL (7). Gene-specific primers for the ACT2 gene (At3g18780) were used to normalize the amount of template in each RT-PCR reaction.

Transcriptome Analysis—Microarray analysis using Affymetrix ATH1 genome arrays was carried out by the Nottingham Arabidopsis Stock Centre (nasc.nott.ac.uk) under the auspices of the Genomic Arabidopsis Resource Network (www.york.ac.uk/res/garnet/garnet.htm). The procedures are Minimum Information about a Microarray Experiment compliant, and data are available on the NASC website.

Biochemical Analysis—For enzyme assays, tissue extracts were prepared from Arabidopsis seedlings as described in Eastmond et al. (7). MLS and ICL assays were carried out as described in Cooper and Bevers (18). A coupled assay for serine-glyoxylate aminotransferase and hydroxypyruvate reductase activity was carried out using a modified version of the method described by Nakamura and Tolbert (19), in which 1 mM glyoxylate was used to start the reaction. Protein content was determined as reported by Bradford (20) using bovine serum albumin as the standard. Total fatty acids were extracted and quantified by gas chromatography-mass spectroscopy using the method described by Browse et al. (21), with C20:1 as a marker for tricylglycerol (TAG). 1H NMR was used to analyze major polar metabolites in 2-day-old seedlings as reported by Ward et al. (22) under the auspices of the Genomic Arabidopsis Resource Network (as above). The levels of glucose (Glc), fructose (Fru), and sucrose (Suc) were quantified in ethanol-soluble extracts as described in Nielsen et al. (23).

RESULTS

Isolation of mls Knock-out Mutants—ICL and MLS are both encoded by single genes in Arabidopsis. To test the hypothesis that Arabidopsis mls mutant seedlings are capable of lipid utilization and gluconeogenesis from acetate, two independent T-DNA insertion knock-out mutants of the MLS gene (At5g03860) were identified (see “Experimental Procedures”). Homozygous individuals lacking MLS (mls-1 and mls-2; Fig. 2A) and wild type segregants (designated MLS-1 and MLS-2) were isolated and analyzed by RT-PCR. This confirmed that neither mls mutant contains a MLS transcript (Fig. 2B and data not shown). RT-PCR using ICL gene-specific primers indicates that the mls mutants have no apparent change in the level of ICL transcripts. Furthermore, although icl-2 mutant seedlings have no ICL transcripts, they have normal levels of MLS transcripts (Fig. 2B). MLS enzyme activity was not detectable in mls seedlings (Fig. 2C and data not shown), whereas the wild type segregants showed a peak of enzyme activity 1.5 days post-imbibition (Fig. 2C). mls mutant seedlings contain ICL enzyme activity at the same level as in wild type seedlings (Fig. 2C and data not shown). The absence of MLS mRNA and
MLS enzyme activity confirms that we have isolated two null mutants.

The expression patterns for genes encoding a number of key enzymes of lipid metabolism were analyzed in mls and icl-2 mutant lines: 3-keto-acyl CoA thiolase (β-oxidation), two peroxisomal isofoms each of citrate synthase and malate dehydrogenase (glyoxylate cycle), and two isofoms of phosphoenolpyruvate carboxykinase (gluconeogenesis) showed no apparent changes in the patterns of the transcripts for these genes in the mls-2 mutant lines as they are in the same Col-0 ecotype as Arabidopsis wild types (Fig. 4). The data reveal that there are many more RNAs were each hybridized to Affymetrix ATH1 genome chips.

Transcriptome Analysis Reveals Major Differences in the Two Glyoxylate Cycle Mutants—To investigate differences in gene expression in the two glyoxylate cycle mutants, RNA was isolated from triplicate batches of 2-day-old, light-grown mutant seedlings to wild type growth in the light than are required to rescue icl-2 seedlings (data not shown). A further phenotype observed in icl-2 mutants was the failure of seedlings to establish into plantlets with true leaves under conditions of limited light (Fig. 3B; Ref. 7). In contrast, the establishment frequency of mls-2 seedlings is similar to wild type levels and only becomes compromised when seedlings are grown in short days (Fig. 3B).

