Tricarboxylic Acid Cycle Activity Regulates Tomato Root Growth via Effects on Secondary Cell Wall Production[1][W][OA]

Margaretha J. van der Merwe[2,3], Sonia Osorio[2], Wagner L. Araújo, Ilse Balbo, Adriano Nunes-Nesi, Eugenia Maximova, Fernando Carrari[4], Victoria I. Bunik, Staffan Persson, and Alisdair R. Fernie*

Max-Planck-Institut für Molekulare Pflanzenphysiologie, 14476 Potsdam-Golm, Germany (M.J.v.d.M., S.O., W.L.A., I.B., A.N.-N., E.M., F.C., S.P., A.R.F.); and A.N. Belozersly Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia (V.I.B.)

Transgenic tomato (*Solanum lycopersicum* 'Moneymaker') plants independently expressing fragments of various genes encoding enzymes of the tricarboxylic acid cycle in antisense orientation have previously been characterized as exhibiting altered root growth. In this study, we evaluate the rates of respiration of roots from these lines in addition to determining their total dry weight accumulation. Given that these features were highly correlated, we decided to carry out an evaluation of the cell wall composition in the transformants that revealed a substantial reduction in cellulose. Since the bulk of cellulose is associated with the secondary cell walls in roots, we reasoned that the transformants most likely were deficient in secondary wall cellulose production. Consistent with these findings, cross-sections of the root collar (approximately 15 mm from the junction between root and stem) displayed reduced lignified secondary cell walls for the transformants. In contrast, cell and cell wall patterning displayed no differences in elongating cells close to the root tip. To further characterize the modified cell wall metabolism, we performed feeding experiments in which we incubated excised root tips in [U-^{14}C]glucose in the presence or absence of phosphonate inhibitors of the reaction catalyzed by 2-oxoglutarate dehydrogenase. Taken together, the combined results suggest that restriction of root respiration leads to a deficit in secondary cell wall synthesis. These data are discussed in the context of current models of biomass partitioning and plant growth.

Metabolism is arguably the best characterized of all molecular interaction networks in biology. From pioneering studies defining key metabolic pathways, such as the Calvin cycle, through decades of enzymology and more recent genetic studies of metabolism, there is an unprecedented density of both mechanistic and descriptive data relating to metabolic behavior (Sweetlove et al., 2008). However, many pathways and processes have been studied in isolation, and the relative importance of primary plant metabolism to the sustenance and regulation of either anabolic polymer production or secondary metabolism has received relatively scant attention. Indeed, publications directly addressing such questions are few and instead tend to concentrate on the direct (primary) precursors of specific pathways (Amor et al., 1995; Cronin and Hay, 1996; German et al., 2003; Ruan et al., 2003; Reiter, 2008). For example, coordination of primary metabolism and cell wall synthesis has largely focused on the immediate precursors, such as different nucleotide sugars (Amor et al., 1995; Ruan et al., 2003; Reiter, 2008).

The importance of a basic understanding for cell wall synthesis is reflected in the recent financial incentives to maximize the conversion of solar energy into ethanol-based biofuels (Somerville, 2006; Carpita and McCann, 2008; Heaton et al., 2008; Lopez-Casado et al., 2008; Pauly and Keegstra, 2008; Yuan et al., 2008; Carroll and Somerville, 2009). Current efforts are largely focused on the conversion of the lignocellulosic matrix to ethanol by improving the extraction of sugar-based molecules or through reductions of the polyphenolic polymer lignin (Carroll and Somerville, 2009). However, it appears that only limited success will be obtained through genetic manipulations of enzymes directly associated with specific cell wall polymers. For example, substantial reductions in one polymer typically result in unwanted growth phenotypes or compensation by other polymers (His et al., 2001; Pena et al., 2007; Persson et al., 2007; Schilmiller et al., 2009). Such outcomes are usually unwanted

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[2] These authors contributed equally to the article.

[3] Present address: Institute for Plant Biotechnology, University of Stellenbosch, Stellenbosch 7602, South Africa.

[4] Present address: Instituto de Biotecnología, Instituto Nacional de Tecnología Agropecuaría, and Consejo Nacional de Investigaciones Científicas y Técnicas, P.O. Box 25, B1712WAA Castelar, Argentina.

* Corresponding author; e-mail fernie@mpimp-golm.mpg.de.

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traits and moreover are difficult to predict. An alternative route is to alter whole cassettes of genes or perturb the ratio of precursor levels for the different cell wall polymers. Such changes may be achieved, for example, by alterations in nucleotide-sugar ratios (Seifert, 2004; Sharples and Fry, 2007). That said, it can be envisaged that these types of changes could also be obtained by changes in upstream metabolic fluxes.

While it may be difficult to directly link changes in biomass with single primary metabolites, it is evident that certain metabolic signatures can signify changes in plant biomass (Meyer et al., 2007; Sulpice et al., 2009). However, no large-scale study has assessed the role of cell wall composition in this context. Several reverse genetic studies have revealed clear correlations between energy metabolism and plant growth. For example, manipulations of apoplastic and cytosolic apyrase and plastidial adenylate kinase activities have all recently been documented to yield clear growth phenotypes (Regierer et al., 2002; Riewe et al., 2008a, 2008b). In addition, suppression of several enzymes associated with the tricarboxylic acid (TCA) cycle also affects aerial plant growth (Carrari et al., 2003; Studart-Guimarães et al., 2007; Sienkiewicz-Porzucek et al., 2008; van der Merwe et al., 2009). These data, and those coming from plants exhibiting deficiencies in photosynthesis per se (Gifford et al., 1984; Stitt and Schulze, 1994; Henkes et al., 2001) as well as data documenting conditional growth deficiencies of mutants of photorespiration (Somerville and Ogren, 1980; Wingler et al., 2000; Voll et al., 2006; Timm et al., 2008; Foyer et al., 2009), suggest that plant growth is restricted by cellular energy availability. Interestingly, tuber-specific down-regulation of plastidial adenylate kinase (Regierer et al., 2002) or cytosolic UMP synthase (Geigenberger et al., 2005) in potato (*Solanum tuberosum*) both resulted in increased tuber growth. In the latter case, the reduction in UMP synthase also led to elevated cell wall constituents. Consequently, a possible explanation for reduced root growth in TCA cycle-deficient tomato (*Solanum lycopersicum*) plants may be that the root cannot produce enough cell wall to sustain growth. To test this, we took two independent approaches. First, we evaluated a range of previously characterized transgenic lines (Nunes-Nesi et al., 2005, 2007; Studart-Guimarães et al., 2007; Sienkiewicz-Porzucek et al., 2008) as well as one novel set of transgenics that affect different enzymes of the TCA cycle. Second, we utilized a phosphonate analog of 2-oxoglutarate, which has been demonstrated to produce a rapid inhibition of the TCA cycle through a specific effect on the reaction catalyzed by the 2-oxoglutarate dehydrogenase complex (Bunik et al., 2005; Araújo et al., 2008), in order to ascertain which altered secondary cell wall phenotype could directly be ascribed to the inhibition of the TCA cycle itself. The results are discussed in terms of current models linking plant energy metabolism with biosynthesis and growth.

