Transcriptomic Analysis of the Role of Carboxylic Acids in Metabolite Signaling in Arabidopsis Leaves

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The transcriptional response to metabolites is an important mechanism by which plants integrate information about cellular energy and nutrient status. Although some carboxylic acids have been implicated in the regulation of gene expression for select transcripts, it is unclear whether all carboxylic acids have the same effect, how many transcripts are affected, and how carboxylic acid signaling is integrated with other metabolite signals. In this study, we demonstrate that perturbations in cellular concentrations of citrate, and to a lesser extent malate, have a major impact on nucleus-encoded transcript abundance. Functional categories of transcripts that were targeted by both organic acids included photosynthesis, cell wall, biotic stress, and protein synthesis. Specific functional categories that were only regulated by citrate included tricarboxylic acid cycle, nitrogen metabolism, sulfur metabolism, and DNA synthesis. Further quantitative real-time polymerase chain reaction analysis of specific citrate-responsive transcripts demonstrated that the transcript response to citrate is time and concentration dependent and distinct from other organic acids and sugars. Feeding of isocitrate as well as the nonmetabolizable citrate analog tricarballylate revealed that the abundance of selected marker transcripts is responsive to citrate and not downstream metabolites. Interestingly, the transcriptome response to citrate feeding was most similar to those observed after biotic stress treatments and the gibberellin biosynthesis inhibitor paclobutrazol. Feeding of citrate to mutants with defects in plant hormone signaling pathways did not completely abolish the transcript response but hinted at a link with jasmonic acid and gibberellin signaling pathways. Our results suggest that changes in carboxylic acid abundances can be perceived and signaled in Arabidopsis (Arabidopsis thaliana) by as yet unknown signaling pathways.

Several types of metabolites have been shown to act as regulators of gene expression in various prokaryotic and eukaryotic organisms (Sellick and Reece, 2005). More than 20 years ago, it was demonstrated that the promoter activities of selected photosynthetic genes are repressed by sugars and acetate while they are induced by nitrate and several amino acids and carboxylic acids (Sheen, 1990). Since then, a large number of metabolic genes whose expression is regulated by altered concentrations of key nutrient metabolites such as sugars and nitrate have been reported (Stitt, 1999; Coruzzi and Zhou, 2001; Price et al., 2004; Usadel et al., 2008).

Sheen and coworkers demonstrated that, in addition to its enzymatic role, hexokinase also functions as a key enzyme in sugar signaling, acting as a direct metabolite sensor in plants that senses hexose concentrations in order to control sugar-regulated gene expression (Jang et al., 1997; Moore et al., 2003).

Intracellular kinases, such as the evolutionarily conserved SUCROSE NONFERMENTING1-related kinases, regulate transcription under energy or nutrient deficiency to restore metabolic homeostasis. However, the identity of the signal that is perceived by these kinases still remains to be elucidated (Baena-González and Sheen, 2008). Indeed, it is increasingly apparent that there may be many more metabolites that are able to initiate transcriptomic changes in plants (Sheen, 1990; Templeton and Moorhead, 2004; Lancien and Roberts, 2006; van Schooten et al., 2006).

Recently, signaling functions were discovered for different tricarboxylic acid (TCA) cycle intermediates such as citrate, succinate, fumarate, 2-oxoglutarate (2-OG),...
and the closely related metabolite 2-hydroxyglutarate (in its reduced form) in human cells (Hewitson et al., 2007; Wellen et al., 2009; Gomez et al., 2010; Yang et al., 2012). While succinate, fumarate, and 2-hydroxyglutarate were shown to inhibit several chromatin modifiers and consequently affect transcription (Yang et al., 2012), citrate has been discussed to be of particular importance for histone acetylation via cleavage to acetyl-CoA by ATP-citrate lyase to regulate the expression of glycolytic genes in liver cells (Wellen et al., 2009). Furthermore, iron citrate was shown to competitively inhibit the activities of protein Tyr phosphatases and thereby to enhance mitogen-activated protein kinase (MAPK) signaling (Gomez et al., 2010).

Evidence that TCA cycle intermediates themselves act in regulating transcript abundances first came from a genome-wide expression analysis of yeast mutants with TCA cycle defects (McCammon et al., 2003). Stepwise inactivation of 15 genes encoding the eight TCA cycle enzymes in yeast revealed changes in expression patterns of genes responding to the TCA cycle defects, which correlated with altered levels of citrate, 2-OG, succinate, and malate (McCammon et al., 2003). In plants, supply of exogenous TCA cycle organic acids, such as citrate, led to a strong accumulation of ALTERNATIVE OXIDASE1 (AOX1) transcripts in tobacco (Nicotiana tabacum) as well as in Arabidopsis (Arabidopsis thaliana) cell suspension cultures, while AOX2 transcript abundance was decreased (Gray et al., 2004; Clifton et al., 2005). Moreover, Muller et al. (2001) reported that the NITRATE REDUCTASE transcript (NIA) was decreased in abundance after 4 h of feeding tobacco leaves with 40 mM malate or citrate but highly induced by 40 mM 2-OG. As these are unlikely to be the only gene products regulated by the abundance of TCA cycle organic acids, we speculated that the induction of AOX1 and repression of the NIA transcript belong to a general response of a certain set of nucleus-encoded genes to a changed abundance in organic acid levels. Intermediates of the TCA cycle are good candidate signaling molecules, as they reflect both the metabolic and redox status of the cell and are known to be transported between compartments. The aim of this study was to establish the role of carboxylic acids in regulating nuclear gene expression in plants.

RESULTS

Citrate Has a Stronger Effect on Transcript Abundances Than Malate

To establish whether carboxylic acids have a general role in the regulation of transcript abundances, the influence of exogenously supplied malate and citrate on the Arabidopsis transcriptome was analyzed in leaf slices. The leaf slice system has been used for gene expression analysis in several previous studies (Raven and Farquhar, 1981; Horling et al., 2003). It allows a fast and homogenous application of effector solutions. Often, cell cultures or protoplasts are also used to allow homogenous application of metabolites to cells (Sheen, 1990; Clifton et al., 2005; Baxter et al., 2007; Ho et al., 2008); however, they have the disadvantage that they contain high amounts of sugars that can also have strong effects on the expression of metabolic genes.