Introduction of the MLS cDNA driven by the cauliflower mosaic virus 35 S promoter into the mls-1 knock-out resulted in complementation of the observed phenotypes (data not shown). Subsequent experiments were performed only with mls-2 and MLS-2 lines as they are in the same Col-0 ecotype as icl-2.

The mls Mutants Have a Less Severe Phenotype than the icl Mutant—The mls seedling phenotypes were examined alongside the icl-2 mutant line isolated previously together with wild type revertant ICL-2 (7). The icl-2 mutant has a stunted phenotype during post-germinative growth, which can be rescued by the addition of exogenous Suc or by growth in high light conditions (Fig. 3A; Ref. 7). However, mls-1 and mls-2 mutants have only a slightly stunted phenotype when grown in the absence of exogenous sugar in the light (Fig. 3A). The mls phenotype is more obvious in the dark where hypocotyl elongation is inhibited, and little root development takes place (Fig. 3A). mls mutant seedlings can be rescued by the addition of exogenous Suc in all conditions (Fig. 3A). Furthermore, lower concentrations of Suc are able to restore the mls-2 mutant seedlings to wild type growth in the light than are required to rescue icl-2 seedlings (data not shown). A further phenotype observed in icl-2 mutants was the failure of seedlings to establish into plantlets with true leaves under conditions of limited light (Fig. 3B; Ref. 7). In contrast, the establishment frequency of mls-2 seedlings is similar to wild type levels and only becomes compromised when seedlings are grown in short days (Fig. 3B).
and lysine in icl-2. In contrast, the glucose levels in mls-2 did not differ appreciably from MLS-2, although the Gln level in mls-2 was also appreciably reduced (Fig. 5, B and C). A further significant finding in this analysis was that mls-2 seedlings have appreciably increased levels of serine (Ser) and glycine (Gly) relative to all other lines (Fig. 5C). There were no changes in organic acids detectable using this method in any genotypes (data not shown).

The Rate of Lipid Breakdown and Sugar Accumulation in mls Seedlings Is Similar to Wild-type Seedlings, whereas icl Seedlings Are Compromised in Both—During the heterotrophic stages of seedling development, TAG stored in the embryo is converted to sugar to fuel seedling growth (25). Accordingly, in wild type seedlings TAG levels decline rapidly during the first few days post-imbibition, reaching undetectable levels after 5 days (Fig. 6A). The absence of MLS in mls-2 mutant seedlings has relatively little effect on the rate of TAG utilization, which after an initial lag period declines at a similar rate to wild type and is undetectable by day 7 (Fig. 6A). In contrast, the initial TAG content of icl-2 seeds is lower than that of other lines, and its rate of utilization in seedlings is much slower, such that TAG is still present 7 days post-imbibition (Fig. 6Aii; Ref. 7).

Measurement of total soluble sugar levels shows that the sugar content of mls-2 seedlings is appreciably greater than that of icl-2 seedlings (Fig. 6, B and C). Levels of Suc decline to very low amounts in all genotypes within the first 2 days post-imbibition, as Suc is used to fuel this stage of growth (Fig. 6B). Thereafter, the Suc content remains very low in the dark (Fig. 6B, iii and iv) but increases slowly in the light in all genotypes, presumably as a result of photosynthetic activity (Fig. 6B, i and ii). However, after 8 days in the light icl-2 seedlings contain half as much Suc as mls-2 seedling and wild types (Fig. 6Bii). The hexose levels detected reveal that in the light, mls-2 seedlings are able to generate more Glc and Fru than icl-2 seedlings. Hexose content increases during the first 2 days in wild type and mls-2 seedlings in the light but not in icl-2 seedlings (Fig. 6C, i and ii). After day 2, the hexose content increases progressively in all genotypes in the light, although more slowly in icl-2. In the dark (Fig. 6C, iii and iv), the hexose contents of wild type seedlings increase rapidly up to day 2 and later decline. During this period, the hexose contents of both mutants are lower than their respective wild types. Together with the observation that TAG is utilized more rapidly in icl-2 than in icl-2 mutants (Fig. 6A), these observations support our original hypothesis that in the light mls-2 seedlings are capable of gluconeogenesis from acetate after lipid β-oxidation, whereas icl-2 seedlings are not (7).