Table I. *Characterization of enzyme activities in roots of 5-week-old mMDH, FL, SCoAL, ACO, and CS antisense transgenic plants*

Values are presented as means ± SE of six individual plants per line. Values marked with different letters differ significantly from each other ($P < 0.05$). ANOVA was done between transgenic lines and their corresponding wild type (WT).

Genotype	Enzyme Activities
	μmol min^{-1} mg^{-1} protein
WT_{mMDH}	3,420 ± 310[a]
mMDH7	1,540 ± 140[b]
mMDH8	1,330 ± 140[b]
mMDH21	1,780 ± 160[b]
WT_{FL}	40 ± 5[a]
FL11	9 ± 1[b]
FL41	7 ± 1[b]
FL63	22 ± 2[c]
WT_{SCoAL}	450 ± 81[a]
SCoAL18	300 ± 40[b]
SCoAL25	221 ± 30[b]
SCoAL47	76 ± 10[c]
WT_{ACO}	110 ± 11[a]
ACO19	7 ± 1[b]
ACO26	42 ± 5[c]
ACO38	71 ± 1[d]
WT_{CS}	97 ± 13[a]
CS22	54 ± 3[b]
CS25	56 ± 3[b]
CS45	24 ± 2[c]

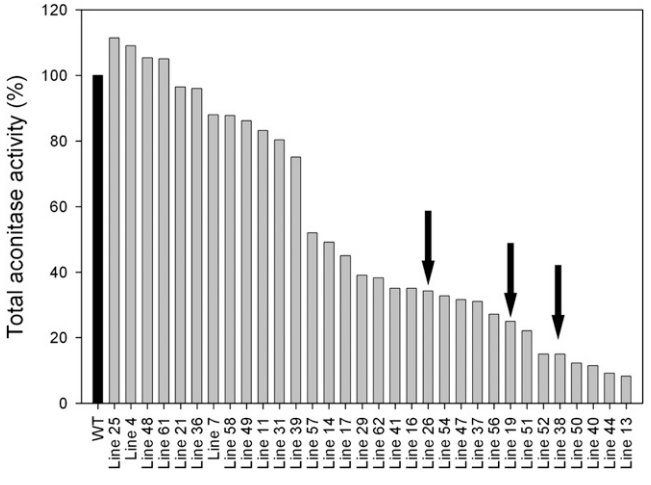

Figure 1. Analysis of antisense aconitase tomato lines. Screening of total aconitase activity in primary transformants in extracts of tissue leaf-sampled 5-week-old plants. Black arrows represent the selected lines for further studies. WT, Wild type.

RESULTS

Generation of Aconitase Antisense Lines

Although we have previously shown that the *aco1* mutant of the wild species tomato *Solanum pennellii*

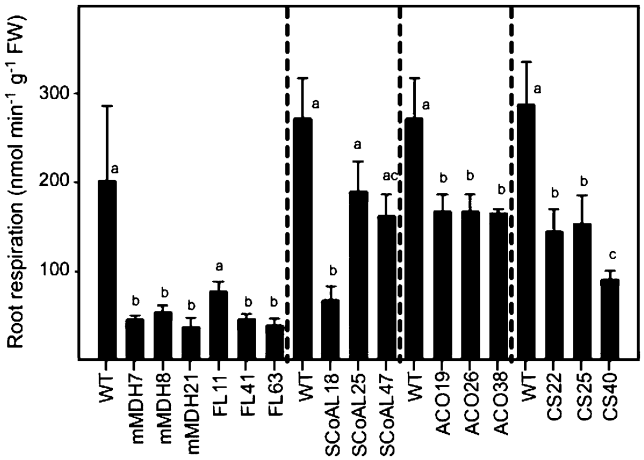

Figure 2. Respiratory parameter in the antisense transgenic lines of 5-week-old plants: mMDH, FL, SCoAL, ACO, and CS. Values are means ± SE of a representative determination on six individual plants. Respiration experiments were carried out as described by Palmieri et al. (2008). Values marked with different letters differ significantly from each other ($P < 0.05$). ANOVA was done between transgenic lines and their corresponding wild type (WT). FW, Fresh weight.

displays reduced root growth (Carrari et al., 2003), which was similar to the subsequent phenotypes observed by reducing the activities of malate dehydrogenase, fumarase, succinyl-CoA ligase, and citrate synthase in cultivated tomato (Nunes-Nesi et al., 2005, 2007; Studart- Guimarães et al., 2007; Sienkiewicz-Porzucek et al., 2008), we decided to create aconitase-deficient transgenics of the cultivated tomato to afford a more uniform comparison. For this purpose, we cloned an 800-bp fragment of the *S. lycopersicum* aconitase antisense orientation into the vector pBINAR (Liu et al., 1999) between the cauliflower mosaic virus 35S promoter and the *octopinsynthase* (*ocs*) terminator and transformed this into plants using an *Agrobacterium tumefaciens*-mediated protocol. Primary transformants were selected on kanamycin and transferred to the greenhouse, where they were screened at the level of leaf aconitase activity, with 32 lines being chosen for further studies, and propagated in tissue culture (Fig. 1). Having generated these additional lines, we then grew them and the previously generated transformants in the greenhouse alongside wild-type controls across a series of four different plantings. Characterization of the roots of the aconitase lines (ACO) and the antisense lines for succinyl-CoA ligase (SCoAL), fumarase (FL), mitochondrial citrate synthase (CS), and mitochondrial malate dehydrogenase (mMDH), confirmed a reduction in the maximal catalytic activities of the enzyme of interest within the tissue (Table I).

Characterization of Root Respiration and Root Growth

Having established that the transgenic material was appropriate for our biological questions, we next characterized the rate of respiration in the roots. For this purpose, we took two complementary approaches. First, we directly characterized the rate of oxygen consumption in excised root tips (the terminal 10 mm of the root tips) of 5-week-old plants in a Clarke-type electrode. These experiments demonstrated that oxygen consumption was significantly reduced in all transgenic lines, with the exception of

Table II. *Label enrichments in 5-week-old mature root tips incubated in unilabeled [^{13}C]Glc for antisense transgenic lines*

Roots for mitochondrial mMDH, FL, SCoAL, ACO, and CS plants were incubated for 5 h. Two representative lines of each transgenic set were characterized. Values are presented as means ± SE of six individual plants per line. Values marked with different letters differ significantly from each other ($P < 0.05$). Negligible enrichment is indicated with "nd."