Based on published data on the organic acid-dependent induction of the AOX1 gene of tobacco (Gray et al., 2004), 1 mM citrate and 1 mM malate were chosen for initial treatments of 2, 4, and 8 h. Reverse transcriptase PCR analysis revealed that the AOX1a (At3g22370) transcript of Arabidopsis was increased to a peak level after an 8-h treatment with 1 mM citrate and malate (Fig. 1A). Thus, these two treatments were selected for further microarray analysis (Arabidopsis 29k Oligonucleotide Microarrays, Galbraith laboratory; Zhang et al., 2008). From about 14,000 to 16,000 transcripts that were detected in each of four independent biological replicates, 1,876 transcripts showed a significant change in abundance after citrate treatment in comparison with leaf slices infiltrated with control buffers only. After malate treatment, only 327 transcripts were significantly differentially regulated (Fig. 1B; Cyber-T test, Bayes $P < 0.05$). A nearly equal number of up- and down-regulated transcripts were found in both treatments (Supplemental Table S1). Although a large number of transcripts were changed by more than 10% after citrate treatment, only 21 transcripts were increased by more than 2-fold (Table I). Six of the these transcripts, ATPase AHA2 (At4g30190), EXORDIUM (EXO; At4g08950), SAM-DEPENDENT METHYLTRANSFERASE (SAM-MT; At2g41380), LEUCINE-RICH REPEAT PROTEIN KINASE (At1g1850), PEROXIDASE (At5g39580), and MERISTEM5 (At4g30270), as well as three of the down-regulated transcripts, two UNKNOWN PROTEINS (At1g76960 and

![Figure 1](image)

Figure 1. Effect of 1 mM malate and 1 mM citrate on transcriptome changes in Arabidopsis leaf slices. A, Time-dependent effect of the exogenous application of malate and citrate on AOX1a transcript abundance as analyzed by reverse transcriptase PCR. Expression levels were normalized according to UBIQ1O and ACT1N2 expression levels. Relative expression values (treatment/control) are expressed as log2IF. B, Venn diagram of transcripts detected in the malate and citrate microarrays. Leaf slices from 4-week-old plants were infiltrated and incubated for 8 h with 1 mM malate or 1 mM citrate (both in 1 mM MES, pH 5.5) or 1 mM MES buffer (pH 5.5) only (control). A total of 327 and 1,876 transcripts were significantly regulated by 8-h malate and citrate treatment, respectively ($n = 4$; Cyber-T test, Bayes $P < 0.05$; Supplemental Table S1). A significant overlap of 84 transcripts was detected under both conditions ($X^2$ test), of which 36 transcripts were inversely regulated with citrate or malate.
Table 1. List of transcripts altered more than 2-fold by 1 mM citrate treatment for 8 h

| Identifier   | Description                          | 1 mM Malate | 1 mM Citrate |
|--------------|--------------------------------------|-------------|--------------|
| At1g73120    | Unknown protein                       | n.d.        | 1.98***      |
| At2g43660    | Glycosyl hydrolase family protein17   | n.d.        | 1.51**       |
| At4g08950    | Exorvidium                           | 0.38+       | 1.34***      |
| At2g31350    | NRAMP3                               | 0.01        | 1.30***      |
| At5g17330    | Glu decarboxylase1                   | n.d.        | 1.27***      |
| At2g41380    | SAM-dependent methyltransferase      | −0.08       | 1.17***      |
| At5g01600    | Ferritin1                            | −0.10       | 1.10***      |
| At4g30270    | Xyloglucan:xyloglucosyl transferase SEN4 | 0.31        | 1.05***      |
| At4g30670    | Putative membrane lipoprotein        | −0.01       | 1.05***      |
| At1g51850    | Putative Leu-rich repeat protein kinase | 0.29*   | 1.01***      |
| At4g30190    | Hydrogen-exporting ATPase AHA2       | 0.09        | 1.01***      |
| At5g39580    | Putative peroxidase                  | n.d.        | 1.01**       |
| At2g47280    | Pectinesterase                       | n.d.        | 1.00***      |
| At1g19960    | Putative transmembrane receptor      | 0.04        | −0.01***     |
| At2g34430    | LHCBI.4                              | −0.03       | −0.03***     |
| At5g44430    | Plant defensin1.2c                   | −0.10       | −0.06***     |
| At1g76960    | Unknown protein                       | 0.00        | −0.08***     |
| At2g6010     | Plant defensin1.3                    | −0.24*      | −1.29***     |
| At1g75830    | Plant defensin1.1                    | −0.24*      | −1.45***     |
| At5g44420    | Plant defensin1.2a                   | −0.20       | −1.48***     |
| At2g26020    | Plant defensin1.2b                   | −0.16       | −1.50***     |

At1g19960) and LIGHT-HARVESTING CHLOROPHYLL PROTEIN-COMPLEXII SUBUNIT B1 (LHCBI.4; At2g34430), were also changed in the same directions after treatment of Arabidopsis with an avirulent Pseudomonas syringae strain (Supplemental Fig. S1A; P < 0.05, Genevestigator database; Zimmermann et al., 2004).

The strongest up-regulated transcript after citrate treatment was At1g73120 (about 4-fold increased), which encodes a protein of unknown function that we refer to here as CITRATE-INDUCED1 (CI1). CI1 was also more than 3-fold up-regulated in iron-deficient roots as well as by a 3-h abscisic acid (ABA) treatment and an 8-d treatment with brassinolide and boric acid (P < 0.05, Genevestigator database; Supplemental Fig. S1B). Two more genes that are involved in iron homeostasis, FERRITIN1 (FER1; At5g01600) and NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN3 (NRAMP3; At2g23150), were also more than 2-fold up-regulated after citrate treatment and strongly induced by ABA treatment but, on the contrary, not by iron deficiency (Supplemental Fig. S1B; P < 0.05, Genevestigator database).

When comparing the lists of significant genes from the malate and citrate experiment, a significant overlap of 84 transcripts (compared with an expected 36 transcripts if the overlap is random) was found that changed under both conditions (Fig. 1B). Among these 84 transcripts, 33 and 15 were up- and down-regulated in both treatments, respectively, and 36 transcripts were inversely regulated (Supplemental Table S1). The group of transcripts that showed a similar response to an increased citrate and malate availability most likely responded to the same signal produced under both conditions, which could be a response to a general increase in carbon availability. This is supported by the observation that many of these genes are also regulated by known sugar signaling pathways (Baena-González and Sheen, 2008). However, given that the transcriptional responses to increased malate or citrate availability were generally very different from one another, the overlap of inversely regulated transcripts most likely responded specifically to either citrate or malate availability and included transcripts coding for the glucan-water dikinase STARCH EXCESS1 (SE1; At1g10760), OUTER-ENVELOPE PROTEIN16-1 (OEP16-1; At2g28900), ALTERNATIVE NAD(P)H DEHYDROGENASE2 (NDA2; At2g29990), PHOTOSYSTEM I LIGHT HARVESTING PROTEIN COMPLEX GENES3 (LHC3; At1g61520), DREB SUBFAMILY A4 of ETHYLENE RESPONSE FACTOR/APETALA2 TRANSCRIPTION FACTOR (DREBA4-TF; At2g44940), and several stress-responsive proteins, such as DEHYDROASCORBATE REDUCTASE1 (DHAR1; At1g19570), TRYPsin INHIBITOR (At1g73260), and PLANT DEFENSIN1.2B (PDF1.2B; At2g26020; Supplemental Table S1).