**mls Seedlings Carry Out Gluconeogenesis from [14C]Acetate, whereas icl Seedlings Do Not**—To directly test the hypothesis that mls-2 seedlings are capable of gluconeogenesis from acetate, 2-day-old light-grown seedlings were fed with [2,14C]acetate and fractionated, and the amount of 14C was incorporated into each fraction determined (Fig. 7). mls-2 and wild type seedlings incorporated the same total amount of 14C during the experiment (123 ± 6 and 122 ± 4 × 10^4 dpm, respectively); the pattern of incorporation in the two genotypes was similar in all fractions except that mls-2 seedlings incorporate 60% of the amount of 14C into sugars and release twice as much 14CO2 compared with wild type seedlings. In contrast, icl-2 seedlings incorporated less total 14C during the experiment (75 ± 5 × 10^4 dpm), and significantly less 14C was detected in all ethanol-soluble fractions than in wild type seedlings. Only 20% of 14C is detected in the sugar fraction of icl-2 seedlings compared with wild type relative to total incorporation (5% in absolute terms). icl-2 seedlings release more than three times the percentage of 14CO2 compared with wild type seedlings (Fig. 7). These data
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Upper, the top 20 genes underexpressed more than 2-fold in icl-2 compared with ICL-2. Lower, the top 20 genes induced in icl-2 compared with ICL-2. The fold change in gene expression in mls-2 is also shown for comparison. NA, not applicable because the signals for the corresponding genes in MLS-2 and mls-2 are less than 100.

### TABLE 1
Differences in the transcriptomes of 2-day-old icl-2 mutant seedlings compared to ICL-2 seedlings

| Gene number | Description* | Signal | Fold induction |
|-------------|--------------|--------|----------------|
| At3g21720   | isocitrate lyase | 5919 49 | 120.5 0.9 |
| At3g16060   | Class I non-symbiotic hemoglobin | 468 26 | 17.7 NA |
| At4g32810   | Putative protein | 296 26 | 8.6 NA |
| At5g17850   | Senescent-associated protein | 122 14 | 5.9 NA |
| At1g71720   | Alcohol dehydrogenase | 209 35 | 5.5 NA |
| At1g53070   | Protein kinase b | 171 31 | 5.5 NA |
| At1g48680   | Hypothetical protein | 553 110 | 5.0 NA |
| At5g46900   | exTA (emb CAA47807.1) | 153 31 | 4.9 1.9 |
| At5g44440   | Berberine bridge enzyme-like protein | 1298 265 | 4.7 NA |
| At1g242020  | Pollen allergen-like protein | 269 57 | 4.7 1.3 |
| ndhG        | NADH dehydrogenase (ND6) | 2374 508 | 4.5 1.6 |
| At1g76790   | O-Methyltransferase family protein | 162 35 | 4.5 1.2 |
| At1g78370   | Glutathione S-transferase | 199 44 | 4.4 1.1 |
| At5g80000   | Glyceraldehyde (like β,1,3-glucanase) | 407 91 | 4.3 0.9 |
| At5g88880   | 3-Keto acyl-CoA thiolase 2 | 133 30 | 4.2 1.1 |
| At1g06350   | Hypothetical protein | 250 60 | 4.2 0.9 |
| At4g12510   | pEARLI 1-like protein | 814 204 | 4.0 1.4 |
| At1g11600   | Putative cytochrome P450 | 109 29 | 3.8 NA |
| At2g28900   | Membrane channel protein b | 367 100 | 3.7 1.1 |
| At5g15120   | Putative protein | 229 64 | 3.6 NA |