Genotype	Citrate	Fumarate	Isocitrate ($\times 10^{-4}$)	Malate	2-Oxoglutarate
			μmol label accumulated expressed as hexose equivalents		
Wild type	31.7 ± 10.3	0.7 ± 0.1	2.7 ± 0.9	4.7 ± 0.9	2.7 ± 1.2
mMDH7	15.6 ± 4.7	0.3 ± 0.2	1.1 ± 0.3[a]	3.3 ± 1.8	5.4 ± 1.9
mMDH8	69.0 ± 21.4	1.1 ± 0.6	2.9 ± 1.0	8.9 ± 1.6[a]	17.6 ± 3.4[a]
mMDH21	7.3 ± 2.7[a]	0.2 ± 0.1[a]	3.3 ± 1.3	2.6 ± 0.8	1.0 ± 0.3
FL11	7.5 ± 2.0[a]	0.3 ± 0.2	7.5 ± 2.1[b]	0.9 ± 0.3[b]	1.8 ± 1
FL41	5.2 ± 2.6[a]	0.5 ± 0.1	4.4 ± 2.3	0.5 ± 0.2[b]	2.8 ± 1.1
FL63	26.2 ± 12.6	0.2 ± 0.1[a]	18.1 ± 9.3	4 ± 1.3	18.4 ± 2.1[a]
SCoAL18	38.8 ± 4.8	0.8 ± 0.3	nd	2.3 ± 0.3[c]	7.7 ± 1.0[b]
SCoAL25	33.7 ± 3.7	0.4 ± 0.1	nd	3.8 ± 1.1	3.5 ± 1.1
SCoAL47	26.5 ± 5.7	0.7 ± 0.3	nd	5.5 ± 2.5	3.5 ± 2.0
ACO19	20.6 ± 5.3	0.5 ± 0.1	nd	4.3 ± 1.0	2.4 ± 0.7
ACO26	25 ± 8.3	0.8 ± 0.3	nd	6.6 ± 1.5	1.3 ± 0.2
ACO38	14.6 ± 3.2[b]	0.7 ± 0.4	nd	5.8 ± 2.0	2.4 ± 1.1
CS22	32.9 ± 10.2	1.2 ± 0.5	2.6 ± 1.1	2.4 ± 0.6[c]	4.2 ± 2.3
CS25	27.1 ± 7.1	1.2 ± 0.4	nd	4.1 ± 2.1	nd
CS40	33.5 ± 4.2	0.5 ± 0.3	nd	3.5 ± 0.7	1.3 ± 1.1

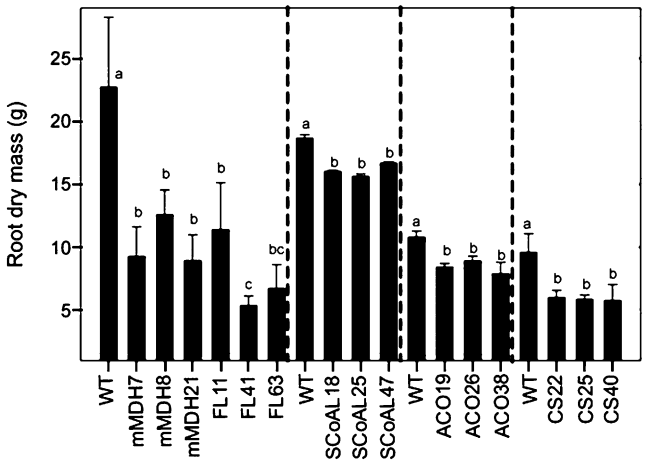

Figure 3. Root biomass parameters in the antisense transgenic lines of 5-week-old plants: mMDH, FL, SCoAL, ACO, and CS. Values marked with different letters differ significantly from each other ($P < 0.05$). ANOVA was done between transgenic lines and their corresponding wild type (WT).

lines FL11 and SCoAL25, as assessed using ANOVA across the entire data set (Fig. 2). In a complementary approach, we performed tracer experiments following the incubation of similarly aged root tips in unilabeled [^{13}C]Glc. Following quenching of metabolism and extraction, the enrichment of individual metabolites was determined by the quantification of ^{13}C enrichment following incubation in [^{13}C]Glc for 5 h using gas chromatography-mass spectrometry (GC-MS). These studies revealed a significant decrease in citrate labeling in the lines FL11 and FL41, mMDH21, and ACO38, while a decrease in the fractional enrichment of malate was additionally observed in FL11 and FL41 (Table II). Perhaps surprisingly, these were the only observable changes in the level of enrichment of organic acids. However, this may be due to the well-known extensive compartmentation of these metabolites (Stitt et al., 1989; ap Rees and Hill, 1994).

To characterize the effect of the down-regulated TCA cycle on root growth, we evaluated the root biomass (Fig. 3; Supplemental Fig. S1). The data revealed a clear decrease in total root biomass for the transgenics compared with controls when assessed by ANOVA across the entire data set. Interestingly, the variance in root respiration and root biomass strongly correlated when plotted against each other (i.e. the reduction in root biomass appears to be directly related to the rate of respiration; Fig. 4).

Metabolite Levels and Cell Wall Composition of the Transformants

We next decided to evaluate the content of carbohydrates in the roots from 5-week-old plants. Soluble sugars and starch were extracted and measured (Table III). These studies revealed that the aconitase lines had increased levels of Fru, Suc, and Glc (significantly only in lines ACO19 and ACO38), and succinyl-CoA ligase lines had increased levels of Glc (significantly in lines SCoAL25 and SCoAL47). By contrast, the mitochondrial citrate synthase lines were invariant in all these parameters, with only a single significant difference being observed. However, these changes were not consistent across the lines and therefore cannot be the cause for the reduction in growth, since we have, by contrast, previously reported that mitochondrial malate dehydrogenase and fumarase transgenic lines

