Enhanced Photosynthesis and Growth in atquac1 Knockout Mutants Are Due to Altered Organic Acid Accumulation and an Increase in Both Stomatal and Mesophyll Conductance

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Stomata control the exchange of CO₂ and water vapor in land plants. Thus, whereas a constant supply of CO₂ is required to maintain adequate rates of photosynthesis, the accompanying water losses must be tightly regulated to prevent dehydration and undesired metabolic changes. Accordingly, the uptake or release of ions and metabolites from guard cells is necessary to achieve normal stomatal function. The AtQUAC1, an R-type anion channel responsible for the release of malate from guard cells, is essential for efficient stomatal closure. Here, we demonstrate that mutant plants lacking AtQUAC1 accumulated higher levels of malate and fumarate. These mutant plants not only display slower stomatal closure in response to increased CO₂ concentration but are also characterized by improved mesophyll conductance. These responses were accompanied by increases in both photosynthesis and respiration rates, without affecting the activity of photosynthetic and respiratory enzymes and the transport of inorganic ions (e.g. K⁺, Cl⁻, and NO₃⁻) as well as metabolites such as the phytohormone abscisic acid (ABA), Suc, and malate, are important players controlling stomatal movements (Hetherington, 2001; Roelfsema and Hedrich, 2005; Pandey et al., 2007; Blatt et al., 2014; Kollist et al., 2014). In this context, although organic acids in plants is known to support numerous regulating the flow of gases between plants and their surrounding atmosphere. Accordingly, the majority of water loss from plants occurs through stomatal pores, allowing plant transpiration and CO₂ absorption for the photosynthetic process (Bergmann and Sack, 2007; Kim et al., 2010). The maintenance of an adequate water balance through stomatal control is crucial to plants because cell expansion and growth require tissues to remain turgid (Sablowski and Cariñier Dornelas, 2014), and minor reductions in cell water volume and turgor pressure will therefore compromise both processes (Thompson, 2005). As a result, the high sensitivity of plant tissues to turgor has prompted the use of reverse genetic studies in attempt to engineer plants with improved performance (Cowan and Troughton, 1971; Xiong et al., 2009; Borland et al., 2014; Franks et al., 2015).

In most land plants, not only redox signals invoked by shifts in light quality (Busch, 2014) but also the transport of inorganic ions (e.g. K⁺, Cl⁻, and NO₃⁻) as well as metabolites such as the phytohormone abscisic acid (ABA), Suc, and malate, are important players controlling stomatal movements (Hetherington, 2001; Roelfsema and Hedrich, 2005; Pandey et al., 2007; Blatt et al., 2014; Kollist et al., 2014). In this context, although organic acids in plants is known to support numerous
and diverse functions both within and beyond cellular metabolism, only recently have we obtained genetic evidence to support that modulation of guard cell malate and fumarate concentration can greatly influence stomatal movements (Nunes-Nesi et al., 2007; Araújo et al., 2011b; Pennell et al., 2012; Medeiros et al., 2015). Notably, malate, in particular, has been considered as a key metabolite and one of the most important organic metabolites involved in guard cell movements (Hedrich and Marten, 1993; Fernie and Martinoia, 2009; Meyer et al., 2010). During stomatal aperture, the flux of malate into guard cells coupled with hexoses generated on starch breakdown lead to decreases in the water potential, and consequently, water uptake by the guard cells ultimately opens the stomata pore (Roelfsema and Hedrich, 2005; Vavasseur and Raghavendra, 2005; Lee et al., 2008). On the other hand, during stomatal closure, malate is believed to be converted into starch, which has no osmotic activity (Pennell et al., 2012) or, alternatively, is released from the guard cells to the surrounding apoplastic space (Lee et al., 2008; Negi et al., 2008; Vähisalu et al., 2008; Meyer et al., 2010). The role of organic acids on the stomatal movements has been largely demonstrated by studies related to

**Figure 1.** Phenotype, growth, and morphological parameters in WT-like and atquac1 mutant plants under normal growth conditions. A, Representative images of 5-week-old Arabidopsis plants observed in at least four independent assays. Plants with reduced expression of AtQUAC1 were compared with plants that genotyped as wild-type (WT-like) during homozygous screening by PCR (for details, see Meyer et al., 2010). In all analyses performed, atquac1-1 and atquac1-2 mutant lines were directly compared with the corresponding WT lines (WT-like-1 and WT-like-2, respectively). B, RDM. C, LA. D, SLA. E, RGR. F, Stomatal index and stomatal density. Values are presented as means ± SE (n = 8) obtained in two independent assays (four in each assay); values in bold in atquac1 plants were determined by Student’s t test to be significantly different (P < 0.05) from their corresponding WT-like.
malate transport (Lee et al., 2008; Meyer et al., 2010; Sasaki et al., 2010). In the last decade, two protein families were identified and functionally characterized to be directly involved with organic acid transport at the guard cell plasma membrane and to be required for stomatal functioning (Lee et al., 2008; Meyer et al., 2010; Sasaki et al., 2010). In summary, AtABCB14, a member of the ABC (ATP binding cassette) family, which is involved in malate transport from apoplast to guard cells, was described as a negative modulator of stomatal closure induced by high CO2 concentration; notably, exogenous application of malate minimizes this response (Lee et al., 2008). In addition, members of a small gene family, which encode the anion channels SLAC1 (slow anion channel 1) and four SLAC1-homologs (SLAHs) in Arabidopsis (Arabidopsis thaliana), have been described to be involved in stomatal movements. SLAC1 is a well-documented S-type anion channel that preferentially transports chloride and nitrate as opposed to malate (Vahisalu et al., 2008, 2010; Geiger et al., 2010; Du et al., 2011; Brandt et al., 2012; Kusumi et al., 2012). Lack of SLAC1 in Arabidopsis and rice (Oryza sativa) have been described to be involved in stomatal movements. SLAC1 is a well-documented S-type anion channel that preferentially transports chloride and nitrate as opposed to malate (Vahisalu et al., 2008, 2010; Geiger et al., 2010; Du et al., 2011; Brandt et al., 2012; Kusumi et al., 2012). Lack of SLAC1 in Arabidopsis and rice (Oryza sativa) culminated in a failure in stomatal closure induced by high CO2 levels, low relative humidity, and dark conditions (Negi et al., 2008; Vahisalu et al., 2008; Kusumi et al., 2012). Although mutations in AtSLAC1 impair S-type anion channel functions as a whole, the R-type anion channel remained functional (Vahisalu et al., 2008). Indeed, a member of the aluminum-activated malate transporter (ALMT) family, AtALMT12, an R-type anion channel, has been demonstrated to be involved in malate transport, particularly at the plasma membrane of guard cells (Meyer et al., 2010; Sasaki et al., 2010). Although AtALMT12 is a member of ALMT family, it is not activated by aluminum, and therefore Meyer et al. (2010) proposed to rename it as AtQUAC1 (quick-activating anion channel 1; Imes et al., 2013; Mumm et al., 2013). Hereafter, we will follow this nomenclature. Deficiency of a functional AtQUAC1 has been documented to lead to changes in stomatal closure in response to high levels of CO2, dark, and ABA (Meyer et al., 2010). Taken together, these studies have clearly demonstrated that both S- and R-type anion channels are key modulators of stomatal movements in response to several environmental factors.