Functional Gene Categories Affected by Citrate and Malate Treatment

To identify significantly changed functional classes rather than single transcripts that responded specifically...
to malate and citrate availability, the microarray data were analyzed using a Wilcoxon rank-sum test (Benjamini-Hochberg corrected) integrated within the MapMan software (Thimm et al., 2004). Categories that were revealed to be significantly regulated under both conditions were photosynthesis (PSI), cell wall, biotic stress, and protein synthesis (Supplemental Table S2). However, most of the functional categories were specific to one or the other treatment, such as the TCA cycle, nitrogen metabolism, sulfur assimilation, and DNA synthesis and signaling for citrate as well as glycolysis and abiotic stress for malate (Supplemental Table S2). In summary, the results from the array experiments revealed that citrate feeding leads to widespread changes in transcript abundances that are distinct from those identified after malate feeding. These findings support the hypothesis that different organic acids might have unique roles in metabolite signaling.

Citrate-Dependent Transcript Regulation Is Time and Concentration Dependent

To confirm the expression data from the microarray analysis and also to investigate whether citrate has a concentration-dependent effect, the abundances of 18 transcripts selected from the microarray analysis were analyzed by quantitative real-time (QRT)-PCR. Citrate-regulated transcripts were selected on the basis that they showed a significant induction (CI1, EXO, FER1, PHOSPHOENOLPYRUVATE CARBOXYKINASE1 [PCK1]; At4g37870, GLUTAMATE DEHYDROGENASE2 [GDH2]; At5g07440, and PHOSPHOGLYCERATE MUTASE [PGM; At3g60420]) or suppression (PDF1.2b and TRANSFER RNA-PSEUDOURIDINE SYNTHASE [tRNA-PUS; At2g30320]) following citrate treatment or in the case that they showed an inverse regulation by malate and citrate (LHCA3, SEX1, DEFENSIN-LIKE FAMILY PROTEIN [DEFL; At2g43550], OEP16-1, DREBA4-TF, and DHAR1). NICOTIANAMINE SYNTHASE4 (NAS4; At1g56430), SUCROSE PHOSPHATE SYNTHASE1 (SPS1; At5g20280), and GLUTAMINE DUMPER4 (GDU4; At2g24762) showed no significant regulation in the microarray experiments with low levels of citrate. A concentration series of 0, 1, 10, and 30 mM citrate was selected and incubated with the leaf slices for 2, 4, and 8 h. Relative transcript abundances were calculated using SYBR Green PCR Master Mix. Expression levels were normalized to the UBQ10 and ACTIN2 transcripts for each treatment and time point. Relative expression values (treatment/control) are expressed as log2IF (mean values ± s.e.; n = 3). Accession numbers of the transcripts are as follows: CI1, At1g73120; DEFL, At2g43550; DHAR1, At1g19570; DREBA4-TF, At2g44940; EXO, At4g08950; FER1, At5g01600; GDH2, At5g07440; GDU4, At2g24762; LHCA3, At1g61520; NAS4, At1g56430; OEP16-1, At2g28900; PCK1, At4g37870; PDF1.2b/PDF1.3, At2g26020/At2g26010; PGM, At3g60420.1/0.2; SAM-MT, At2g41380; SEX1, At1g10760; SPS1F, At5g20280; tRNA-PUS, At2g30320. Full names of transcripts and primer sequences are given in Supplemental Table S4.

Figure 2. Concentration- and time-dependent effects of externally applied citrate on the abundance of 18 transcripts selected from the microarray analysis. Arabidopsis leaf slices were incubated in 0, 1, 10, and 30 mM citrate for 2 h (black bars), 4 h (gray bars), and 8 h (dark gray bars) as described in “Materials and Methods.” QRT-PCR analysis was carried out in triplicate (technical and biological) using SYBR Green PCR Master Mix. Expression levels were normalized to the
for all 18 transcripts relative to the respective control treatment from the same time points (Fig. 2). The expression data from the microarray analysis was confirmed for 17 of the marker transcripts by QRT-PCR at the 8-h time point with 1 mM citrate. The transcript level of CI1 was increased to a much greater extent than detected on the microarray (about 15-fold [log₂ = 3.89] induced in the QRT-PCR analysis and only 4-fold in the microarray analysis). All transcripts showed a qualitatively similar response to the microarray except for DREBA4-TF, which did not respond to low concentrations of citrate, but similar to the array it was altered in abundance in response to higher concentrations of citrate (10 and 30 mM; Fig. 2). For most transcripts, the expression levels were most strongly regulated by 10 mM citrate at the 4- and 8-h time points. Changes in most transcripts could also be detected as early as 2 h (Fig. 2). Treatment with 30 mM citrate did not lead to a further induction for most of the transcripts, and for CI1, DREBA4-TF, FER1, and PCK1, the induction was actually less than with 10 mM citrate (Fig. 2). In summary, the kinetic analysis of the citrate marker transcripts clearly showed a time- and concentration-dependent effect of citrate on gene expression level.

Metabolic Perturbations Resulting from Feeding of Malate and Citrate

Feeding malate and citrate to Arabidopsis leaves led to a substantial perturbation in the transcriptome. However, it cannot be concluded that this is a direct response, since these metabolites will affect related metabolic pathways in various subcellular compartments. To gain a better insight into the metabolic perturbations that might have been caused by citrate and malate feeding, a gas chromatography-mass spectrometry (GC-MS)-based metabolite profile analysis from leaf slices treated for 2, 4, and 8 h was performed (Fig. 3; Supplemental Table S3).

The metabolite profile from malate-treated leaf slices was not significantly different from control leaf slices at the time points 2 and 4 h. Only after 8 h were a marginally significant increase in malate content ($P = 0.058$) and slight decreases in benzoic acid, glycerol, and Gly content detected (Supplemental Table S3). More changes in metabolite contents were observed after citrate feeding, especially after 4 and 8 h, with significant increases in sugars (Suc, Glc, Fru, and Gal), amino acids (Glu, Tyr, and Phe), and organic acids (citrate and succinate) contents (Fig. 3). However, since gene expression changes were already apparent after 2 h (Fig. 2), the number of other possible candidate metabolites involved in signaling remains low. It cannot be ruled out that some of the alterations in gene expression relate to local subcellular changes in metabolite concentrations that were not detected by the GC-MS method employed here.