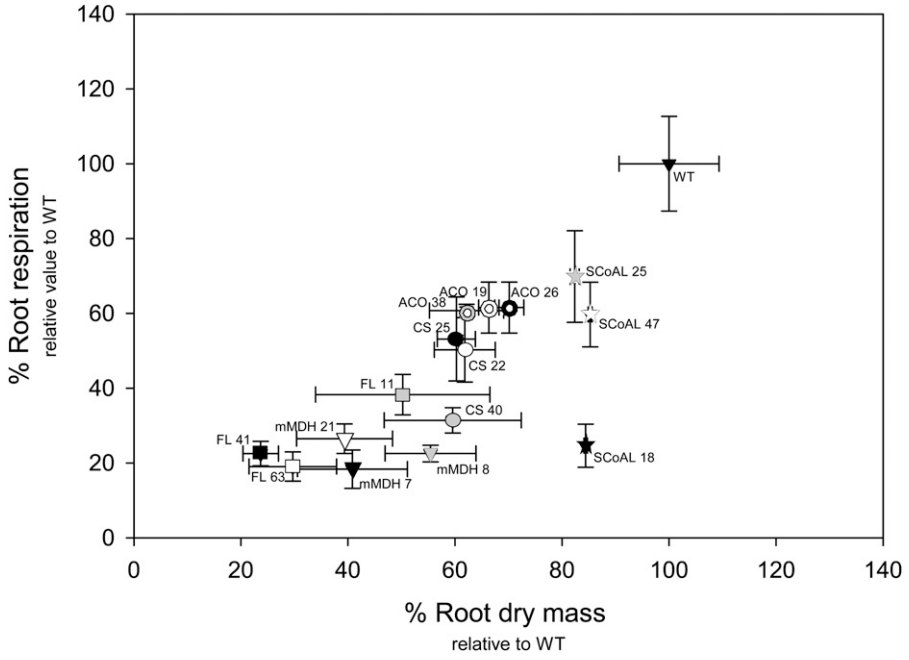

Figure 4. Correlation analysis (Pearson's test) of root biomass and respiration parameters in the lines mMDH ($r = 0.962$), FL ($r = 0.989$), SCoAL ($r = 0.732$), ACO ($r = 0.985$), and CS ($r = 0.953$). Correlations were significant at $P < 0.05$ for all antisense lines except SCoAL. WT, Wild type.

Table III. *Storage carbohydrate, starch, and protein contents of 5-week-old mature antisense root tips*

Data are presented as means ± SE of six individual plants per line. Values marked with different letters differ significantly from each other ($P < 0.05$).

Genotype	Glc	Fru	Suc	Starch	Protein
	μmol g^{-1} fresh wt				*μg g^{-1} fresh wt*
Wild type	76.1 ± 8.1	56.7 ± 8.9	15.8 ± 1.1	20.7 ± 1.0	190.1 ± 0.5
SCoAL18	60.9 ± 6.4	48.2 ± 10.7	18.6 ± 0.8	8.6 ± 0.5[a]	185.6 ± 3.4
SCoAL25	95.1 ± 10.1[a]	79.4 ± 12.0[a]	29.8 ± 2.1[a]	14.7 ± 2.2[b]	190.3 ± 3.3
SCoAL47	129.3 ± 13.7[b]	57.3 ± 8.0	14.0 ± 2.2	18.7 ± 3.4	189.6 ± 1.7
ACO19	114.1 ± 10.5[a,b]	79.4 ± 11.6[a]	30.0 ± 2.2[a]	17.3 ± 3.4	190.8 ± 3.9
ACO26	90.3 ± 10.6	85.1 ± 9.8[a]	26.8 ± 2.1[a]	18.2 ± 3.5	196.0 ± 3.4
ACO38	129.3 ± 13.7[a]	93.5 ± 24.9[a]	23.6 ± 1.6[a]	15.9 ± 2.8	202.2 ± 3.3[a]
CS22	68.5 ± 7.2	50.5 ± 9.8	17.3 ± 2.0	18.1 ± 1.2	195.7 ± 5.3
CS25	82.9 ± 8.8	59.5 ± 6.2	22.1 ± 1.5[b]	17.3 ± 3.2	190.1 ± 3.2
CS40	98.9 ± 10.5[a]	81.1 ± 5.3[a]	17.5 ± 1.1	13.8 ± 3.6[b]	186.1 ± 2.3

were characterized by reduced levels of sugars (van der Merwe et al., 2009).

Interestingly, the level of cellulose was substantially reduced in the fumarase and mitochondrial malate dehydrogenase lines both when assessed by ANOVA across the entire data set (Fig. 5). Similar trends were also observed for all the transgenic lines (Fig. 5). In addition, the neutral sugar composition was unaltered in the mutant lines as assessed by alditol acetate analyses using GC-MS (Table IV), suggesting no changes in hemicellulose polymer.

The substantial decrease in cellulose suggests a dramatic reduction in cell wall synthesis. To assess whether these reductions altered the cell morphology or cell wall integrity, we made root tips and root collar cross-sections of the transgenic tomato lines and compared these with the wild type. We used the dye phloroglucinol to visualize polyphenolic polymer lignin, which is a major component of the secondary cell wall (Fig. 6; Ros-Barcelo, 1997). Interestingly, the wall for the mMDH and FL lines appeared to have a reduction in lignin content, particularly prominent in the case of lines MDH8 and FL11 (Fig. 6). In addition, several transgenic root cross-sections displayed irregular xylem vessels, which have previously been described in secondary cell wall-deficient mutants (Brown et al., 2005; Fig. 6). However, no, or very minimal, effects were observed in the CS, ACO, and SCoAL lines (data not shown). It is important to know that the mMDH and FL enzymes comprise the last steps of the TCA cycle and are responsible for the regeneration of acetyl-CoA for subsequent use in gluconeogenesis. In contrast, the CS, ACO, and SCoAL enzymes represent the first three steps in the cycle and therefore draw on the acetyl-CoA pool. It is evident, therefore, that despite the reduced root biomass and the underdeveloped xylem, the root is still able to sustain growth.

Analysis of the Effects of Chemical Inhibition of the TCA Cycle on Cellular Metabolism in Roots

To further characterize metabolism in the transgenics, we next performed feeding experiments in which we incubated excised root tips in a buffered solution containing 10 mM [U-^{14}C]Glc in the presence and absence of phosphonate inhibitors of the reaction catalyzed by 2-oxoglutarate dehydrogenase for a period of 2 h. The rationale of this experiment was that the phosphonate inhibitors succinyl phosphonate and carbonyl ester of succinyl phosphonate have been documented to be potent and specific inhibitors of 2-oxoglutarate dehydrogenase in widely divergent species (Bunik et al., 2005; Bunik and Fernie, 2009). Moreover, inhibition of this activity has previously been reported to have dramatic consequences on plant respiration, even following short-term application (Araujo et al., 2008), which would not be complicated by secondary effects of a long-term inhibition of energy metabolism. In order to assess whether a sudden block of the metabolite flux through the TCA cycle would result in reduced cell wall material, we applied

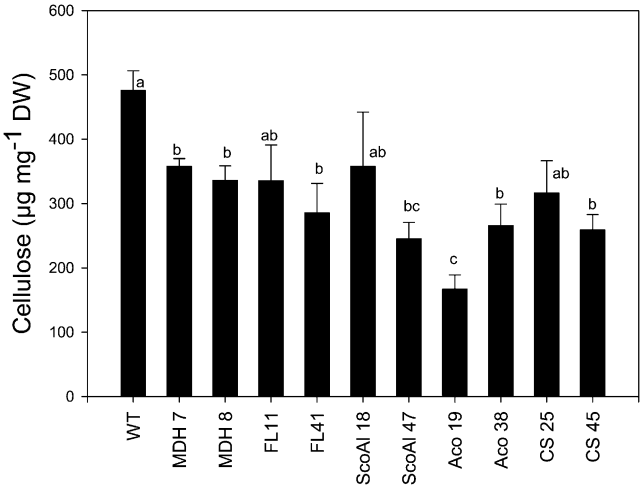

Figure 5. Cellulose content in the transgenic lines. Cellulose content of the root collar of the mature roots is shown. Two representative lines of each transgenic set were characterized. Values are means ± SE of a representative determination on six individual plants per line. Values marked with different letters differ significantly from each other ($P < 0.05$). DW, Dry weight; WT, wild type.