Despite a vast number of studies involving the above-mentioned anion channels, little information concerning the metabolic changes caused by their impairment is currently available. Such information is important to understand stomatal movements, mainly considering that organic acids, especially the levels of malate in apoplastic/mesophyll cells, have been highlighted as of key importance in leaf metabolism (Fernie and Martinoia, 2009; Araújo et al., 2011a, 2011b; Lawson et al., 2014; Medeiros et al., 2015). Here, we demonstrate that a disruption in the expression of AtQUAC1, which leads to impaired stomatal closure (Meyer et al., 2010), was accompanied by increases in mesophyll conductance ($g_{m}$), which is defined as the conductance for the transfer of CO2 from the intercellular airspaces ($C_i$) to the sites of carboxylation in the chloroplastic stroma ($C_c$). By further...
Table 1. Gas exchange and chlorophyll a fluorescence parameters in WT-like and atquac1 plants

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT-Like-1</th>
<th>atquac1-1</th>
<th>WT-Like-2</th>
<th>atquac1-2</th>
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</thead>
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<tr>
<td>A_s (μmol CO_2 m^{-2} s^{-1})</td>
<td>6.23 ± 0.49</td>
<td>8.74 ± 0.20</td>
<td>6.53 ± 0.30</td>
<td>7.65 ± 0.35</td>
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<td>g_s (mol H_2O m^{-2} s^{-1})</td>
<td>0.15 ± 0.03</td>
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<td>0.15 ± 0.01</td>
<td>0.20 ± 0.02</td>
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<td>WUEi (A_s/g_s)</td>
<td>41.0 ± 5.5</td>
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<td>41.1 ± 3.0</td>
<td>40.3 ± 3.3</td>
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<tr>
<td>R_n (μmol CO_2 m^{-2} s^{-1})</td>
<td>0.85 ± 0.10</td>
<td>1.29 ± 0.17</td>
<td>0.66 ± 0.14</td>
<td>1.28 ± 0.13</td>
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<tr>
<td>F_v/F_m</td>
<td>0.79 ± 0.01</td>
<td>0.78 ± 0.02</td>
<td>0.77 ± 0.03</td>
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<tr>
<td>F_v/F_m*</td>
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<td>0.58 ± 0.007</td>
<td>0.56 ± 0.006</td>
<td>0.57 ± 0.005</td>
</tr>
<tr>
<td>J_{tr} (μmol m^{-2} s^{-1})</td>
<td>70.8 ± 2.12</td>
<td>79.8 ± 1.8</td>
<td>71.0 ± 4.4</td>
<td>75.7 ± 2.6</td>
</tr>
</tbody>
</table>

*a_s, Net photosynthesis rate; g_s, stomatal conductance; WUEi, intrinsic water use efficiency; F_v/F_m, maximum PSII photochemical efficiency; F_v/F_m*, actual PSII photochemical efficiency; J_{tr}, electron transport rate estimated by chlorophyll fluorescence parameters.

characterization of atquac1 knockout plants, we demonstrated that reduced diffusive limitations resulted in higher photosynthetic rates and altered respiration that, in turn, led to enhanced biomass accumulation. Overall, the results obtained are discussed both in terms of the importance of organic acid transport in plant cell metabolism and with regard to the contribution that it plays in the regulation of both stomatal function and growth.

RESULTS

atquac1 Plants Exhibited Slightly Elevated Leaf Growth

Given that stomata are the main gate to control CO_2 influx into leaves, we investigated whether mutations in AtQUAC1 affected growth parameters in the two independent atquac1 T-DNA lines (atquac1-1 and atquac1-2) described in detail by Meyer et al. (2010). We first confirmed the absence of AtQUAC1 transcripts in leaves of the mutants by reverse transcription PCR (Supplemental Fig. S1). The mutant lines, which had no visible aberrant phenotypes during the vegetative growth phase (Fig. 1A), displayed enhanced rosette dry mass (Fig. 1B) and relative growth rate (RGR; Fig. 1E), coupled with increased total leaf area (LA; Fig. 1C) and specific leaf area (SLA; Fig. 1D). Although we observed clear differences between the two mutant lines in their RGR, when compared to their respective WT-like (for genotyped as wild type) plants, we noticed that the enhancement observed in RGR was proportionally similar between atquac1 T-DNA lines. We additionally observed that stomatal density and stomatal index (Fig. 1F) were unaltered in both mutant lines.

Closing Kinetics, Water Loss, and Sensitivity to Drought Are Affected in atquac1 plants

The absence of AtQUAC1 has been previously demonstrated to impact stomatal closure in response to both CO_2, dark, and ABA (Meyer et al., 2010). To further assess the impact of the lack of a functional AtQUAC1 on stomatal conductance (g_s) and water loss in Arabidopsis plants, we next adopted three complementary approaches. First, we confirmed the duration of stomatal responses following dark-to-light and light-to-dark transitions as well as normal-to-high and high-to-normal CO_2 concentrations (Supplemental Fig. S2). Our results confirmed the deficient stomatal regulation in mutant plants, which showed slower stomatal closing kinetics in response to both light-to-dark transitions (Supplemental Fig. S2, A and B) and normal-to-high CO_2 concentrations (Supplemental Fig. S2, C and D). In contrast, the light-stimulated opening kinetics was less affected, albeit we also observed a relative tendency of faster opening and higher g_s even in response to high CO_2 levels. Given that atquac1 plants have slower stomatal closing, despite similar stomatal density (Fig. 1F), we next performed a time scale water loss experiment from excised rosettes by analyzing fresh weight loss. Consistent with slower stomatal closure, water loss was similar in both WT-like and atquac1 plants during the beginning of the experiment. However, after 240 min, water loss from atquac1 plants resulted in 32% fresh weight loss against 28% in WT-like plants (Fig. 2, A and B). These data suggest that atquac1 plants most likely exhibit higher sensitivity to stress conditions. However, given that fresh weight loss in the detached rosette might not reflect the situation in planta, we next decided to analyze the response of those plants following water restriction in plants growing on soil. Indeed, after suspension of irrigation, atquac1 plants showed earlier symptoms of chlorosis and leaf wilting, i.e. 4 to 5 d after withholding watering against 6 to 7 d in both WT-like plants (Fig. 2C). Thus, absence of AtQUAC1 in Arabidopsis plants is likely to increase its sensitivity to drought episodes.

