Regulation of gene expression by photosynthetic signals triggered through modified CO₂ availability

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Abstract

Background: To coordinate metabolite fluxes and energy availability, plants adjust metabolism and gene expression to environmental changes through employment of interacting signalling pathways.

Results: Comparing the response of Arabidopsis wild-type plants with that of the mutants adg¹, pgr¹ and vtc¹ upon altered CO₂-availability, the regulatory role of the cellular energy status, photosynthetic electron transport, the redox state and concentration of ascorbate and glutathione and the assimilatory force was analyzed in relation to the transcript abundance of stress-responsive nuclear encoded genes and psaA and psbA encoding the reaction centre proteins of photosystem I and II, respectively. Transcript abundance of Bap¹, Stp¹, psaA and psbB was coupled with seven metabolic parameters. Especially for psaA and psbB, the complex analysis demonstrated that the assumed PQ-dependent redox control is subordinate to signals linked to the relative availability of 3-PGA and DHAP, which define the assimilatory force. For the transcripts of sAPx and Csd2 high correlations with the calculated redox state of NADPH were observed in pgr¹, but not in wild-type, suggesting that in wild-type plants signals depending on thylakoid acidification overlay a predominant redox-signal. Strongest correlation with the redox state of ascorbate was observed for 2CPA, whose transcript abundance regulation however was almost insensitive to the ascorbate content demonstrating dominance of redox regulation over metabolite sensing.

Conclusion: In the mutants, signalling pathways are partially uncoupled, demonstrating dominance of metabolic control of photorespiration centre expression over sensing the redox state of the PQ-pool. The balance between the cellular redox poise and the energy signature regulates sAPx and Csd2 transcript abundance, while 2CPA expression is primarily redox-controlled.

Background
Photosynthesis provides plant cells with assimilates, reducing power and ATP. To coordinate their supply and demand, plants respond to environmental changes on
stimulate ROS formation [8-10]. PQH2 and ROS are redox electron transport, like plastoquinone pool (PQ), and tophosphorylation reduce the carriers of intersystem separation of a high trans-thylakoid [22]. In anthin de-epoxidation [21] and cyclic electron transport and PsbS protonation [20], and may be limited in violaxanthin cycle, which can support biosynthesis of the plant carbohydrate metabolism. In contrast to pgr1, in adg1 photosynthetic electron transport is affected only indirectly by carbohydrate-induced feedback inhibition [23]. The third selected mutant, vtc1, carries a point mutation in GDP-mannose pyrophosphorylase [17], which catalyzes precursor formation in the biosynthesis of the low-molecular weight antioxidant ascorbate. vtc1 was included in this analysis to investigate the importance of ascorbate availability and ascorbate-related redox processes on nuclear and plastid gene expression.

Arabidopsis wt and the mutants were compared under conditions of limiting, ambient and saturating CO2 concentrations. Below the CO2 compensation point, Rubisco preferentially catalyzes oxygenation of ribulose-1,5-bisphosphate and activates photorespiration, which triggers an extra demand for ATP. Per reaction cycle, chloroplastic NADPH consumption is low under photosynthetic electron transport is affected only indirectly by carbohydrate-induced feedback inhibition [23].

The hypothesis of the work was that the combination of working with defined mutants and altering CO2 availability for modulation of photosynthesis allows addressing the question of how selected nuclear and plastidic genes are regulated in Arabidopsis in response to redox, metabolic and energy signals.

Results

The work aimed at differentiating signals involved in the control of gene expression. To this end Arabidopsis wt and mutants defective in chloroplast starch biosynthesis (adg1; [16]), thylakoid acidification (pgr1; [15]) and the biosynthesis of the major low-molecular-weight antioxidant ascorbate (vtc1; [17]) were compared in relation to their metabolite patterns, photosynthetic performance and transcript amount regulation. Following growth at ambient conditions, the CO2 availability was decreased for 6 h to levels below the CO2 compensation point or increased to saturation of RuBisCO to establish contrast-
ing conditions for photosynthesis with high and low acceptor availability and concomitant high and low reduction pressure.