The Effect of Sugars and Other Organic Acids on Citrate Marker Transcripts

The repression of transcripts involved in photosynthesis as observed after citrate feeding is also known to occur after sugar or acetate feeding (Sheen, 1990). As feeding of citrate also resulted in increased sugar levels, it is possible that at least part of the observed transcript response is related to increased sugar levels. To compare the effects of sugars (Suc and Glc) and other carboxylic acids (acetate, pyruvate, isocitrate, 2-OG, succinate, fumarate, malate, and oxaloacetate) with the response to citrate treatment, a comparative QRT-PCR analysis for selected marker transcripts was performed. The above-mentioned metabolites (10 mM each) were fed to Arabidopsis leaf slices for 4 h in order to obtain a maximal transcript response at an early time point. Similar concentrations of various organic acids (5 mM) were used by Sheen (1990) to study the expression of the pyruvate phosphokinase in maize (Zea mays) protoplasts. As citrate and malate are the most abundant carboxylic acids in plant cells, with concentrations of about 1 to 5 mM in the cytosol (Martinoia and Rentsch, 1994), external feeding of 10 mM carboxylic acid should also be high enough for the other selected carboxylic acids.

The transcript of LHCA3 was selected as a down-regulated marker transcript for citrate (Fig. 2) but also for Suc ($P < 0.05$, Genevestigator database), and AOX1a was selected as an up-regulated marker transcript for organic acids in general (Gray et al., 2004). Furthermore, four genes were selected that showed a strong regulation on transcript level even with low levels of citrate: CI1, FER1, OEP16-1, and SAM-MT (Fig. 2). In the QRT-PCR analysis, LHCA3 was down-regulated by the following metabolites with decreasing strength: acetate > Suc > isocitrate > citrate (Fig. 4). No regulation of the LHCA3 transcript was observed after Glc treatment (Fig. 4), which is consistent with the data from the Genevestigator database, where no transcript regulation occurred after treatment of seedlings for 4 h with 3% Glc (Li et al., 2006).

A slight but significant up-regulation of LHCA3 transcript was observed for pyruvate ($P < 0.05$), and a marginally significant up-regulation was also observed for 2-OG treatment ($P < 0.1$). An up-regulation of LHCA3 transcript is also known to occur after nitrate treatment, while nitrate starvation led to a strong decrease in LHCA3 transcript level ($P < 0.05$, Genevestigator database; data not shown). As organic acid metabolism is ultimately linked to nitrate assimilation, a connection of organic acid and nitrate signaling is conceivable.

The up-regulation of AOX1a transcript and protein levels is known to occur as a general response to increased levels of TCA cycle organic acids (Gray et al., 2004). In the QRT-PCR analysis, AOX1a transcript level was strongly induced after incubation of leaf slices with acetate (11-fold) and to a slightly lesser extent with citrate, Glc, oxaloacetate, and pyruvate.
Up-regulation of AOX1a after incubation with malate and Suc was only marginally significant after 4 h. OEP16-1 was selected as a citrate-specific down-regulated marker transcript (Fig. 2; Supplemental Table S1). The OEP16-1 transcript level was also significantly decreased by acetate, malate, and oxaloacetate and only marginally significantly decreased by isocitrate and 2-OG treatments. However, a significant up-regulation of OEP16-1 was observed for Suc, Glc, and pyruvate treatments (Fig. 4). Gonzali et al. (2006) also observed a strong and concentration-dependent up-regulation of OEP16-1 by Suc and Glc, while Fru and turanose had no such strong effect.

Interestingly, two of the citrate marker transcripts, CII and FER1, only responded to citrate with a significant up-regulation in transcript level but not to isocitrate or any other organic acid or sugar treatment. On the contrary, both transcripts were significantly down-regulated by most of the other treatments (Fig. 4).

The transcript of SAM-MT was also significantly up-regulated by citrate and to a lesser extent by Glc and isocitrate. A significant down-regulation in SAM-MT transcript level was observed for pyruvate, 2-OG, succinate, malate, and oxaloacetate (Fig. 4). These results indicate that the transcript response to citrate is specific for selected transcripts and that citrate can be distinguished from isocitrate by the cell.

Tricarballylate Feeding Resembles the Citrate Response of Marker Transcripts

To get a better insight into the discrimination between isocitrate and citrate on marker gene expression, the effect of feeding the nonmetabolizable citrate analog tricarballylate (Wolffram et al., 1993) was also tested. Tricarballylate can be transported into cells and mitochondria via citrate transporters. The molecular structure of tricarballylate is very similar to that of citrate and isocitrate, but the hydroxyl group at the C3 atom of citrate and at the C2 atom of isocitrate is missing. However, tricarballylate and citrate are structurally distinct from the chiral isocitrate molecule, which will ultimately impact their binding affinities to protein recognition sites.

Similar to citrate, and in contrast to isocitrate, tricarballylate was also able to induce (with slightly less efficiency) the transcript levels of the solely citrate-induced genes CII and FER1 (Figs. 4 and 5). Tricarballylate also displayed a comparable effect to citrate on the transcript levels of OEP16-1 and SAM-MT, which were also regulated by isocitrate, albeit to a lesser extent (Figs. 4 and 5). Tricarballylate might partially interfere with citrate sensing, as the transcript induction was slightly less efficient than after citrate feeding (Fig. 5).

To compare the effects of tricarballylate and citrate on metabolite abundances, a GC-MS-based metabolite...
profiling analysis was performed (Supplemental Table S4). Again, levels of Fru and Suc were significantly increased after feeding of 10 mM citrate (as with 1 mM citrate; Fig. 3) but not after feeding with tricarballylate. The only metabolite that was changed significantly in the same direction in both treatments was citrate itself. Increases in cellular citrate levels following tricarballylate feeding are most likely due to a competitive inhibition of aconitase (Gawron and Jones, 1977). Together, these results strongly indicate that citrate itself rather than a downstream product is sensed by the plant cell.

The Transcriptome Response to Citrate Is Most Similar to Biotic Stress Treatments

To identify physiological conditions or treatments that most closely resemble the transcriptome response to citrate, a comparative data analysis was performed between all highly significantly citrate-regulated transcripts ($P$, 0.001; 38 transcripts up- and down-regulated greater than 1.5-fold) and all publicly available Affymetrix microarray data sets dealing with environmental perturbations (5,178 data sets) using the Signature tool in Genevestigator (data not shown). The highest similarity was revealed between citrate and three microarray experiments dealing with *Pseudomonas syringae* infection for 24 h compared with mock-treated leaf samples for 24 h (Wang et al., 2008) as well as leaf samples treated with bacterial flagellin22 (flg22) and leaf samples treated with the GA biosynthesis inhibitor paclobutrazol (PBZ; both Gene Expression Omnibus accession no. GSE17464).