AtQUAC1 Deficiency Results in Increased g_s and Enhanced Photosynthesis Rate

Considering that most of plant biomass is derived from photosynthesis, we fully characterized the
photosynthetic performance by analyzing diffusional, photochemical, and biochemical constraints to photosynthesis. Compared with their respective WT control, mutant plants displayed higher net photosynthetic rates ($A_N$) and $g_s$ whereas no differences in intrinsic water use efficiency ($WUE_i$) were observed (Table I). Dark respiration ($R_d$) was higher (approximately 40%) in *atquac1* plants than in their respective WT-like counterparts (Table I). The differences in $A_N$ were unlikely to have been related to photochemical constraints given that both the maximum quantum efficiency of photosystem II (PSII; $F_v/F_m$) and capture efficiency of excitation energy ($F_v'/F_m'$) remained invariant. Additionally, the electron transport rate ($J_{fl}$) was marginally increased only in *atquac1-1* (Table I).

By further analyzing gas exchange under photosynthetically active photon flux density (PPFD) that ranged from 0 to 1000 μmol m$^{-2}$ s$^{-1}$, we observed that mutant plants exhibited unaltered $A_N$ irrespective of the irradiance. Indeed, the saturation irradiance ($I_s$) and the light-saturated $A_N$ ($A_{PPFD}$) were increased only in *atquac1-2* plants with no changes both in the compensation irradiance ($I_c$) and light use efficiency (Supplemental Table S1; Supplemental Fig. S3). Additionally, the response of $A_N$ to the internal CO$_2$ concentration ($A_N/C_i$ curves; Fig. 3, A and B) was obtained, which were further converted into responses of $A_N$ to chloroplastic CO$_2$ concentration ($A_N/C_c$ curves; Fig. 3, C and D). Interestingly, under ambient CO$_2$ concentration (400 μmol mol$^{-1}$), $C_i$ estimations were similar between *atquac1* and WT-like plants, whereas $C_c$ was increased in *atquac1* plants (Table II). $g_m$, estimated using a combination of gas exchange and chlorophyll $a$ fluorescence parameters via two independent methods, was significantly higher (29%) in *atquac1* plants in comparison to their respective WT-like (Table II). In addition, the maximum carboxylation velocity ($V_{cmax}$) and maximum capacity for electron transport rate ($J_{max}$) were higher in both mutant lines only when estimated on a $C_i$ basis, whereas on a $C_c$ basis $J_{max}$ was increased only in *atquac1-1* line (Table II). Moreover, the similarities in the $J_{max}/V_{cmax}$ ratios suggest that although differences in $A_N$ were observed an adequate functional balance between carboxylation and electron transport rates probably occurred.

The overall photosynthetic limitations were next partitioned into their functional components: stomatal

Figure 3. Net photosynthesis ($A_N$) curves in response to substomatal ($C_i$) or chloroplastic ($C_c$) CO$_2$ concentrations in WT-like and *atquac1* plants. A to D, $A_N/C_i$ curves (A and B) and $A_N/C_c$ curves (C and D) to WT-like-1/1 *atquac1* and WT-like-2/2 *atquac1*, respectively. Asterisk indicates that from this point and above, the $A_N$ in *atquac1* plants were statistically higher than WT-like ones by Student’s $t$ test ($P < 0.05$). Values are presented as means ± s.e. ($n = 10$) obtained using the ninth leaf totally expanded from ten different plants per genotype in two independent assays (five plants in each assay).
Diel changes in the levels of organic acids (malate and fumarate) were similar to those observed for sugars and starch, with higher values being consistently observed in mutant plants. Remarkably, atquac1 plants showed increases in both malate and fumarate content mainly at the middle of the light period (Fig. 5; Supplemental Fig. S3).

We next decided to extend this study to major primary pathways of plant photosynthetic metabolism by using an established gas chromatography-mass spectrometry (GC-MS) protocol (Lisec et al., 2006). This analysis revealed, however, that among the 40 successfully annotated compounds related to primary metabolism, only a relatively small number of changes were evident (Fig. 5). By analyzing 13 individual amino acids, we observed increases only in Gln, Pro, Gly, and Ala, which were moreover only significantly different in atquac1-1 plants (Fig. 5). When considering the levels of the organic acids, we also observed that only the levels of maleic acid, malate, fumarate, succinate (only in atquac1-1), and glyceraldehyde (only in atquac1-2) increased in mutant lines. Other changes of note in the metabolite profiles were the significant increases in glycerol and myoinositol (in both lines, Fig. 5).

We next investigated whether the metabolic perturbation observed could also affect the activity of important enzymes related to photosynthetic and respiratory metabolism (Table III). Although changes in both photosynthesis and respiration were observed in atquac1 plants, there were no changes in either Rubisco or NADP-dependent malate dehydrogenase (NADP-MDH). Moreover, increases in transketolase activity were observed in both atquac1 lines, whereas glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

(lₐ), mesophyll (lₘ), and biochemical (lₜ', Table II). The photosynthetic rates were mainly constrained by lₘ (64% and 54% in WT-like and atquac1 plants, respectively), whereas lₚ accounted for, on average, 19% in both WT-like and atquac1 plants, and lₘ contributed with 16% and 26% in WT-like and atquac1 plants, respectively. These analyses demonstrated that atquac1 plants exhibits lower lₘ compared to the WT-like plants in close agreement with the higher gₘ observed (Table II).

Mutations in AtQUAC1 Affect Mainly Carbon Metabolism without Strong Effects in Activity of Related Enzymes

To explore the consequences of changes in photosynthetic capacity among the genotypes, we further conducted a detailed metabolic analysis in leaves of the mutants and WT-like plants. Evaluation of compounds related to nitrogen metabolism revealed that there were no changes in the levels of nitrate, chlorophylls, total amino acids, and soluble proteins in a consistent manner with the altered expression of AtQUAC1 (Supplemental Figs. S4 and S5). During the light period, mutant lines accumulated more Gic (Fig. 4, A and B), Suc (Fig. 4, E and F), and starch (Fig. 4, G and H), reaching higher values at the end of this period. Notably, the mutant lines were able to fully degrade these metabolites by the end of the dark period, reaching similar values to those observed in WT-like plants (Fig. 4), corroborating the increased Rₜ observed in atquac1 plants (Table I). Suc was the main storage sugar in all genotypes reaching, on average, 3 and 15 times higher contents than those of Glc and Fru, respectively. It is important to note that the higher concentrations of starch and sugars observed in atquac1 plants were accompanied by higher Aₜ.
activity was increased only in atquac1-1 plants. Regarding enzymes related to respiratory metabolism, no significant changes were observed for the activities of Suc synthase (Susy), phosphoglycerate kinase (PGK), or NAD-dependent malate dehydrogenase (NAD-MDH).