Table IV. *Cell wall analyses of the transgenic lines*

Monosaccharide composition of TFA hydrolysates of root cell walls was measured by alditol acetate derivatives (Blakeney et al., 1983). Two representative lines of each transgenic set were characterized. Values are means ± SE of a representative determination on six individual plants per line. Values marked with different letters differ significantly from each other ($P < 0.05$).

Genotype	Alditol Acetate Derivative						
	Glc	Gal	Man	Xyl	Ara	Fuc	Rha
				μg mg^{-1} dry wt			
Wild type	12.5 ± 3.2	32.1 ± 4.8	5.7 ± 0.2	60.6 ± 4.8	49.7 ± 11.2	11.3 ± 4.0	11.5 ± 1.6
mMDH7	15.4 ± 2.1	33.7 ± 2.4	6.0 ± 0.5	86.6 ± 3.4[a]	51.7 ± 3.1	7.2 ± 1.4	10.8 ± 0.3
mMDH8	11.6 ± 0.9	29.4 ± 5.6	5.6 ± 0.9	67.4 ± 10.0	44.6 ± 6.9	6.0 ± 0.3	12.6 ± 0.6
FL11	13.0 ± 2.1	35.5 ± 6.2	4.5 ± 0.9	60.6 ± 11.9	49.8 ± 4.9	9.0 ± 2.1	12.2 ± 1.1
FL41	13.7 ± 1.4	36.0 ± 3.44	5.0 ± 0.9	69.8 ± 3.2	54.1 ± 4.4	8.1 ± 0.7	14.6 ± 1.6
ScoAl18	12.0 ± 0.9	31.2 ± 3.3	5.7 ± 0.2	53.2 ± 2.6	47.7 ± 5.1	6.1 ± 0.6	12.3 ± 0.3
ScoAl47	16.6 ± 0.9	35.7 ± 1.2	8.0 ± 0.9[a]	64.6 ± 6.1	60.1 ± 1.7	7.7 ± 1.1	12.7 ± 1.5
ACO19	12.6 ± 0.7	29.8 ± 3.4	6.9 ± 1.4	66.4 ± 6.9	57.9 ± 6.2	7.6 ± 0.7	13.3 ± 1.9
ACO38	10.8 ± 0.6	28.1 ± 1.6	5.6 ± 0.5	52.7 ± 3.2	44.2 ± 1.0	5.8 ± 0.2	8.9 ± 0.6
CS25	11.5 ± 1.4	31.7 ± 4.8	5.3 ± 0.3	48.7 ± 9.2	44.7 ± 7.3	6.1 ± 1.2	11.4 ± 1.8
CS45	12.4 ± 1.8	29.7 ± 3.7	6.2 ± 0.8	47.5 ± 0.5	42.1 ± 6.8	5.6 ± 0.5	11.4 ± 0.1
				mol %			
Wild type	6.1 ± 0.4	5.8 ± 0.8	28.5 ± 1.4	35.4 ± 1.9	2.8 ± 0.3	15.5 ± 1.0	5.9 ± 0.4
mMDH7	4.9 ± 0.1	3.3 ± 0.4	25.7 ± 0.5	43.2 ± 0.4[a]	2.5 ± 0.2	14.0 ± 0.4	6.4 ± 0.7
mMDH8	6.9 ± 0.4	3.3 ± 0.1	26.5 ± 1.9	40.2 ± 4.1	2.8 ± 0.3	14.5 ± 1.6	5.8 ± 0.5
FL11	6.6 ± 0.9	4.8 ± 0.8	28.7 ± 1.3	34.6 ± 2.8	2.2 ± 0.2	16.9 ± 0.8	6.3 ± 0.5
FL41	7.0 ± 0.5	3.9 ± 0.1	28.4 ± 0.7	36.7 ± 0.7	2.2 ± 0.2	15.7 ± 0.8	5.9 ± 0.3
SCoAL18	7.1 ± 0.4	3.5 ± 0.3	30.0 ± 1.0	33.6 ± 0.7	3.0 ± 0.1	16.3 ± 0.4	6.3 ± 0.0
SCoAL47	6.0 ± 0.4	3.6 ± 0.4	31.1 ± 1.4	33.3 ± 1.3	3.4 ± 0.2	15.4 ± 0.4	7.1 ± 0.1
ACO19	6.6 ± 0.5	3.8 ± 0.2	31.3 ± 0.9	36.0 ± 1.8	3.1 ± 0.3	13.6 ± 1.4	5.7 ± 0.3
ACO38	5.5 ± 0.3	3.6 ± 0.0	30.0 ± 0.4	35.7 ± 0.9	3.1 ± 0.1	15.9 ± 0.8	6.1 ± 0.2
CS25	7.2 ± 1.6	3.7 ± 0.3	29.7 ± 0.7	32.3 ± 1.4	3.0 ± 0.2	17.6 ± 0.5	6.4 ± 0.5
CS45	6.3 ± 0.6	3.5 ± 0.2	26.4 ± 0.8	38.2 ± 1.2	3.0 ± 0.2	15.9 ± 1.5	6.6 ± 0.2

these inhibitors to wild-type plants at final concentrations of 50 and 100 μM. Consistent with previous data obtained following inhibition of 2-oxoglutarate dehydrogenase in potato tubers (Araujo et al., 2008), there were reductions in [U-^{14}C]Glc uptake (Fig. 7A), metabolism (Fig. 7B), and $^{14}CO_2$ evolution (Fig. 7C), which were more severe in incubations in which a higher concentration of inhibitor was used. Moreover, despite unaltered accumulation in organic acids (Fig. 7D), similar reductions of radiolabel redistribution to protein (Fig. 7E) and cell wall (Fig. 7F) and increase of radiolabel redistribution to phosphoesters (Fig. 7G) to those observed in potato tuber were apparent. In this study, we estimated absolute fluxes following calculation of the specific activity of the hexose phosphate pool (Fig. 7H). The results of these calculations revealed that the rates of starch (Fig. 7I) and Suc synthesis (Fig. 7J) were essentially unaltered, as was the rate of glycolysis (Fig. 7K). The rate of cell wall synthesis, however, was significantly reduced (Fig. 7L), thus suggesting that inhibitors of the TCA cycle have a direct and immediate effect on cell wall biosynthesis.