To further analyze this similarity in more detail, a cluster analysis was performed on all significant transcripts after 1 mM citrate feeding ($P$, 0.05) and the experiments dealing with biotic stress, GA, and sugar signaling. For this, the following microarray data sets were selected: 2-h treatment with flg22 or PBZ (GSE17464), 2-h treatment with Glc (E-MEXP-475), 4-h treatment with Suc (NASCARRAYS-315), KIN10 over-expressors versus the wild type (Baena-González et al., 2007), and 1 mM malate for 8 h (this study). The hierarchical cluster analysis confirmed the similarity between the transcript response to citrate and biotic stress, as citrate was most similar to flg22 and *P. syringae* treatment in five out of 10 clusters (Fig. 6, clusters 2, 3, 5, 6, and 7). In three of 10 clusters, each transcript altered after citrate treatment behaved most similarly to PBZ (clusters 4, 5, and 7) and Glc (clusters 1, 2, and 5). Interestingly, transcripts were mostly regulated in opposite directions in KIN10 mutants in comparison with citrate (Fig. 6).

**Figure 4.** Comparison of the effects of different sugars and organic acids on the abundance of LHCA3, AOX1a, CI1, OEP16-1, FER1, and SAM-MT transcripts. Arabidopsis leaf slices were incubated in 0 or 10 mM of each metabolite for 4 h as described in “Materials and Methods.” For a better overview, changes in transcript abundances after citrate treatment are marked in black. QRT-PCR analysis was carried out in triplicate (technical and biological) using SYBR Green PCR Master Mix. Expression levels were normalized to the UBQ10 and ACTIN2 transcripts. Relative expression values (treatment/control) are expressed as log2IF (mean values ± s; $n = 3$). Crosses and asterisks indicate significant differences from control treatments (buffer only; * $P$, 0.05, ** $P$, 0.01, Student’s $t$-test). Accession numbers of transcripts are as follows: AOX1a, At3g22370; CI1, At1g73120; FER1, At5g01600; LHCA3, At1g61520; OEP16-1, At2g28900; SAM-MT, At2g41380.
whether one of these pathways is interlinked with citrate-dependent gene expression: (1) pitI (defect in carbon/nitrogen balance; Ferrario-Méry et al., 2005); (2) abi4 (ABA insensitive; Finkelstein et al., 1998); (3) bak1 (elg; brassinosteroid [BR] hypersensitive; Whippo and Hangarter, 2005); (4) coi1 (jasmonic acid [JA] insensitive; Xie et al., 1998); (5) mlo2-11 NahG (mlo2-11; powdery mildew resistant; Consonni et al., 2006); NahG (suppressed salicylic acid [SA] accumulation; Gaffney et al., 1993); (6) npr1 (for nonexpressor of pathogenesis-related genes1; blocked SA signal transduction; Cao et al., 1997); (7) pad4-1 (inhibited SA signaling; Glazebrook et al., 1997); (8) ctr1 (constitutive ethylene signaling; Kieber et al., 1993); (9) ein2-1 pad4-1 (ein2-1; ethylene insensitive; Guzmán and Ecker, 1990); (10) quadruple DELLA (qDELLA); relief of growth repression, methyl jasmonate, and GA insensitive; Cheng et al., 2004; Navarro et al., 2008); and (11) gai (for GA insensitive; Lee et al., 2002).

The PII protein presented a likely candidate for a carboxylic acid-dependent signaling pathway, as it is an evolutionarily conserved carboxylic acid sensor protein that is localized in chloroplasts in plants (Ferrario-Méry et al., 2005). However, the pII mutant did not show an altered response to citrate in comparison with the wild type for the selected citrate-regulated marker transcripts CI-1, OEP16-1, FER1, and SAM-MT (Fig. 7). The transcript abundance response to citrate was also not completely abolished in any other of the hormone signaling mutants (Fig. 7). However, a significant attenuation in citrate induction of CI-1, FER1, and SAM-MT transcripts was observed in the bak1 (elg) mutant. BAK1 (for BRI1-associated kinase), the coreceptor of BRI1 (a BR receptor) and FLS2 (a flg22 receptor), is a Leu-rich repeat receptor kinase and is involved in restricting programmed cell death in response to pathogen challenge (Chinchilla et al., 2009). The other mutant that also showed an attenuated citrate induction of FER1 and SAM-MT transcripts was qDELLA (Fig. 7). The qDELLA mutant is deficient in four of the five DELLA proteins involved in GA signaling and is insensitive to methyl jasmonate (Navarro et al., 2008). However, a constitutively active DELLA mutant that stabilizes DELLA proteins (gai) was not overall significantly differentially affected by citrate treatment in comparison with the wild type (Fig. 7). Only the SAM-MT transcript was more strongly induced by citrate in the gai mutant. In contrast, the down-regulation of transcripts after citrate treatment might involve different signaling pathways, as another set of mutants showed an altered response toward citrate on the down-regulated marker transcript OEP16-1. Repression of OEP16-1 by citrate was attenuated again in the qDELLA mutant as well as in the SA defense signaling lines NahG and pad4-1, while the npr1 mutant showed a significantly increased repression of OEP16-1 transcript in the presence of citrate in comparison with the wild type (Fig. 7). A slightly increased repression of OEP16-1 was also observed in the coi1 mutant in the presence of citrate (Fig. 7). Thus, our results indicate that BAK1 and possibly also DELLA proteins might play a role in the amplification of the signal that leads to an accumulation of transcripts after citrate treatment. However, overall, our results do not clearly imply that citrate impacts a single plant hormone signaling pathway, which could be due to the fact that signaling pathways are usually not linear, and often, an extensive cross talk branch between different signaling pathways is observed to enable plants to integrate different signals (Genoud et al., 2001).

DISCUSSION

Besides their conserved function as TCA cycle intermediates, carboxylic acids such as citrate have been identified as important players in metabolite signaling in yeast and animal cells (McC amore et al., 2003; Wollen et al., 2009; Yang et al., 2012). Similar functions can also be expected for malate and citrate in plants, as both carboxylic acids can be exported from or imported into mitochondria, plastids, and other subcellular compartments (Fernie et al., 2004; Meyer et al., 2010a). Therefore, they are linked to various metabolic pathways in different compartments, making them ideally placed to function as reporter molecules of metabolic states. The aim of this study was to assess whether malate and citrate have general roles in the regulation
of transcript abundances of nuclear genes and to elucidate whether there exist a general organic acid response to altered levels of TCA cycle metabolites or whether different organic acids might convey a different signal to the cell.