| Genes overexpressed in icl-2 seedlings |
|---------------------------------------|
| At1g46880    | Methionine/cystathionine γ lyase b | 3 129 | 49.6 NA |
| At3g80140    | β-Glucosidase-like protein | 22 521 | 23.9 NA |
| At5g20230    | Blue copper-binding protein | 44 685 | 15.5 NA |
| At3g59970    | Leucinecontaminad dioxgenase-like protein | 17 171 | 9.8 NA |
| At5g06000    | 11-β-Hydroxyoester dehydrogenase-like | 38 359 | 9.5 NA |
| At1g17810    | Tonoplast intrinsic protein b | 24 219 | 9.0 NA |
| At4g07820    | Pathogenesis-related protein b | 81 715 | 8.8 1.8 |
| At1g08630    | 1-Aspartine aldolase-like protein | 186 1501 | 8.1 1.7 |
| At3g7340     | Glutamine-dependent asparagine synthetase | 343 2698 | 7.9 2.4 |
| At2g3620     | Endochitinase b | 23 169 | 7.3 NA |
| At1g10070    | Branched chain amino acid aminotransferase-like protein | 108 726 | 6.7 NA |
| At5g54740    | 2S storage protein-like | 101 666 | 6.6 NA |
| At1g21400    | Branched chain α-keto acid dehydrogenase E1 α subunit | 32 206 | 6.4 NA |
| At2g55540    | Glycine-rich protein b | 666 3036 | 5.8 3.5 |
| At5g49750    | Putative protein | 101 561 | 5.6 NA |
| At5g85730    | Xylolucan endotransglycosylase-like | 157 814 | 5.2 1.0 |
| At2g32150    | Hydrolyase b | 273 1383 | 5.0 1.8 |
| At2g41100    | Calmodulin-like protein | 169 856 | 5.0 1.2 |
| At2g41200    | Late embryogenesis abundant protein | 46 175 | 4.8 NA |
| At1g71050    | Transcription factor b | 96 438 | 4.5 NA |
| At3g80140    | β-Glucosidase-like protein | 22 521 | 23.9 NA |
| At5g20230    | Blue copper-binding protein | 44 685 | 15.5 NA |

* Description from the Munich Information Centre for Protein Sequences or NCBI Arabidopsis genome database.

b Indicates the assignment of function is only putative.

confirm that mls-2 seedlings are capable of glucogenesis from acetate, whereas icl-2 seedlings are not.

\[ ^{14}\text{C} \text{Glycine and L-}^{14}\text{C} \text{Serine Can Act as Gluconeogenic Precursors in 2-Day-old Seedlings} \]

To investigate whether glucogenesis is possible from Gly and Ser, consistent with our hypothesis that glyoxylate may be incorporated into sugars via these metabolites, \(^{14}\text{C}\)-labeled Gly and Ser were fed to 2-day-old seedlings. Because the pools of unlabeled Gly and Ser in these seedlings vary, comparison of the rates of transfer of \(^{14}\text{C}\) to sugar is not informative. Nevertheless, \(^{14}\text{C}\) was detected in sugars of seedlings of all genotypes after feeding, confirming that glucogenesis occurs from these amino acids (data not shown).

**DISCUSSION**

Consistent with our hypothesis, mls seedlings grow faster, use their TAG more rapidly, and are better able to become established as plantlets with true leaves than icl seedlings. The transcriptome data showed that gene expression in mls-2 seedlings differed very little from that of wild type. In contrast, expression of 397 genes differed more than 2-fold in icl-2 seedlings compared with wild type. Genes overexpressed in icl-2 compared with wild type included many that are commonly up-regulated in carbohydrate-limited or senescent plant tissues. These include genes encoding enzymes of protein and amino acid catabolism and various glycohydrolases that might scavenge sugars from diverse carbohydrate sources. A gene encoding Gln-dependent Asn synthetase, which is highly responsive to carbohydrate limitation (24), was found to be induced in icl-2 and to a lesser extent in mls-2 seedlings.