AtQUAC1 Repression Does Not Strongly Affect the Expression of Other Genes Related to Ion Transport in Guard Cells

We next analyzed whether AtQUAC1 repression affected the expression of genes currently known to or putatively related to organic and inorganic ion transport, as well as genes involved in guard cell movements. We felt such experiments were important given that the loss of function of AtSLAC1 was associated with downregulation of several guard-cell-expressed transporters (Laanemets et al., 2013). To extend this molecular characterization, we attempted to look at the expression levels of several ion channels and transporter in guard cells from isolated epidermal fragments, including ALMT6, ALMT9, AtABCB14, AtSLAC1, AHA1, AHA5, KAT1, KAT2, AKT1, AKT2, AtKC1, TPC1, and GORK (for further details, see “Materials and Methods” and Supplemental Table S2). Quantitative real-time PCR (qRT-PCR) analysis of the transcript levels of these genes revealed, in sharp contrast to the situation observed in the case of atslac1 plants (Laanemets et al., 2013), that the vast majority of evaluated genes in atquac1 plants were unaltered.
Thus, although KAT1 and KAT2 were reduced in atquac1-2 plants only and the AHA5 transcript level was increased in atquac1-1 plants only, our results indicate that the stomatal effects observed here were not likely to be mediated by an alteration in the general efficiency of transport of the guard cells.

Figure 5. Relative metabolite content in leaves of WT-like and atquac1 plants. Amino acids, organic acids, and sugars and sugar-alcohols were determined by GC-MS as described in “Materials and Methods.” The full data sets from these metabolic profiling studies are additionally available in Supplemental Table S3. Data are normalized with respect to the mean response calculated for the corresponding WT-like (to allow statistical assessment, individual plants from this set were normalized in the same way). WT-like-1 or -2, Gray bars; atquac1-1 or -2, black bars. Values are presented as means ± SE (n = 5). Asterisks indicate that the values from mutant lines were determined by Student’s t test to be significantly different (P < 0.05) from their corresponding WT-like.
Porter (reduction in not only with the repression of an organic acid transport has signiﬁcantly altered in atquac1 plants involving the elevation of cytosolic Ca\textsuperscript{2+} concentrations, which downregulated the inward K\textsuperscript{+} channel activity. By contrast, stomatal opening kinetics were not signiﬁcantly altered in atquac1 plants (Supplemental Fig. S2). Furthermore, as changes in the activities of key enzymes of photosynthetic and respiratory metabolism were not observed (Table III) and $V_{\text{cmax}}$ on a $C_r$ basis (Table II) was similar between WT-like and atquac1 plants, we contend that diffusive rather than biochemical limitations had a major role explaining the changes in photosynthetic rates. These results, coupled with those obtained by Meyer et al. (2010) and Sasaki et al. (2010), provide strong evidence that AtQUAC1 is essential for an efﬁcient stomatal closure yet does not strongly affect the central primary metabolism.

### Functional Absence of AtQUAC1 Alters Stomatal Movements and Mesophyll Conductance

Given the increased photosynthetic rates and subsequent increases in LA and RGR (Figs. 1 and 3; Table I), we next investigated the mechanisms underlying this positive growth response. Since the changes described above took place independently of changes in the stomatal density and photosynthetic pigment levels (Fig. 1F; Supplemental Fig. S4), it is reasonable to assume that molecular and metabolic mechanisms occurred enabling a reprogramming in response to impaired stomatal closure in atquac1 plants under our experimental conditions. The results presented here provide further evidence that the functional lack of AtQUAC1 leads to slower stomatal closure in response to dark and high CO\textsubscript{2} concentrations (Supplemental Fig. S2). Additional compelling evidence supporting the role of AtQUAC1 on the regulation of stomatal function was due to the dramatic reductions of inward K\textsuperscript{+} channel currents (Laanemets et al., 2013). In this study, the authors also identiﬁed a compensatory feedback control in atslac1 plants regulating those highly specialized cell types, as yet, remains elusive. Here, by using a combination of physiological and biochemical approaches, we provide evidence that the genetic manipulation of organic acid transport has signiﬁcant potential to biotechnological applications (Martinoia et al., 2012; Schroeder et al., 2013; Medeiros et al., 2015). Both the data we provide and the recent molecular characterization of Arabidopsis plants deﬁcient in the expression of AtQUAC1 (Meyer et al., 2010; Sasaki et al., 2010) and data concerning the regulation of AtQUAC1 (Imes et al., 2013; Mumm et al., 2013) add further support to the importance of this anion channel regulating stomatal movements. Importantly, we showed that other aspects of photosynthetic and respiratory metabolism, the GC-MS-based metabolite proﬁle (Fig. 5; Supplemental Table S3), and the transcript levels of some key channels and transporters involved in guard cell ion transport (Fig. 6) all displayed relatively few and mild changes. Such observations likely indicate that AtQUAC1 plays little part in terms of total cellular homeostasis. It is important to note here that impairments in the AtSLAC1 activity also reduced stomatal opening kinetics that were associated not only with the repression of an organic acid transporter (reduction in AtABCB14 expression), but mainly

### DISCUSSION

Ion transport from guard cells to their surroundings has been proven essential to stomatal movements. Indeed, it is well known that the efflux of malate from guard cells can regulate the activity of anion channels on guard cell membrane (Hedrich and Marten, 1993; Hedrich et al., 1994; Raschke, 2003; Lee et al., 2008; Negi et al., 2008; Kim et al., 2010), suggesting that the organic acid accumulation on the apoplast space might inﬂuence stomatal movements. Indeed, the role of the organic acids (e.g. malate and fumarate) on the regulation of stomata movements was recently conﬁrmed (Nunes-Nesi et al., 2007; Meyer et al., 2010; Aratuo et al., 2011b; Medeiros et al., 2015). However, the metabolic hierarchy regulating those highly specialized cell types, as yet, remains elusive. Here, by using a combination of physiological and biochemical approaches, we provide evidence that the genetic manipulation of organic acid transport has signiﬁcant potential to biotechnological applications (Martinoia et al., 2012; Schroeder et al., 2013; Medeiros et al., 2015). Both the data we provide and the recent molecular characterization of Arabidopsis plants deﬁcient in the expression of AtQUAC1 (Meyer et al., 2010; Sasaki et al., 2010) and data concerning the regulation of AtQUAC1 (Imes et al., 2013; Mumm et al., 2013) add further support to the importance of this anion channel regulating stomatal movements. Importantly, we showed that other aspects of photosynthetic and respiratory metabolism, the GC-MS-based metabolite proﬁle (Fig. 5; Supplemental Table S3), and the transcript levels of some key channels and transporters involved in guard cell ion transport (Fig. 6) all displayed relatively few and mild changes. Such observations likely indicate that AtQUAC1 plays little part in terms of total cellular homeostasis. It is important to note here that impairments in the AtSLAC1 activity also reduced stomatal opening kinetics that were associated not only with the repression of an organic acid transporter (reduction in AtABCB14 expression), but mainly

### Table III. Enzyme activities in WT-like and atquac1 plants

Activities were determined in whole rosettes (5-week-old) harvested at middle of the photoperiod. Values are presented as means ± s (n = 5); values in bold type in atquac1 plants were determined by Student’s t test to be signiﬁcantly different (P < 0.05) from their corresponding WT-like. FW, Fresh weight.