DISCUSSION

While considerable research effort has been expended on defining the importance of nucleotide sugar precursors in the regulation of cell wall biosynthesis (Amor et al., 1995; Cronin and Hay, 1996; German et al., 2003; Reiter, 2008), relatively few studies have directly focused on the general role that primary metabolism, and more specifically energy metabolism, plays in this process. Our recurrent observations that tomato plants deficient in the expression of genes encoding enzymes of the mitochondrial TCA cycle exhibited stunted root growth (Carrari et al., 2003; Studart-Guimarães et al., 2007; van der Merwe et al., 2009) have driven us to address the question of whether cell wall biosynthesis is energy limited in roots. While there are precedents to suggest that this may be the case, these are somewhat indirect; for example, tuber-specific down-regulation of UMP synthase in potato led to elevated flux to, and accumulation of, cell wall constituents, while Arabidopsis (*Arabidopsis thaliana*) knockouts of plastidial adenylate kinase and of the mitochondrial adenylate transporter ANT1 were characterized by enhanced and reduced growth, respectively (Carrari et al., 2005; Palmieri et al., 2008). In this paper, we assess root respiration rates, root growth, metabolite levels, metabolic fluxes, and cell wall characteristics both at the levels of chemical composition and cell morphology of a battery of transgenics displaying deficient expression of different constituent enzymes of the TCA cycle. In addition, we evaluate the effect of short-term inhibition of respiratory activity following the application of phosphonate analogs of one of the key intermediates of the TCA cycle, 2-oxoglutarate.

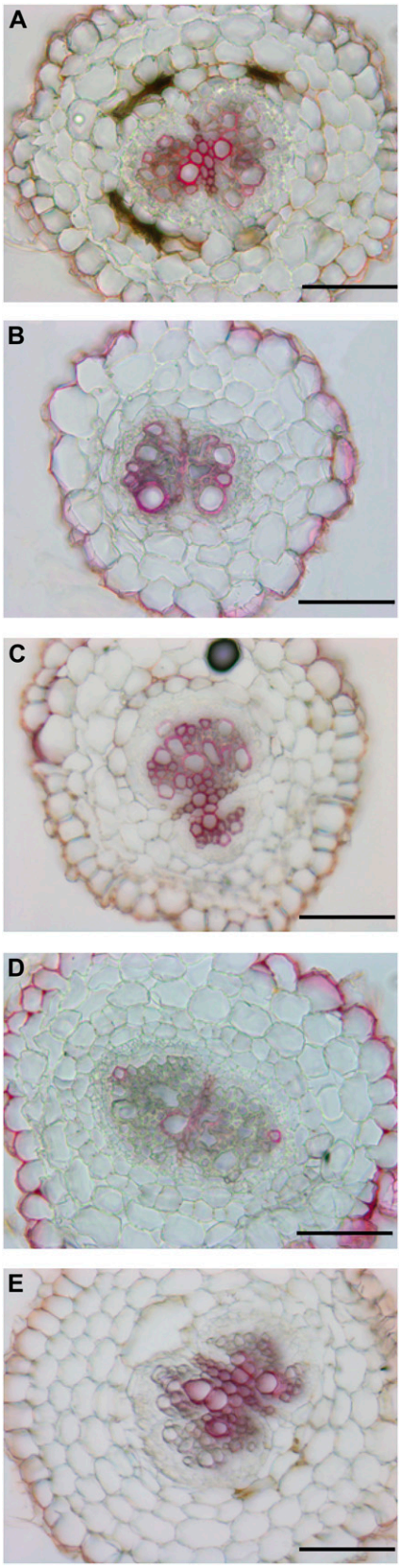

Figure 6. Phloroglucinol staining for lignin content in the transgenic mMDH and FL lines. Mature root collars embedded in agarose were sectioned (50 μm). A, Wild type. B, mMDH8. C, mMDH21. D, FL11. E, FL41. The samples shown were chosen as representative of four to six replicates per line. Bars = 100 μm.

Deficiency in Expression of Genes Encoding Enzymes of the TCA Cycle Results in Dramatic Decreases in Respiration and in Root Growth

Unsurprisingly, reduction in the activity of TCA cycle in the root has a far greater impact on respiration in the root than in the leaf, which is able to compensate for deficiencies in the TCA cycle by means of both photosynthetic and photorespiratory processes (Krömer et al., 1993; Krömer, 1995). It is possible that alternative electron donors of the mitochondrial electron transport chain exist in heterotrophic tissues, such as the reactions catalyzed by galactolactone dehydrogenase and the electron transfer flavoprotein complex. However, evidence of their operation in plants is, as yet, confined to photosynthetic tissues (Ishizaki et al., 2005, 2006; Nunes-Nesi et al., 2005; Alhagdow et al., 2007). That said, it would appear likely that the respiratory bypass pathways function in heterotrophic tissues even if the pathways of photosynthesis and photorespiration do not. The results from the direct measurement of oxygen consumption were largely, although not entirely, in keeping with flux determinations, which suggested a decreased redistribution of label to both citrate and malate. However, evaluation of such data is extraordinarily difficult, given the complex subcellular distribution of the organic acid pools of plants (Stitt et al., 1989; ap Rees and Hill, 1994). The reduced rate of respiration observed in the transformants was closely coupled to a decreased root biomass when assessed on a dry weight basis, a fact that we believe is most likely due to the decreased energy status and production of precursor metabolites to support biosynthesis.

Deficiency in Expression of Genes Encoding Enzymes of the TCA Cycle Results in Metabolic Shifts That Influence Cell Wall Accumulation and Composition

We document here that the restriction in TCA cycle activity in the transformants provokes considerable changes in the levels of cellulose suggestive of a markedly altered secondary cell wall. In keeping with this hypothesis, cross-sections of the root collar of the mMDH and FL lines, MDH8 and FL11, clearly contained less lignin as assessed by phloroglucinol staining. However, differences were neither observed in cell organization nor in cell wall patterning of cells in the root elongation zone. It is conceivable that these reductions in cellulose and lignin lead to a decreased root dry biomass, such as that previously described in Arabidopsis cellulose synthase mutants (Taylor et al., 1999, 2000, 2003); however, further experiments would be required to categorically prove this hypothesis.