NMR analysis of polar solutes showed that icl-2 seedlings have low levels of Glc, whereas mls-2 seedlings contain Glc at wild type levels (Fig. 5B). Both icl-2 and mls-2 seedlings have low Gln (Fig. 5C), which is consistent with both having elevated expression of a Gln-dependent asparagine synthetase gene, but Asn levels were not elevated. The elevated levels of valine, isoleucine, and lysine in icl-2 seedlings may result from in-
TABLE II
Differences in the transcriptomes of mls-2 mutant seedlings compared to MLS-2 seedlings

| Genes underexpressed in mls-2 seedlings | Signal | Fold induction |
|-----------------------------------------|--------|---------------|
| At5g03860 Malate synthase                | 767    | 142.9         |
| At3g14210 Myrosinase-associated protein^b | 168    | 7.0           |
| At1g45220 Putative peptidase            | 208    | 3.6           |
| At5g09220 Amino acid transport protein, AAP2 | 138    | 3.5           |
| At1g52070 Jasmonate-inducible protein^b | 136    | 3.3           |
| At5g64170 Putative protein              | 100    | 2.9           |
| At1g09240 Nicotinamide synthase^b       | 179    | 2.8           |
| At4g04830 Putative protein              | 341    | 2.4           |
| At1g18980 Germin^a                      | 118    | 2.4           |
| At5g12110 Elongation factor 1B α subunit| 140    | 2.3           |
| At5g26270 Putative protein              | 387    | 2.1           |
| At1g52060 Jasmonate-inducible protein^b | 106    | 2.0           |

| Genes overexpressed in mls-2 seedlings | Signal | Fold induction |
|---------------------------------------|--------|---------------|
| At2g41850 Polygalacturonase-like protein | 38    | 4.9           |
| At2g05540 Glycine-rich protein^a       | 203    | 3.5           |
| At5g39580 Peroxidase ATP24a            | 209    | 2.8           |
| At3g43450 1-Aminocyclopropane-1-carboxylic acid oxidase | 86    | 2.4           |
| At1g80180 Expressed protein            | 60     | 2.4           |
| At3g59590 Putative protein             | 59     | 2.3           |
| At4g18910 Major intrinsic protein-like protein | 190   | 2.3           |
| At5g45450 1-Aminoacyclopropane-1-carboxylic acid oxidase | 58    | 2.2           |
| At1g78830 Expressed protein            | 52     | 2.1           |
| At1g73260 Trypsin inhibitor^a          | 406    | 2.1           |

^a Description from the Munich Information Centre for Protein Sequences or NCBI Arabidopsis genome databases.

^b Indicates the assignment of function is only putative.

cincreased protein catabolism, as suggested by transcriptome data. Changes in amino acids and increased expression of genes encoding enzymes of branched-chain amino acid catabolism in icl-2 are likely to be causally related.

The greater rate of breakdown of TAG in mls relative to icl seedlings (Fig. 6A) and the accumulation of sugars in mls seedlings as they grow (Fig. 6, B and C) imply that mls seedlings are able to convert fatty acids from TAG into sugars. This was confirmed by the transfer of 14C from acetate to sugars in mls-2 at a level of 60% that of wild type seedlings (Fig. 7). In contrast to icl-2 seedlings, the pattern of 14C labeling in mls-2 seedlings differed from wild types only in the small increase in level of radioactivity released as CO2 and the small decrease incorporated into sugars (Fig. 7). This is entirely consistent with gluconeogenesis from acetate in mls-2 seedlings, which is less efficient than in wild type, such that more of the [14C]acetate is respired in mls-2 but is far more efficient than in icl-2 seedlings. The observation that seedlings lacking MLS are capable of gluconeogenesis from acetate in mls-2 seedlings, which is in marked contrast to the observation that ICL is required for such gluconeogenesis in Arabidopsis seedlings (7) and fulfills our original hypothesis. This observation also contrasts with the observation that both MLS and ICL are essential for gluconeogenesis and growth of microorganisms on ethanol, acetate, or fatty acids (2–5).