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<thead>
<tr>
<th>Enzymes</th>
<th>WT-Like-1</th>
<th>atquac1-1</th>
<th>WT-Like-2</th>
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<td>Rubisco initial</td>
<td>946.4 ± 69.2</td>
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<td>Transketolase</td>
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<td>NADP-GAPDH</td>
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<td>Susy</td>
<td>63.8 ± 4.5</td>
<td>65.9 ± 4.2</td>
<td>62.9 ± 2.4</td>
<td>61.4 ± 3.9</td>
</tr>
<tr>
<td>PGK\textsuperscript{b}</td>
<td>62.1 ± 3.9</td>
<td>58.8 ± 5.8</td>
<td>51.9 ± 2.9</td>
<td>48.7 ± 4.7</td>
</tr>
<tr>
<td>NAD-MDH\textsuperscript{a}</td>
<td>512.7 ± 15.9</td>
<td>548.4 ± 22.9</td>
<td>458.1 ± 19.4</td>
<td>444.9 ± 3.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Activation state expressed in percentage (%). \textsuperscript{b}Values expressed in μmol min\textsuperscript{-1} g\textsuperscript{-1} FW.
AtQUAC1 Modulates Stomatal Closure and Metabolism

Figure 6. Relative transcript responses of genes involved in organic and inorganic ion transport in guard cell. Transcript abundance of Arabidopsis plasma membrane H+-ATPases AHA1 and AHA5, transporter AtABC14, and ion channels ALMT6, ALMT9, SLAC1, KAT1, KAT2, AKT1, AKT2, TPC1, AtKCI1, and GORK was determined. RNA was isolated from epidermal fragments. Data are normalized with respect to the mean response calculated for the corresponding WT-like. atquac1-1, Black bars; atquac1-2, gray bars. Values are presented as means ± se (n = 4); asterisks indicate values that were determined by Student’s t test to be significantly different (P < 0.05) from their corresponding WT-like.

Plant photosynthetic capacity was considered for a long time to be limited only by the rate of CO₂ diffusion through the stomata and by the capability of the photosynthetic machinery to convert the light energy into biomass at the biochemical one (Flexas et al., 2012). However, it is now recognized that the pathway to CO₂ diffusion from the mesophyll to the Rubisco carboxylation sites in the chloroplasts can become an important limiting factor to the photosynthetic process due to the several resistances in the gas and liquid phases during this way. Thus, gas exchange measurements have attempted to explain both stomata physiology and variations in photosynthesis (Farquhar et al., 1980), has recently turned out to be a key point in explaining limitations during this process (Bernacchi et al., 2002; Flexas et al., 2007, 2012, 2013; Warren, 2008b; Bown et al., 2009; Niinemets et al., 2009; Jin et al., 2011; Scafaro et al., 2011; Martins et al., 2013). Noteworthy, gs and gₛ are very often coregulated (Flexas et al., 2012), which can reflect either a strong coordination between Aₛ and gs or a compensatory mechanism, where gₛ tends to compensate changes in gs particularly under suboptimal conditions (e.g. drought). In such cases, where gs seems to be more affected than gₛ, this coregulation has the purpose of optimizing Aₛ (Warren, 2008a; Duan et al., 2009; Vrábl et al., 2009; Galmés et al., 2011; Flexas et al., 2012; Galmés et al., 2013). Accordingly, photosynthetic limitations were estimated and revealed that the mesophyll fraction had a greater contribution to the lower Aₛ observed in WT-like compared to atquac1 plants (Table II). Collectively, the results presented here demonstrate that the higher capacity of CO₂ fixation in atquac1 plants was associated with a higher Cₛ due to increased gₛ in atquac1 mutant plants that can, at least partially, explain the increased growth presented by those plants (Fig. 1). The observed effects on gₛ, which were followed by increases in gₛ (Tables I and II) without changes in the stomatal density (Fig. 1F), indicate that the diffusional component was the main player controlling Aₛ. Detailed biochemical and physiological analyses delimit this response as a consequence of perturbation of stomatal function; however, the exact mechanisms underlying this phenomenon are not immediately evident. Although several studies have attempted to explain both stomata physiology and variations in gm (Kollist et al., 2014; Lawson et al., 2014), which may rely on anatomical properties (Peguero-Pina et al., 2012), our understanding of this subject remains far from complete. Accordingly, a parameter commonly used to characterize the physical limitation inside the leaves is the leaf dry mass per unit area (LMA) that increase as a function of increases in cell wall thickness, potentially decreasing the velocity of CO₂ diffusion (Flexas et al., 2008; Niinemets et al., 2009; Flexas et al., 2012). The LMA is considered a key trait in plant growth and performance, allowing plants to cope with different environmental conditions most likely because the amount of light absorbed by a leaf and the diffusion pathway of CO₂ through the leaf depend partially on its thickness (Vile et al., 2005; Poorter et al., 2009, Villar et al., 2013). The inverse of LMA is the ratio of leaf area to leaf mass or SLA; thus, a reduction in LMA is translated into increments in SLA and, in turn, increases in gm. Indeed, the values of SLA found here are in agreement with this hypothesis once we observed higher values of both SLA and gm in atquac1 plants (Fig. 1D; Table II). In this vein, although we have not observed changes in stomatal density but increased SLA, studies related to the leaf anatomy of these mutants might help to explain whether the increases in gm are governed by anatomic traits or by further investments of nitrogen to mesophyll proteins involved in increasing gm such as carbonic anhydrases, aquaporins, Rubisco, and others (Buckley and Warren, 2014). Accordingly, it is not without precedence to suggest that changes in gm will eventually correlate with changes in sugars, which are cell wall precursors, and, to a lower extent, to organic acids, as observed here. In this sense, it seems plausible that an interaction between these compounds may exist and, thus, directly or indirectly be associated with enhancement of gm and by extension Aₛ. It will be important to establish the functional significance of this observation in future studies.