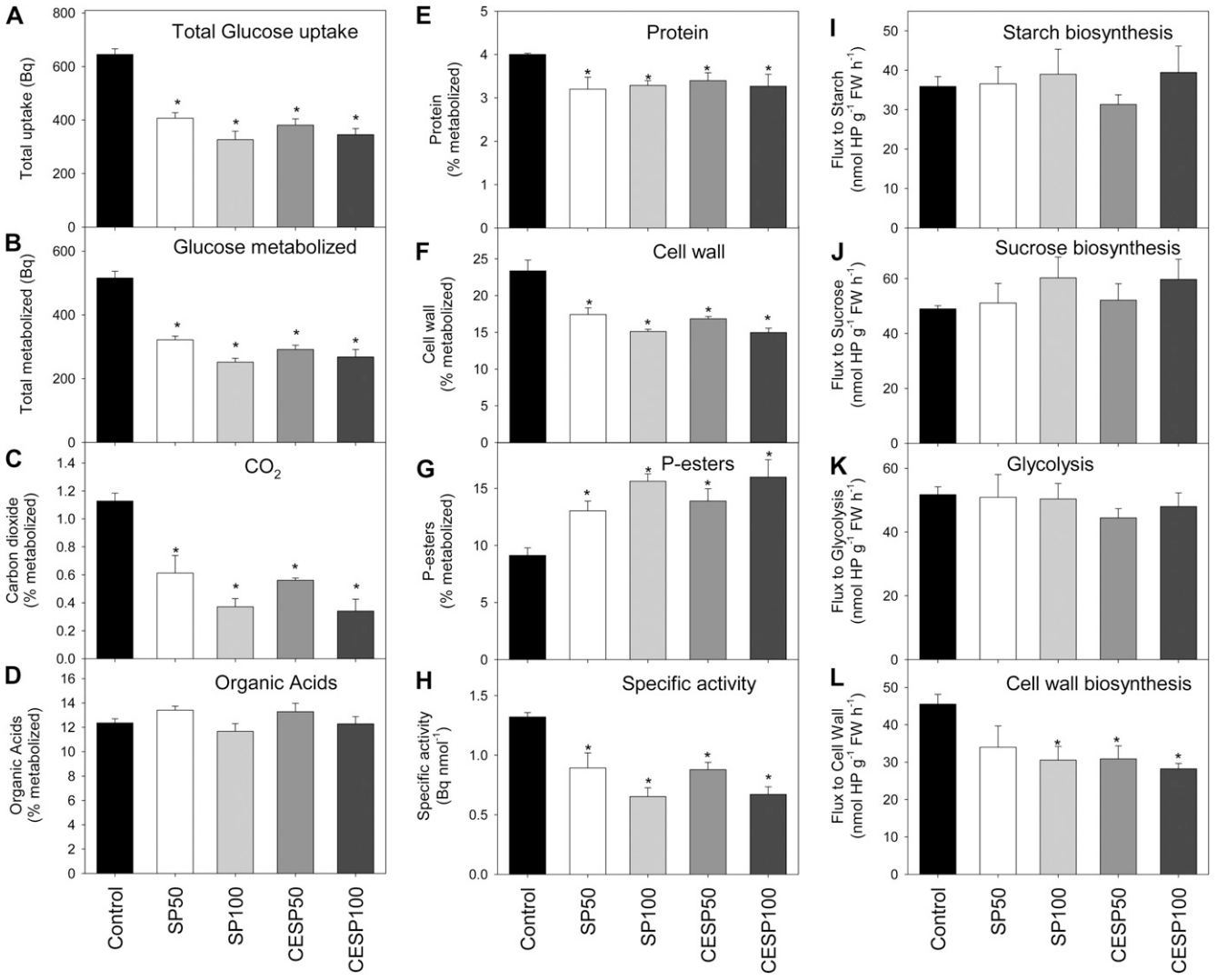

Figure 7. Effect of SP or CESP on metabolism of [U-^{14}C]Glc by root tips. Root tips were preincubated in 10 mM MES-KOH buffer, pH 6.5, containing 2 mM Glc in the absence (control) or presence of varying concentrations of succinyl phosphonate (SP) or carbonyl ester of succinyl phosphonate (CESP) for 1 h, then [U-^{14}C]Glc (specific activity, 8.11 MBq mmol^{-1}) was added and incubated for 2 h. Each sample was extracted with boiling ethanol, and the amount of radioactivity in each metabolic fraction was determined as described in "Materials and Methods." Values are expressed as percentages of the total radiolabel metabolized and are means ± SE of four biological replicates. Asterisks demarcate values that were judged to be significantly different from the control ($P < 0.05$). FW, Fresh weight.

In order to further investigate this observation, we followed the metabolic fate of [U-^{14}C]Glc supplied exogenously to excised root tips of the wild-type tomato in the presence or absence of potent inhibitors of the reaction catalyzed by 2-oxoglutarate dehydrogenase. The short-term nature of this inhibition effectively allows us to discriminate between the primary and secondary features of the transgenics, since it removes the aspect of long-term adaptations. As was previously observed in potato tubers (Araujo et al., 2008), the inhibitor clearly results in a dramatic reduction of respiration. Intriguingly, estimating absolute fluxes in the root system revealed a depressed rate of cell wall synthesis but unaltered rates of glycolysis, starch, and Suc biosynthesis. This finding suggests that at times when energy is scarce, the root cell prioritizes maintenance of primary metabolic pathways that support energy generation and storage above those that support other processes. This finding is not without precedence, since studies of the metabolic architecture of both tomato and Arabidopsis cell suspension cultures came up with similar conclusions (Rontein et al., 2002; Williams et al., 2008). Our results thus provide strong evidence that the inhibition of secondary cell wall biosynthesis in the transgenics is a direct consequence of the reduced TCA cycle activity and support our initial contention that this process is energy limited in heterotrophic plant tissues. Further-

more, we believe that this work highlights a novel link between primary metabolism and secondary cell wall synthesis that nicely complements prior work focusing on nucleotide sugar partitioning. This fact notwithstanding, it is clear that teasing out the precise mechanisms linking these processes will be of high future interest at both fundamental and applied levels.

MATERIALS AND METHODS

Plant Material

Previously characterized tomato (*Solanum lycopersicum* 'Moneymaker') seeds, exhibiting reduced expression of mitochondrial malate dehydrogenase, fumarase, succinyl-CoA ligase, and citrate synthase (Nunes-Nesi et al., 2005, 2007; Studart-Guimarães et al., 2007; Sienkiewicz-Porzucek et al., 2008), were sterilized and germinated on MS medium (Murashige and Skoog, 1962). Plants were grown in a long-day regime at day/night temperatures of 22°C/20°C and a relative humidity of 50% on a vermiculite medium supplemented with slow-release fertilizer (Lewatit HD 50; Beyer). The presence of the transgene was verified by PCR using standard protocols, prior to analysis of enzyme activities of the protein in question.

Chemicals

Unless stated otherwise, all chemicals, cofactors, and enzymes were purchased either from Sigma-Aldrich or Merck. Phosphate analogs were synthesized at the Chemical Department of Moscow Lomonosov State University according to Bunik et al., (2005).