**Metabolic regulation**

**The energy status**

In *wt* during the 6 h fumigation period the ADP content increased 1.5-2-fold irrespective of the treatment (Table 1). In parallel, the ATP accumulated only in 0 ppm CO$_2$, but decreased in 350 and 2000 ppm CO$_2$. The ATP/ADP ratio was nearly unchanged around 6 in 0 ppm, but decreased to 2.2 and 1.8, respectively, in 350 and 2000 ppm CO$_2$ (Table 1). Mutant metabolism resulted in specific modifications of the *wt*-pattern:

In *adgl*, ATP and ADP accumulated 2.2- and 5.5-fold in 0 ppm CO$_2$ and 1.3- and 1.9-fold in 350 ppm CO$_2$, indicating an increase in the total adenylate concentration, but a decrease in the ATP/ADP ratio. In 2000 ppm CO$_2$, the ATP content increased insignificantly, while the ADP content decreased resulting in an ATP/ADP ratio similar to the initial values obtained before the treatment (Table 1).

The ATP content of *vtcl* slightly but steadily decreased with increasing CO$_2$ availability, while the ADP content increased from the lowest levels observed prior to the fumigation experiment 6.2-fold in 0 ppm CO$_2$, 2.8-fold in 350 ppm CO$_2$ and 3.3-fold in 2000 ppm CO$_2$ (Table 1). The high ATP and especially the low ADP contents after 6

### Table 1: Energetisation and phosphorylation state under influence of different acceptor availabilities: ATP content, ADP content, 3-PGA content and DHAP content in *wt, adgl, pgr1* and *vtcl* under different CO$_2$ regimes (0 ppm, 350 ppm and 2000 ppm CO$_2$).

|       | Start 0 ppm CO$_2$ | 350 ppm CO$_2$ | 2000 ppm CO$_2$ |
|-------|--------------------|----------------|-----------------|
| **wt** |                   |                |                 |
| ATP   | 97.6 ± 15.6        | 127.9 ± 31.9 a | 79.1 ± 14.7     |
| ADP   | 17.5 ± 2.8         | 29.7 ± 4.5 b   | 36.2 ± 10.4 b   |
| 3PGA  | 294.0 ± 149.2 abc  | 117.0 ± 73.3 c | 404.1 ± 44.4 a  |
| DHAP  | 100.0 ± 10.4 abc   | 71.7 ± 40.2 c  | 36.1 ± 15.3 b   |
| ATP/ADP | 5.6                | 5.8            | 2.2             |
| [NADPH/NADP$^+$]$_{calc}$ | 23.0                | 34.0            | 16.7            |
| Assimilatory Force | 333.0                | 600.4           | 87.6            |
| **adgl** |                   |                |                 |
| ATP   | 68.2 ± 8.7 e       | 149.6 ± 25.6 abc | 88.8 ± 5.1 c     |
| ADP   | 20.6 ± 10.1 bc     | 113.6 ± 25.0 a  | 39.7 ± 24.5 bc  |
| 3PGA  | 341.2 ± 123.3 b ab | 113.7 ± 37.5 c  | 487.6 ± 81.3 a  |
| DHAP  | 46.0 ± 15.0 b      | 54.0 ± 38.8 c   | 45.3 ± 24.2 ab  |
| ATP/ADP | 3.3                | 1.3             | 2.2             |
| [NADPH/NADP$^+$]$_{calc}$ | 16.6                | 63.9            | 16.9            |
| Assimilatory Force | 132.1                | 456.7           | 91.0            |
| **pgr1** |                   |                |                 |
| ATP   | 71.1 ± 12.4 ef     | 57.5 ± 9.3 ef   | 84.6 ± 4.4 d    |
| ADP   | 24.5 ± 10.3 bc     | 29.5 ± 7.4 b    | 44.3 ± 12.7 b   |
| 3PGA  | 511.9 ± 142.0 ab   | 137.2 ± 49.2 c  | 522.5 ± 14.3 a  |
| DHAP  | 90.0 ± 13.0 a      | 43.5 ± 27.3 c   | 46.1 ± 11.8 a   |
| ATP/ADP | 2.9                | 1.9             | 1.9             |
| [NADPH/NADP$^+$]$_{calc}$ | 22.9                | 44.4            | 18.4            |
| Assimilatory Force | 172.3                | 311.0           | 86.5            |
| **vtcl** |                   |                |                 |
| ATP   | 116.0 ± 21.6 bc    | 101.6 ± 38.0 bcdg | 96.8 ± 9.8 cdg |
| ADP   | 11.9 ± 4.5 bc      | 74.8 ± 10.3 a   | 33.1 ± 14.1 b   |
| 3PGA  | 355.1 ± 35.9 ab    | 144.3 ± 96.5 c  | 392.2 ± 130.9 b |
| DHAP  | 40.0 ± 15.0 ab     | 56.4 ± 23.2 c   | 50.9 ± 24.3 b   |
| ATP/ADP | 9.8                | 1.4             | 2.9             |
| [NADPH/NADP$^+$]$_{calc}$ | 5.3                | 58.5            | 17.9            |
| Assimilatory Force | 110.4                | 383.4           | 127.3           |