Further obtained from assays of fresh weight water loss and drought stress (Fig. 2). Collectively, these analyses showed that the mutant lines lost water faster than their respective WT-like, characterizing a water-spending phenotype and likely more sensitive to drought events (Fig. 2C). Thus, these data clearly document the importance of AtQUAC1 and, by extension, organic acid transport in guard cell function.
Carbon Metabolism Is Changed as Consequence of the Higher Photosynthetic Rates in atquac1 Plants

The organic acid levels in the apoplast exhibited a negative correlation between the malate and fumarate content and $g_s$, with a greater contribution of malate (Nunes-Nesi et al., 2007; Araújo et al., 2011b). This information, coupled to the results obtained with the malate transporter AtABCB14 (Lee et al., 2008), provides strong evidence that the apoplastic content of malate and fumarate can modulate the functioning of guard cells and, in turn, affect the stomatal movements with effects on leaf metabolism. The results presented here demonstrate that impaired organic acid transport, which culminates with alteration in $g_s$ and $g_m$, promote minor changes in primary metabolism, mainly in carbon metabolism, under normal growth conditions. This fact notwithstanding, the increases in the hexose contents in atquac1 plants were observed not to be primarily due to increases in Suc or starch breakdown since these metabolites accumulated over the course of the light period (Fig. 4). In good agreement with the higher $A_N$ observed in atquac1 plants (Table I), higher levels of sugars were also observed, which were properly remobilized during the dark period supporting higher $R_d$ (Table I) and, in turn, leading to the enhanced growth observed in those plants (Fig. 1). However, it is important to mention that caution should be taken when analyzing such results obtained under well-controlled under conditions. This is particularly true given that the growth benefit observed here (Fig. 1A), which was obtained under well-watered conditions, is not maintained under drought situations (Fig. 2C) most likely associated with a higher $g_s$ and $g_m$ observed in atquac1 mutant plants. In contrast to the situations observed here, reductions in another stomatal channel protein (SLAC1) in rice were associated with increments in both $A_N$ and $g_s$ without any growth benefit under well-watered conditions (Kusumi et al., 2012) but culminated in lower productivity and yield of rice plants under field conditions. Taken together, these data indicate that an increased sensitivity to water limitations episodes associated with higher $g_s$ and $g_m$ can exceed the enhanced $CO_2$ assimilation under less favorably environments. Accordingly, our understanding of plant responses to water limitation is still fragmentary, most likely due to the complex responses involving adaptive changes and/or deleterious effects. Under field conditions, the responses can be synergistically or antagonistically modified by the interaction with other plants and/or superimposition of other stresses and therefore caution should be taken when interpreting the results described here and as such further investigation should be performed within the context of understanding the stomata responses to water stress.

Regarding to the nitrogen metabolism, it has been demonstrated an inverse relationship between amino acid contents and growth, given that even under nitrogen starvation (which leads to reduced growth), the amino acid levels have been shown to increase without changes in Rubisco activation, total protein, and chlorophyll contents (Tschoep et al., 2009). Interestingly, increases
in both \( A_N \) and growth in \( atquac1 \) plants were neither followed by changes in chlorophyll, amino acid, or total soluble protein content (Supplemental Figs. S4 and S5), nor by changes in Rubisco activation state (Table III), highlighting that the increases in \( A_N \) were indeed associated with lower diffusional limitations (Table II). Furthermore, similar values of \( I_{max}:V_{cmax} \) ratio (Table II) and unchanging activities of some enzymes related to photosynthetic metabolism (e.g. Rubisco) are consistent with a photosynthetic functional balance since plants are able to adjust Rubisco content/activation and other photosynthetic machinery components to maintain the balance among the enzymatic reactions (e.g. Rubisco) and light harvesting (e.g. chlorophylls; Stitt and Schulze, 1994). Collectively, our results suggest that inefficient regulation of the stomatal closure via repression of \( AIQUAC1 \) culminates in higher growth and photosynthetic rates through increased \( g_m \) and \( g_t \) albeit promoting minor changes on carbon metabolism. This hypothesis is illustrated in Figure 7 and would hence explain why the accumulation of organic acids, in special malate within the guard cells, will culminate with a longer stomatal aperture in \( atquac1 \) plants. This model further suggests that increased \( A_N \) is likely related to the maintenance of a high chloroplastic \( CO_2 \) concentration, ultimately leading to growth enhancement. It should be borne in mind that the changes observed in several sugars, as well as in dark respiration in \( atquac1 \) plants can, at least partially, explain the higher growth rates. The exact mechanism by which changes in organic acid transport induced simultaneous changes in both \( g_m \) and \( g_t \) remains as yet unclear; however, it seems reasonable to anticipate this might be related to an as-yet-unknown signaling compound associated with higher photosynthetic rates.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (Arabidopsis thaliana) plants used here were of the Colombia ecotype (Col-0) background. Seeds were surface-sterilized and imbibed for 2 d at 4°C in the dark on agar plates containing half-strength Murashige and Skoog media (Murashige and Skoog, 1962). Seeds were subsequently germinated and grown in a growth chamber under short-day conditions (8 h/16 h of light/dark) with 150 \( \mu \)mol m\(^{-2}\)s\(^{-1}\) white light, 22°C/20°C throughout the day/night cycle, and 60% relative humidity. The T-DNA mutant lines \( atquac1-1 \) (SM_3_38992) and \( atquac1-2 \) (SM_3_37173) were obtained from the John Innes Centre JIC collection (Tissier et al., 1999) and were previously described (Meyer et al., 2010). Plants with reduced expression of \( AIQUAC1 \) were compared with plants that genotyped as wild-type (WT-like) during homozygous screening by PCR (for details, see Meyer et al., 2010). In all analyses performed, \( atquac1-1 \) and \( atquac1-2 \)-mutant lines were directly compared with the corresponding WT lines (WT-like-1 and WT-like-2, respectively). The abundance of transcripts was confirmed by semiquantitative PCR using specific primers pairs designed to span the T-DNA insertion site of the two mutant loci (for details, see Supplemental Fig. S1).

Growth Analysis

Whole rosettes from 5-week-old plants were harvested and the RDW, LA, SLA, RGR were evaluated. LA was measured by digital image method using a scanner (Hewlett-Packard Scanjet G2410) and the images were after processed using the Rosette Tracker software (De Vylder et al., 2012). SLA and RGR, which is the net dry weight increase per unit dry weight per day (g g\(^{-1}\) day\(^{-1}\)), were calculated using the classical approach described by Hunt et al. (2002) with the following equations:

\[
SLA (m^2 \text{ kg}^{-1}) = \frac{LA}{DW} \cdot \text{LDW} = \text{Leaves dry weight}
\]

RDW was measured 20 d after germination when the rosettes are expected to be with 20% of its final size (Boyce et al., 2001).

Stomatal Density and Stomatal Index

After 2 h of illumination in the night-day cycle described above, leaf impressions were taken from the abaxial surface of the ninth leaf totally expanded with dental resin imprints (Berger and Altmann, 2000). Nail polish copies were made using a colorless glaze (Von Groll et al., 2002), and the images were taken with a digital camera (AxioCam MRC) attached to a microscope (Zeiss, model AX10). The measurements were performed on the images using the Cell \(^\text{B} \) software (Soft Imaging System). Stomatal density and stomatal index (the ratio of stomata to stoma plus other epidermal cells) were determined in at least 10 fields of 0.04 mm\(^2\) per leaf from eight different plants.