Enzyme Analyses

Enzymes were extracted as described previously (Tauberger et al., 2000) and assayed exactly as described in the literature (Gibon et al., 2004; Nunes-Nesi et al., 2005).

cDNA Cloning and Expression

An 800-bp fragment of aconitase was cloned in antisense orientation into the vector pBINAR (Liu et al., 1999) between the cauliflower mosaic virus 35S promoter and the *ocs* terminator. This construct was introduced into plants by an *Agrobacterium tumefaciens*-mediated transformation protocol, and plants were selected and maintained as described in the literature (Tauberger et al., 2000). Initial screening of 32 lines was carried out using a combination of activity and mRNA measurements. These screens allowed the selection of three lines, which were chosen for subsequent physiological characterization.

Root Respiration

Root tips (25 mg) were cut with a sharp blade, washed, and incubated in 10 mM MES-KOH, pH 6.5, in a Clark-type oxygen electrode. The rate of oxygen consumption was calculated as described by Geigenberger et al. (2000).

Root Staining and Visualization

About 5-mm root collars (approximately 15 mm from the junction between root and stem) from 5-week-old plants were embedded in 4% (w/v) agarose. Cross-sections (50 μm thick) were cut by vibrotome (Leica VT 1000S) and stained with 0.1% (w/v) phloroglucinol (Sigma-Aldrich).

Serial cross-sections were observed with an epifluorescence motorized microscope (Olympus BX 61). Images were captured by Cell^P Software (Olympus).

Metabolism of [^{13}C]Glc or [^{14}C]Glc by Tomato Roots

Wild-type mature roots (approximately 500 mg) were cut directly from 5-week-old plants, washed three times with 10 mM MES-KOH buffer, pH 6.5, following a 1-h preincubation in the presence or absence of either 50 or 100 μM of the inhibitor succinyl phosphate or carboxyethyl ester of succinyl phosphate, and then incubated for 2 h in 10 mM MES-KOH buffer, pH 6.5, containing 2 mM Glc in a 100-mL Erlenmeyer flask shaken at 90 rpm containing 1.00 μCi of [^{14}C]Glc (specific activity of 8.11 MBq mmol^{-1}). The $^{14}CO_2$ liberated was captured (in hourly intervals) in a KOH trap, and the amount of radiolabel was subsequently quantified by liquid scintillation counting.

In the case of [^{13}C]Glc, the procedure was similar but roots were incubated with 10 mM unilabeled [^{13}C]Glc or unlabeled Glc.

Extraction and Fractionation of Radiolabeled Material

Tissue was fractionated exactly as described by Fernie et al. (2001b), with the exception that hexoses were fractionated enzymatically rather than utilizing thin-layer chromatography. Labeled Suc levels were determined after 4 h of incubation of 200 μL of total neutral fraction with 4 units mL^{-1} hexokinase in 50 mM Tris-HCl, pH 8.0, containing 13.3 mM $MgCl_2$ and 3.0 mM ATP at 25°C. For labeled Glc and Fru levels, 200 μL of neutral fraction was incubated with 1 unit mL^{-1} Glc oxidase and 32 units mL^{-1} peroxidase in 0.1 M potassium phosphate buffer, pH 6, for a period of 6 h at 25°C. After the incubation time, all reactions were stopped by heating at 95°C for 5 min. The label was separated by ion-exchange chromatography as described by Fernie et al. (2001a). The reliability of these fractionation techniques has been thoroughly documented previously (Runquist and Kruger, 1999; Fernie et al., 2001b; Araujo et al., 2008). Fluxes were calculated as described by Geigenberger et al. (2000).

Extraction and Fractionation of Stable Isotope-Labeled Material

Stable isotope-labeled material was extracted and prepared for GC-MS-based analysis as described previously (Roessner et al., 2001). The GC-MS was run using the exact same settings as detailed by Lisec et al. (2006). Fractional enrichment of metabolites was calculated by directly comparing replicate samples incubated in unilabeled [^{13}C]Glc with those incubated in Glc of natural abundance as previously described by Roessner-Tunali et al. (2004).

Cell Wall Analyses

Cell walls were isolated from about 5-mm root collars (approximately 15 mm from the junction between root and stem) from 5-week-old plants preceded by washing 500 mg of homogenized tissue with 1 mL of 70% ethanol. After vigorous centrifugation, the supernatant was mixed with an equal volume of a chloroform:methanol (1:1, v/v), the supernatant was decanted, and this procedure was repeated three times. Finally, the pellet was resuspended in 1 mL of acetone and centrifuged, and the pellet was dried under vacuum. Starch was removed by incubating with 40 units of α-amylase (from porcine pancreas; Sigma-Aldrich) and β-amylase (from sweet potato [*Ipomoea batatas*]; Sigma-Aldrich) in 20 mL of Tris-HCl, pH 7.0, for 24 h. After incubation, the cell wall material was precipitated in 70% ethanol by adding an appropriate volume of 96% ethanol to the reaction mixture and washed with acetone.

Plant cell walls were hydrolyzed for monosaccharide composition analysis (Selvendran et al., 1979). Cell wall material (1 mg) was incubated in 250 μL of trifluoroacetic acid (TFA) in a screw-capped borosilicate test tube for 1 h at 121°C. The suspension was diluted with 300 μL of 2-propanol and evaporated at 40°C. This step was repeated three times and subsequently resuspended in 200 μL of water, vortexed, and centrifuged, and the TFA-insoluble residues were separated from the solution to measure the cellulose content.

The hydrolysate was used for neutral sugar composition determined by GC according Blakeney et al. (1983) using 30 μg of myoinositol internal standard. The hydrolysate was evaporated in order to prepare the alditol acetates. Reduction to alditols was achieved by resuspending the pellet in reducing agent (250 μL of sodium borohydride in 1 M ammonium hydroxide) and incubating for 1 h at room temperature. The mixture was neutralized by the addition of 20 μL of glacial acetic acid and subsequently thoroughly washed with acetic acid:methanol (1:9, v/v) followed by methanol washes. The mixture was acetylated by the addition of 50 μL of acetic anhydride and 50 μL of pyridine and incubated for 20 min at 121°C. The mixture was washed with toluene. Cleanup proceeded by adding 500 μL of distilled water and 500 μL of methylene chloride and vortexing thoroughly. The apolar phase was removed, evaporated, and resuspended in 100 μL of acetone. The hydrolyzed

monosaccharide composition was determined by GC-MS on an Agilent 6890 Series GC system equipped with a 5975B inert XL MSD and an SP-2380 fused silica capillary column (30 m × 0.25 mm i.d. × 20 μm film thickness; Supelco).

The preparation and hydrolysis of crystalline cellulose from the pellet left after TFA hydrolysis were performed as described (Updegraff, 1969). After hydrolysis, the cellulose-derived Glc was determined using the anthrone assay (Dische, 1962), with Glc as standard.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Root biomass accumulation in a second independent trial.

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