Alterations in Citrate and Malate Levels Result in Unique Transcriptome Responses

To investigate the transcript response to alterations in organic acid levels, an established leaf slice system was applied (Raven and Farquhar, 1981; Muller et al., 2001; Horling et al., 2003) that allows quick and efficient feeding of organic acids and other effector solutions to leaves. The microarray experiments from feeding low levels of citrate and malate (1 mM each) revealed that both organic acids convey an overall very different transcriptome response and that only a small set of transcripts such as AOX1a is regulated by both organic acids (Fig. 1). Even at a higher concentration of 10 mM, which lies well above the usual apoplastic malate concentrations under low and high energy and hormone signaling mutants (black bars) and wild-type (WT; white bars) Arabidopsis accessions. Significant differences between the wild type and mutants are indicated (*P < 0.05, **P < 0.1, Student’s t test). QRT-PCR analysis was carried out in triplicate (technical and biological) using SYBR Green PCR Master Mix. Expression levels were normalized to the UBQ10 and ACTIN2 transcripts. Relative expression values (citrate/control) are expressed as log2IF (mean values ± SE; n = 3). Accession numbers of transcripts are as follows: CI1, At1g73120; FER1, At5g01600; OEP16-1, At2g28900; SAM-MT, At2g41380.
CO₂ concentrations (0.4–3 mM; Hedrich et al., 1994), the transcript response to malate and citrate was overall very different for selected marker transcripts (Fig. 4). Hence, from these results, we conclude that there is only a small general cellular response to low levels of weak acids and that most of the transcript response is specific to one or the other carboxylic acid. In addition to citrate being the stronger effector of transcript abundances, several transcripts from different functional categories were regulated in opposite directions to malate and citrate in comparison with control treatments (Supplemental Table S1). Many of the regulated transcripts encoded metabolism-associated proteins, demonstrating that there is metabolite-signaled feedback regulation of enzyme abundance. Distinct effects on gene expression have also been recently reported for the phosphonate analogs of 2-OG, which inhibit the 2-OG complex (Araújo et al., 2012). Seventy-six transcripts were significantly regulated after treatment of leaf samples with succinyl phosphate for 4 h, which are mostly different from those observed after citrate and malate feeding (data not shown).

Citrate and Malate Have Unique Roles in Cellular Metabolism That Might Be Responsible for the Observed Transcriptome Response

Despite the fact that malate and citrate are both TCA cycle intermediates, they have different chemical properties and also partially different cellular functions. In contrast to malate, citrate acts as a chelator of apoplastic Fe³⁺ in plants to maintain cellular iron homeostasis (Haydon and Cobbett, 2007). Thus, alterations in citrate levels might convey information about cellular iron homeostasis, and 11 transcripts coding for proteins involved in metal handling and transport were significantly up-regulated with increased citrate abundance (Supplemental Table S1). A specific role for iron citrate in cellular signaling was recently demonstrated in macrophage cells, where the mononuclear dicitrato-iron complex specifically inhibits protein Tyr phosphatases in vitro as well as in vivo and stimulates MAPK signaling (Gomez et al., 2010).

Different functions for malate and citrate also concern the redox status of cells, as malate that is converted to oxaloacetate produces either NADH or NADPH through malate dehydrogenases, which are localized in cytosol, mitochondria, peroxisomes, and chloroplasts. Malate derived from chloroplasts has often been discussed in the context of redox signaling, since excess reducing equivalents produced by the photosynthetic electron transport chain can be exported as malate: the so-called “malate valve” (Scheibe, 2004). An increase in apoplastic malate concentration was recently demonstrated to regulate the closure of stomata via the guard cell-specific R-type anion channel AtALMT12 (Meyer et al., 2010b). Thus, a feedback response of changes in malate levels of photosynthetic gene expression is plausible, and changes in photosynthetic rates are often observed in TCA cycle mutants (Carrari et al., 2003; Nunes-Nesi et al., 2005, 2007; Sienkiewicz-Porzucek et al., 2010; Sulpice et al., 2010; Araújo et al., 2011).

The observation that citrate had a generally repressive effect on the abundance of photosynthetic transcripts while malate feeding resulted in a slight accumulation of these transcripts is interesting in this context (Supplemental Tables S1 and S2). Similar observations of a coordinated transcript response on photosynthetic transcripts were also made under diverse conditions where mitochondrial functions were impaired (Schwarzländer et al., 2012). Perturbation of mitochondrial function often leads to an oxidative inhibition of the mitochondrial aconitase, which is followed by a rise in cellular citrate levels (Vanlerberghe and McIntosh, 1994; Morgan et al., 2008; Lehmann et al., 2009; Gupta et al., 2012). Mitochondrial citrate was discussed as a possible mediator of mitochondrial retrograde signals in plants as well as in yeast cells, but an in-depth transcriptome study was lacking so far (Vanlerberghe and McIntosh, 1994; Mackenzie and McIntosh, 1999; McCammon et al., 2003; Clifton et al., 2005; Schwarzländer and Finkemeier, 2013). Vanlerberghe and McIntosh (1996) already reported that the AOX1 transcript level responded to increased levels of citrate that were supplied externally or were increased internally through the inhibition of aconitase using monofluoroacetate. While isocitrate could also induce AOX1 (although to a lesser extent than citrate), malate, 2-OG, succinate, pyruvate, or Gly showed no such effect (Vanlerberghe and McIntosh, 1996). AOX1a, SAM-MT, and NDA2, which are marker transcripts of the mitochondrial retrograde response (Schwarzländer et al., 2012; Van Aken and Whelan, 2012), were also induced by citrate in this study. Recently, it was demonstrated in Arabidopsis roots that citrate but not nitric oxide mediates the induction of AOX (transcript as well as activity) upon hypoxia after the inhibition of aconitase (Gupta et al., 2012). However, the induction of AOX1a in our study was not specific to citrate, as it was induced most strongly after acetate treatment as well as after pyruvate, malate, oxaloacetate, and sugar feeding (Fig. 4). High carboxylic acid levels as well as sugar levels are known to report an energy-rich situation regulating glycolysis and nitrogen metabolism by inhibiting phosphofructokinase and phosphoenolpyruvate carboxylase activities, respectively (Plaxton, 1996). In these situations, the risk of overreduction of the mitochondrial and chloroplastic electron transport chain must be decreased by reprogramming metabolism to use the excess energy for biosynthetic reactions and to avoid oxidative damage.