Gas Exchange and Chlorophyll Fluorescence Measurements

Gas exchange parameters were determined simultaneously with chlorophyll \( a \) fluorescence measurements using an open-flow infrared gas exchange analyzer system (LI-6400XT; LI-COR) equipped with an integrated fluorescence chamber (LI-6400-40; LI-COR). Instantaneous gas exchanges were measured after 1 h illumination during the light period under 700 \( \mu \)mol m\(^{-2}\)s\(^{-1}\) at the leaf level (saturation) of PPFD, determined by \( A/PPFD \) curves (net photosynthesis) (\( A_N \) in response to PPFD curves; Supplemental Fig. S3; Supplemental Table S1). The reference \( CO_2 \) concentration was set at 400 \( \mu \)mol CO\(_2\) \( m^2\) air. All measurements were performed using the 2 cm\(^2\) leaf chamber at 25°C, and the leaf-to-air vapor pressure deficit was kept at 1.2 to 2.0 kPa, while the amount of blue light was set to 10% PPFD to optimize stomatal aperture.

The initial fluorescence (\( F_i \)) was measured by illuminating dark-adapted leaves (1 h) with weak modulated measuring beams (0.03 \( \mu \)mol m\(^{-2}\)s\(^{-1}\)). A saturating white light pulse (8000 \( \mu \)mol m\(^{-2}\)s\(^{-1}\)) was applied for 1 s to obtain the maximum fluorescence (\( F_{m_a} \)), from which the variable-to-maximum chlorophyll fluorescence ratio was then calculated: \( F_v/F_m = (F_{m_a} - F_i)/F_{m_a} \). In light-adapted leaves, the steady-state fluorescence yield (\( F_s \)) was measured with the application of a saturating white light pulse (8000 \( \mu \)mol m\(^{-2}\)s\(^{-1}\)) to achieve the light-adapted maximum fluorescence (\( F_{m_s} \)). A far-red illumination (2 \( \mu \)mol m\(^{-2}\)s\(^{-1}\)) was applied after turn off the actinic light to measure the light-adapted initial fluorescence (\( F_{i_s} \)). The capture efficiency of excitation energy by open PSII reaction centers (\( F_{i_s}/F_{m_s} \)) was estimated according Logan et al. (2007) and the actual PSII photochemical efficiency (\( \phi_{PSII} \)) was estimated as \( \phi_{PSII} = (F_{m_s} - F_{i_s})/F_{m_s} \) (Genty et al., 1989).

As the \( \phi_{PSII} \) represents the number of electrons transferred per photon absorbed in the PSII, the electron transport rate (\( I_{b_e} \)) was calculated as \( I_{b_e} = \phi_{PSII} \cdot \alpha \cdot \beta \cdot PPFD \), where \( \alpha \) is leaf absorbance and \( \beta \) reflects the partitioning of absorbed quanta between PSII and PSI, and the product \( \alpha \beta \) was adopted as described in the literature for Arabidopsis as equal to 0.451 (Flexas et al., 2007).

Dark respiration (\( K_{d} \)) was measured using the same gas exchange system as described above after at least 1 h during the dark period and it was divided by two (\( R/P_{2} \)) to estimate the mitochondrial respiration rate in the light (\( K_{c} \);Niinemets et al., 2005, 2006; Niinemets et al., 2009).

\( A/PPFD \) curves were initiated at ambient \( CO_2 \) concentration (\( C_a \)) of 400 \( \mu \)mol mol\(^{-1}\) and \( PPFD \) of 600 \( \mu \)mol m\(^{-2}\)s\(^{-1}\). Then, the PPFD was increased to 1000 \( \mu \)mol m\(^{-2}\)s\(^{-1}\) and after decreased until 0 \( \mu \)mol m\(^{-2}\)s\(^{-1}\) (11 different PPFD steps). Simultaneously chlorophyll \( a \) fluorescence parameters were obtained (Yin et al., 2009). The responses of \( A_{N} \) to \( C_a \) (\( A/C \) curves) were performed at 700 \( \mu \)mol m\(^{-2}\)s\(^{-1}\) at 25°C under ambient \( O_2 \). Briefly, the measurements started at ambient \( CO_2 \) concentration (\( C_a \)) of 400 \( \mu \)mol mol\(^{-1}\) and once the steady state was reached, \( C_a \) was decreased stepwise to 50 \( \mu \)mol mol\(^{-1}\). Upon completion of the measurements at low \( C_a \), \( C_a \) was returned to 400 \( \mu \)mol mol\(^{-1}\) to restore the
Estimation of Mesophyll Conductance ($g_m$), Maximum Rate of Carboxylation ($V_{\text{max}}$), Maximum Rate of Carboxylation Limited by Electron Transport ($J_m$), and Photosynthetic Limitations

The concentration of CO$_2$ in the carboxylation sites ($C_c$) was calculated following Harley et al. (1992) as:

$$C_c = \left(1 \left\{I^* \left(I_{lh} + 8(A_N + R_t)\right)\right\}/I_{lh} - 4(A_N + R_t)\right)$$

where the conservative value of $I^*$ for Arabidopsis was taken from Walker et al. (2013). Then, $g_m$ was estimated as the slope of the $A_N$ versus $C_c$ - $C_c$ relationship as:

$$g_m = A_N/(C_c - C_c)$$

Thus, estimated $g_m$ is an averaged value over the points used in the relationship ($C_c < 300$ mol mol$^{-1}$).

Given that current methods for estimating $g_m$ include several assumptions as well as critical limitations and sources of error that need to be considered to obtain reliable values (Pons et al., 2009), $g_m$ was estimated by the Ethier and Livingston (2004) method, which fits $A_N/C_c$ curves with a nonrectangular hyperbola version Farquhar-von Caemmerer-Berry FvCB model, based on the hypothesis that $g_m$ reduces the curvature of the Rubisco-limited portion of an $A_N/C_c$ curve.

From $A_N/C_c$ and $A_N/C_c$ curves, the maximum carboxylation velocity ($V_{\text{max}}$) and the maximum capacity for electron transport rate ($J_m$) were calculated by fitting the mechanistic model of CO$_2$ assimilation (Farquhar et al., 1980) using the $C_c$ or $C_c$-based temperature dependence of kinetic parameters of Rubisco ($K_c$ and $K_c$; Walker et al., 2013). Then $V_{\text{max}}$, $J_m$, and $g_m$ were normalized to 25°C using the temperature response equations from (Starkey et al., 2007).