Because icl mutant seedlings are essentially incapable of gluconeogenesis from lipid (Fig. 7; Ref. 7), we deduce that ICL is required for gluconeogenesis in the mls mutants. The succinate produced by ICL will be oxidized in the mitochondria and oxaolacetic acid transported to maintain the cycle. ICL can only continue to produce succinate as long as the glyoxylate is metabolized. Furthermore, conversion of this glyoxylate to sugar is required for the net conversion of TAG to sugar to support plant growth; its respiration or degradation would achieve no more than is achieved by the respiration of TAG in the icl
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Fig. 6. Lipid and sugar levels in wild type and mutant seedlings during post-germinative growth. A, total fatty acids were extracted at daily intervals from seedlings grown in continuous light. Symbols are as above. Sucrose in (i) mls-2; (ii) MLS-2; icl-2; (iii) and (iv) ICL-2. The level of C20:1, a marker for TAG, was quantified in mls-2 and MLS-2 seedlings (i) and icl-2 and ICL-2 seedlings (ii). Data presented are the means ± S.D. for three measurement each from two separate experiments. B, Sucrose levels in seedlings. Symbols are as above. Sucrose in (i) mls-2 and MLS-2 and (ii) icl-2 and ICL-2 seedlings, grown in continuous light. Sucrose in mls-2 and MLS-2 (iii) and icl-2 and ICL-2 seedlings (iv), grown in darkness. C, total hexoses (Glc plus Fru) in seedlings. Hexoses in mls-2 and MLS-2 (i) and icl-2 and ICL-2 (ii) seedlings, grown in continuous light. Hexoses in mls-2 and MLS-2 (iii) and icl-2 and ICL-2 (iv) seedlings, grown in darkness. Data presented are the means ± S.D. for triplicate batches of seedlings. DPI, days post-imbibition.

We hypothesized that this glyoxylate would enter the photorespiratory gluconeogenic pathway. Some bacteria can achieve gluconeogenesis from glyoxylate using the “glycerate pathway” employing glyoxylate carboligase, tartronic semialdehyde reductase, and glycerate kinase (26). To our knowledge, glyoxylate carboligase and tartronic semialdehyde reductase have not been detected in plants, and no genes are predicted in the *Arabidopsis* genome. *Micrococcus denitrificans* converts glyoxylate to oxaloacetate via erythro-β-hydroxyaspartate (27), but again, we can find no evidence for the appropriate enzymes in plants.

Final confirmation of our proposed pathway of MLS-independent gluconeogenesis from acetate will depend upon detailed genetic and molecular characterization in the future. However, our hypothesis that glyoxylate will feed into the photorespiratory pathway is supported by elevated levels of Gly and Ser in mls seedlings and by the transfer of 14C from Gly and Ser into sugars. Microarray data show that the genes encoding the enzymes of the photorespiratory pathway are expressed as highly in 2-day-old seedlings as in 7-day-old seedlings, and mature leaves (information obtained from nasc.nott.ac.uk). We have also determined that serine-glyoxylate aminotransferase activity coupled to hydroxypruvinate reductase activity in mls-2 seedlings was 20.8 ± 2.1 and 39 ± 4.3 

\[ \mu \text{mol} \text{min}^{-1} \text{mg of protein}^{-1} \]

to metabolize glyoxylate through the photorespiratory pathway, and the block caused by the lack of MLS is alleviated. At this point TAG breakdown starts to accelerate, and sugars begin to accumulate.

The observation that seeds of icl-2 accumulate less lipid than wild type while mls-2 seeds accumulate normal amounts (Fig. 6A; Ref. 7) provides evidence that ICL has an important function at other stages of the life cycle for which MLS is not required. There are reports of ICL activity in the absence of MLS in green leaves (28, 29, 30), which may thus function in such a pathway.

The phenotypes of glyoxylate cycle mutants clearly indicate that there is a cost to *Arabidopsis* seedlings lacking ICL and MLS. However, although the absence of ICL and thus gluconeogenesis from lipid results in severely compromised seedling growth (Fig. 3; Ref. 7), the absence of MLS results in a much less severe phenotype because the seedlings are able to employ an alternative gluconeogenic mechanism. This novel metabolic pathway from acetate to sugar, potentially employing photorespiratory enzymes in conjunction with glyoxylate cycle enzymes, in *mls* mutant seedlings is an example of the remarkable metabolic flexibility of *Arabidopsis*.

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