The Relationship of the Transcriptome Response to Citrate and Biotic Stress

In our array experiment, 50 transcripts, which belong to the functional category biotic stress and plant defense, were significantly altered in abundance after
citrate treatment (Supplemental Table S1). Furthermore, the cluster analysis revealed a high similarity of the transcript response to citrate in experiments dealing with biotic stress, such as after *P. syringae* infection or flg22 treatment (Fig. 6). Several of the transcripts related to pathogen defense were decreased in expression, such as the *PATHOGENESIS-RELATED5* (PR5) transcript (At1g75040), which is known to be induced by Glc via the catalytic activity of hexokinase (Xiao et al., 2000). Citrate is known to have an inhibitory effect on the growth of gram-negative bacteria due to its action as a chelator of Ca$^{2+}$ and Mg$^{2+}$ ions, which destabilizes the plasma membrane of bacteria (Vaara, 1992; Boziaris and Adams, 1999). Hence, an increased production of citrate could act as a defense mechanism to pathogens in plants. Interestingly, an enhanced resistance to *P. syringae* infection and expression of SA-linked PR genes was observed in knockout lines of the cytosolic NADP-isocitrate dehydrogenase, which accumulated citrate (2-fold) while sugars such as Fru were decreased in abundance and SA levels were not increased (Mhamdi et al., 2010). In contrast to the external citrate treatment, the PR5 transcript level was significantly up-regulated in the icdh lines (2-fold) while it was significantly down-regulated (log$_2$-transformed relative induction factor [log2IF] = −0.41) after citrate treatment (Supplemental Table S1). Differences in transcript regulation between short-term chemical treatments and constitutive knockout lines, however, are not surprising, as mutant plants often adapt their signaling state to changes in metabolism (Schwarzländer et al., 2012). Furthermore, in this study, it was shown that most transcript abundances are regulated depending on citrate concentration and incubation time (Fig. 2). To mimic the same dynamic conditions in a transgenic plant, and thus a one-to-one comparison, will be nearly impossible to achieve, especially as metabolic cross talk in signaling can also be expected (e.g. sugar levels were down-regulated in icdh mutants while they were up-regulated with citrate feeding).

A link between mitochondrial function and pathogen defense was recently demonstrated in the *noxy* mutants, which have a defect in mitochondrial physiology and are affected in mitochondrial retrograde signaling (Vellisillo et al., 2013). Whether the *noxy* mutations also affect mitochondrial TCA cycle activities, and thus citrate export, is currently unknown and will be interesting to investigate.

The Interaction of Citrate with Plant Hormone Signaling Pathways

The comparison with Genevestigator data sets also revealed a similarity of the transcript response to citrate and PBZ treatment that inhibits GA biosynthesis and thus affects GA signaling (Fig. 6). To further investigate the interaction of citrate with plant hormone signaling, several mutants with defects in hormone signaling pathways that convey signals derived from GA, JA, SA, and BR were tested (Fig. 7). The bak1 mutant, which is hypersensitive to BR (Whippo and Hangarter, 2005), as well as *qDELLA*, which is constitutively derepressed in GA signaling, showed the strongest attenuation in the transcript accumulation after citrate treatment. Both proteins play a role in plant hormone signaling pathways involved in the plant response to biotic stress. Thus, citrate might partially repress the GA signaling pathway, which could also explain the similarity to the transcriptome response to pathogens, as GA not only regulates important plant growth responses but also interacts with other plant hormone signaling pathways and, for example, stimulates the production of SA (Alonso-Ramírez et al., 2009). Furthermore, BRs also inhibit flg22 responses and promote the susceptibility of *Arabidopsis* to *P. syringae* (Albrecht et al., 2012; Belkhadir et al., 2012), which may explain why the BR-hypersensitive bak1 mutant has reduced citrate responses.

Interestingly the repression of *OEPI6-1* after citrate treatment was elevated in the *npr1* mutant, which is derepressed in JA signaling. The redox-regulated NPR1 protein usually acts as suppressor of JA-dependent signaling by transcriptional suppression of JA-induced genes and by blocking signaling via the E3 ubiquitin ligase-containing complex SCF$^{npr1}$ containing COI1 (Sporø et al., 2003). Besides its SA-dependent regulation, NPR1 also acts independently and integrates JA and ethylene signals (Feyss and Park, 2000). This was demonstrated by Reuber et al. (1998), who reported that PRI induction was abolished in the *NahG* transgenic line upon pathogen infection with *Erysiphe orontii* but was only partially compromised in the *npr1* mutant background. In the case of *OEPI6-1*, the *npr1* mutant even showed an opposite effect to the *NahG* transgenic line (Fig. 7), indicating that NPR1 might act as a suppressor of citrate-mediated gene repression. Hence, citrate feeding might impact the interaction of these different plant hormone signaling pathways, and it will be important to further dissect this interaction in the future.

Is Citrate Affecting the Transcriptome Directly?

Here, we have demonstrated that feeding of citrate to *Arabidopsis* leaves induced a mild but widespread reprogramming of gene expression. Although some of the observed transcriptome changes might result from an induced accumulation of sugars, some of the transcriptome changes were different from those observed after Glc and Suc feeding (Fig. 6) or even unique to citrate feeding, such as the response of the selected marker transcripts, *CII* and *FER1*, which were solely regulated in abundance with increased citrate and not with other sugars or carboxylic acids (Fig. 4). Only feeding with the nonmetabolizable citrate analog tri-carballylate also resulted in an induced accumulation of these marker transcripts (Fig. 5). The extent of
induction of $CI1$ and $FER1$ marker transcripts, however, was slightly less than after citrate feeding, while internal citrate levels were increased to a similar extent than after citrate feeding. One explanation for the difference in induction could be that citrate is directly sensed at the plasma membrane, and thus the subcellular location of citrate would be important for signaling. However, an alternative explanation could be that tricarballylate competes with citrate for binding sites at putative receptor proteins or enzymes involved in signaling. Despite the structural similarity of citrate and tricarballylate, tricarballylate is much less inhibitory to isocitrate dehydrogenase activity than citrate at high concentrations (Coultate and Dennis, 1969). Another example is the bacterial tricarboxylate chemoreceptor McpS (for methyl-accepting chemotaxis receptor protein), which has much higher binding affinities to citrate than to isocitrate and no binding affinities to tricarballylate, indicating an essential role for the hydroxy group in protein binding and recognition in this case (Lacal et al., 2010). Our results demonstrate that the regulation of transcript abundance by altered citrate level was very much time and concentration dependent (Fig. 2). Taken together, these results strongly indicate that plants cells can specifically recognize the abundance of citrate.