The photosynthetic limitations estimated based on the approach described by Grassi and Magnani (2005). This method uses the values of $A_N$, $L_e$, $V_{\text{max}}$, $I^*$, $C_c$, and $K_c = K_c (1 + O/K_c)$ and permits the partitioning into the functional components of photosynthetic constraints related to stomatal ($I_l$), mesophyll ($I_m$), and biochemical ($I_b$) limitations:

$$I_l = \frac{\left(\frac{C_c}{\Delta l} \times \frac{\Delta l}{\gamma_M + \Delta l} \right)}{\Delta l + \frac{\Delta l}{\gamma_M + \Delta l}}$$

$$I_m = \frac{\left(\frac{\Delta l}{\gamma_M + \Delta l} \times \frac{\Delta l}{\gamma_M + \Delta l} \right)}{\Delta l + \frac{\Delta l}{\gamma_M + \Delta l}}$$

$$I_b = \frac{\left(\frac{C_c}{\Delta l} \times \frac{\Delta l}{\gamma_M + \Delta l} \right)}{\Delta l + \frac{\Delta l}{\gamma_M + \Delta l}}$$

$\gamma_M$ is the total conductance to CO$_2$ from ambient air to chloroplasts ($\gamma_M = 1/[(1/g_c) + (1/g_m)]$). The fraction $\Delta A_N/\Delta C_c$ was calculated as:

$$\frac{\Delta A_N}{\Delta C_c} = \frac{\left(V_{\text{max}} \left(I^* - K_c\right)\right)}{(C_c + K_c)}$$

Stomatal Opening and Closing Kinetics Measurements

The $g_c$ values were recorded at intervals of 1 min using the same gas-exchange system described above. The $g_c$ responses to dark/light/dark transitions were measured in plants adapted to dark, at least for 2 h. The light in the chamber was kept turned off, and then turned on and then off for 10/60/60 min.

The CO$_2$ concentration in the chamber was 400 mol mol$^{-1}$ air. For responses to CO$_2$ concentration, transitions leaves were exposed to 400/800/400 mol CO$_2$ mol$^{-2}$ air for 10/60/40 min under PPDF of 150 mol m$^{-2}$ s$^{-1}$.

Water Loss Measurements

For water loss measurements, the weight of detached rosettes, incubated abaxial side up under the same growth conditions described above, were determined over 4 h at the indicated time points. Water loss was calculated as a percentage of the initial fresh weight (Araújo et al., 2011b).

Determination of Metabolic Levels

Whole rosettes were harvested in different times along of the light/dark cycle (0, 4, 8, 16, and 24 h). Rosettes were flash-frozen in liquid nitrogen and stored at $-80$°C until further analyses. Metabolic extraction was performed by rapid grinding in liquid nitrogen and immediate addition of the appropriate extraction buffer. The levels of starch, Suc, Fru, and Glc in the leaf tissues were determined exactly as described previously (Fernie et al., 2001). Malate and fumarate were determined exactly as detailed by Nunes-Nesi et al. (2007). Proteins and amino acids were determined as described previously (Gibon et al., 2004b). The levels of other metabolites were quantified by GC-MS as described by Roesner et al. (2001), whereas photosynthetic pigments were determined exactly as described before (Porra et al., 1989).

Analyses of Enzymatic Activities

The enzymatic extract was prepared as previously described (Gibon et al., 2004a). Rubisco, PGK, transketolase, NADP-GAPDH, NAD-MDH, NADP-MDH, and SuSy activities were determined as described by Sulpice et al. (2007), Burrell et al. (1994), Gibon et al. (2004a), Leegood and Walker (1980), Jenner et al. (2001), Scheibe and Stitt (1988), and Zrenner et al. (1995), respectively.

Isolation of Guard-Cell-Enriched Epidermal Fragments

The isolation of guard-cell-enriched epidermal fragments was performed as described previously (Fandey et al., 2002) with minor adaptations. Fully expanded leaves from four rosettes per sample were blended for 1 min and then for 30 to 1 min (twice for 30 s) using a warring blinder (Phillips, RI 2044) with an internal filter to clarify the epidermal fragments of mesophyll and fibrous cells. Subsequently, epidermal fragments were collected on a nylon membrane (200 μm mesh) and washed to avoid apoplasm contamination before being frozen in liquid nitrogen. This protocol resulted in a guard cell purity of approximately 98% as assessed by Antunes et al. (2012).

qRT-PCR

qRT-PCR analysis was performed with total RNA isolated from epidermal fragments using the TRIzol reagent (Ambion, Life Technology) following the manufacturer’s manual. The integrity of the RNA was checked on 1% (w/v) agarose gels, and the concentration was measured before and after DNase I digestion using a spectrophotometer. Digestion with DNase I (Amplification Grade DNase I, Invitrogen) was performed according to the manufacturer’s instructions. Subsequently, total RNA was reverse-transcribed into cDNA using Universal RiboClone cDNA Synthesis System (Promega) according to the respective manufacturer’s protocols. For analysis of gene expression, the Power SYBR Green PCR Master Mix was used with the MicroAmp Optical 96-well Reaction Plate (both from Applied Biosystems) and MicroAmp Optical Adhesive Film (Applied Biosystems). The obtained cycle number at threshold was adjusted, and the estimation of the amplification efficiency was calculated using the Real-Time PCR Miner tool (Zhao and Fernald, 2005). The relative expression levels were normalized using the constitutively expressed genes F-BOX and TIP41-LIKE (Czechowski et al., 2005) and calculated using the ΔΔCT method. The primers used for qRT-PCR were designed using the QuantPrime software (Arvidsson et al., 2008) or taken from those described by Laaenem et al. (2013).

Detailed primer information is described in Supplemental Table S2. The following genes were analyzed: ALUMINUM ACTIVATED MALATE TRANSPORTER6 and -9, ALMT6 and ALMT9; ARABIDOPSIS THALIANA ATP-BINDING CASSETTE B14, AABCB14 (Lee et al., 2008); SLACT; H’-ATPASE1 and -5, AHA1 and AHA5 (Ueno et al., 2005); POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA1, KAT1 (Nakamura et al., 1995) and KAT2
Experimental Design and Statistical Analysis

The data were obtained from the experiments using a completely randomized design using all four genotypes (two WT-like genotypes × two T-DNA mutant lines atqat1). Data are expressed as the mean ± se. Data were submitted to ANOVA and tested for significance (p < 0.05) differences using Student’s t tests. All the statistical analyses were performed using the algorithm embedded into Excel (Microsoft).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. AtQUAC1 gene structure and semiquantitative RT-PCR.

Supplemental Figure S2. Stomatal aperture and closure kinetics in response to light and CO2.

Supplemental Figure S3. Net photosynthesis curves in response to PPFD.

Supplemental Figure S4. Total chlorophyll content and chlorophyll a/b ratio.

Supplemental Figure S5. Nitrate, free amino acids, and soluble protein content.

Supplemental Figure S6. Organic acid content.

Supplemental Table S1. Photosynthetic parameters obtained from light-response curves.

Supplemental Table S2. Primers utilized for qRT-PCR.

Supplemental Table S3. Relative metabolite levels determined by GC-MS.

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LITERATURE CITED


Medeiros et al.


Hedrich R, Marten I, Martin I (2012) Malate-induced feedback regulation of plasma membrane anion channels could provide a CO2 sensor to guard cells. EMBO J 12: 897–901


Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification
of the concentration of chlorophyll standards by atomic absorption spectroscopy. BBA - Bioenergetics 975: 384–394