In mammalian cells, different carboxylic acids have been shown to have specific effects on the activity of 2-OG-dependent 2-oxogenases, such as the hypoxia-inducible factor prolyl hydroxylases, JmjC family Lys demethylases, and 5-methylcytosine hydroxylases, and thus affect transcription (for review, see Yang et al., 2012). While fumarate, succinate, and isocitrate were shown to competitively inhibit the PHD2 hypoxia-inducible factor hydroxylase, citrate was also able to bind to the active site of PHD2 but had no inhibitory effect. Furthermore, citrate was the only carboxylic acid to induce the PHD2 apocatalytic formation and protected PHD2 activity from inactivation by actively removing iron (Hewitson et al., 2007). Another specific role for citrate in metabolite signaling was shown for iron dicitrate, which competitively inhibits the SHP1 protein Tyr phosphatase in macrophage cells to regulate MAPK signaling (Gomez et al., 2010). Furthermore, citrate exported from mitochondria and metabolized through the cytosolic ATP-citrate lyase was shown to mediate histone acetylation in liver cells to specifically regulate the expression of glycosylation-related genes (Wellen et al., 2009). Hence, citrate might indeed have specific roles in signaling that differ from other carboxylic acids, especially due to its high affinity to binding divalent cations such as iron or even calcium (for review, see Schwarzländer and Finkemeier, 2013). It will be interesting to investigate whether such specific signaling mechanisms also exist in plants, and the major challenge in future research will be to understand how the abundance of key metabolites such as citrate and other carboxylic acids is perceived and signaled within the plant cell.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

*Arabidopsis (Arabidopsis thaliana)* plants were grown on soil for 4 weeks on compost supplemented with vermiculite at 22°C with a photoperiod of 16 h and a light intensity of 100 to 150 µmol m⁻² s⁻¹. The following mutant lines were obtained from the Nottingham Arabidopsis Stock Center: cop1 (N663405), bki (N159), npr1-1 (N3726), abi1-1 (N8104), pad4-1 (N3806), ein2-1/pad4-1 (N66000), and nilo2-1/nab2 (N9726). The pfl mutant seeds were kindly provided by Sylvie Ferrario-Mery (INRA) and Michael Hodges (Université Paris Sud-XI). gDELLA, gai, and ctri1 mutant seeds were kindly provided by Nicholas Harberd (University of Oxford).

### Effecter Treatments

Ten to 20 fully expanded leaves from 4-week-old Arabidopsis plants were peeled and cut into 2-mm-diameter leaf slices using a razor blade (for each biological replicate). After vacuum infiltration in effector solutions (three times for 5 min each), the leaf slices were incubated at a light intensity of 100 µmol m⁻² s⁻¹ for the indicated time periods. All solutions used for infiltrations were made in 1 mS MES, and pH was adjusted afterward to 5.5 (KOH). All chemicals were purchased from Sigma-Aldrich. At the end of the incubations, leaf material was briefly blotted dry on tissue paper and flash frozen in liquid nitrogen.

### RNA Isolation, cDNA Synthesis, and QRT-PCR

RNA was extracted using Trizol Reagent (Invitrogen) followed by chloroform extraction, isopropanol precipitation, and subsequent LiCl precipitation. The quality and quantity of RNA were confirmed on agarose gels and by spectrophotometric quantitation. Complementary DNA (cDNA) was synthesized from DNase-treated RNA with SuperScriptII reverse transcriptase (Invitrogen) after the manufacturer’s protocol. QRT-PCR was carried out in triplicate in an ABI Prism 3700 using SYBR Green Master Mix (ABI) and gene-specific primers. Levels of selected transcripts in each sample were calculated using the standard curve method (Applied Biosystems; McWatters et al., 2007). Expression levels of each transcript were normalized to *ACTIN2* (At3g18780) and *POLYUBIQUITIN10* (At4g05320) transcripts as housekeeping genes. The amount of transcript in each control treatment (for each time point) was set to 1, and changes in treated samples are expressed as log2IFs (treatment/control). Primer sequences are listed in Supplemental Table S5.

### Microarray Experiments

cDNA labeling was carried out using the 3DNA Array 50 kit (version 2; Genisphere) according to the manufacturer’s instructions. Cy5- or Cy3-labeled cDNA probes were hybridized against the Arabidopsis 29k Oligonucleotide Microarrays (http://ag.arizona.edu/microarray). Microarray hybridization and data analysis were performed as described (Schwarzländer et al., 2012). Significantly regulated transcripts were detected by a Cyber-T test analysis (Baldis and Long, 2001; Bayes $P < 0.05$). The data sets were deposited at the European Bioinformatics Institute ArrayExpress database (accession no. E-MEXP-3110) according to the Minimum Information About Microarray Experiments guidelines. Functional class scoring was implemented using MapMan software (Thimm et al., 2004) applying the Benjamini-Hochberg correction. The Genevestigator database was used to analyze the expression of selected marker genes under various stress treatments as well as to perform a similarity analysis with citrate-regulated transcripts against all available microarray data sets in the database (Zimmermann et al., 2004). Hierarchical cluster analysis was performed using the algorithm for average linkage clustering with a Pearson correlation integrated in the MeV version 4.7.3 microarray software suite (Saeed et al., 2006). Transcript data for the cluster analysis were extracted from the following microarray data sets: ihg22 and PBZ (CSE17464); Gk (E-MEXP-475); Suc (NASCARRAYS-315), and KIN10 overexpressors (Beena-González et al., 2007).

### Determination of Metabolite Levels

For metabolite extraction, 100 mg of leaf material was briefly washed, dried, and flash frozen in liquid nitrogen. Metabolites were extracted and analyzed by GC-MS as described (Roessner et al., 2001), and relative quantification was performed using the TagFinder software package (Luedemann et al., 2008).
Statistical Analysis

Student’s t and χ² tests were performed using Microsoft Excel. Significant differences (P < 0.05) are highlighted with asterisks and marginal significant differences (P < 0.1) with crosses in the figures. Cyber-T analysis was performed using the online platform http://cybertics.uc.edu (Baldi and Long, 2001).

Supplemental Data

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

Supplemental Figure S1. Differential expression of selected citrate-regulated transcripts under biotic and abiotic stress treatments.

Supplemental Table S1. Data table with mean log₂ fold changes and P values from Cyber-T analysis from malate and citrate microarray analysis.

Supplemental Table S2. Functional class scoring results with Hochberg correction from malate and citrate microarray experiments using MapMan software.

Supplemental Table S3. List of regulated metabolites after 1 ms citrate and malate feeding for 2, 4, and 8 h identified by GC-MS analysis.

Supplemental Table S4. List of regulated metabolites after feeding of 10 ms citrate or 10 ms tricarballylate for 4 h identified by GC-MS analysis.

Supplemental Table S5. List of primers used in QRT-PCR experiments.

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