Different letters indicate significant differences. The data are means of n = 4–6, ± SE from at least 4 different experiments. ATP/ADP ratio, [NADPH]/[NADP$^+$]$_{calc}$ and assimilatory force $F_A$ (according to Dietz & Heber 1989). These derived parameters were calculated from the primary data above.
h resulted in a strong decrease in the ATP/ADP-ratio from 9.8 to around 2 (Table 1).

pgr1 is limited in thylakoid acidification [18]. Like in vtc1, the ATP/ADP ratio decreased to values around two during the experiment independent of the CO2 concentration applied (Table 1). However, the relative decrease was much less in pgr1 due to an already much lower initial ATP/ADP ratio (vtc1: 9.8; pgr1: 2.9). In addition to the generally low ATP/ADP ratio, the total adenylate concentration was also low in pgr1 suggesting coupling between adenylate biosynthesis and ADP-phosphorylation efficiency. The ADP contents were similar to that in wt and did not increase in 0 ppm like in vtc1 and adg1 (Table 1), while the ATP content slightly decreased demonstrating a mutation-specific difference in regulation of the adenylate concentration in parallel to the ATP/ADP ratio (Table 1).

**Assimilatory force and the reduction state of the NADP system**

The assimilatory force \( F_A \), i.e. the product of the phosphorylation potential \([ATP]/[ADP][P_i]\) and the ratio of \([NADPH]/[NADP^+]\), was calculated from the DHAP/3-PGA ratio (Table 1) as introduced in [24]. \( F_A \) indicates the energization of metabolism by photosynthetic light reactions as compared to the consumptive demand. The relationship was originally defined for chloroplasts. Due to energy and metabolite coupling it also tentatively describes the energy status of leaves [25]. \( F_A \) was increased in wt and all mutants during 6 h in CO2-free air compared to the values at onset of fumigation (wt: 180%; adg1: 352%; pgr1 180%; vtc1: 347%) (Table 1) indicating a high reduction state of the NADP-system (wt: 148%; adg1: 385%; pgr1 194%; vtc1: 326% increase compared to the onset of illumination) (Table 1). At high CO2, the \([NADPH]/[NADP^+]_\text{calc} \) ratios of the mutants were little increased in wt (175%), adg1 (155%) and pgr1 (123%), but elevated in vtc1 (366%) compared to the values at onset of fumigation (Table 1). The calculated reduction states of NADP at high CO2 were increased in wt (241%), adg1 (152%) and pgr1 (153%), but hardly changed in vtc1 (108%) relative to ambient conditions (Table 1).

The strong decline in the 3-PGA content in CO2-free air indicated an increased assimilatory force in low CO2 (wt: 685%; adg1: 511%; pgr1: 301%; vtc1: 326% relative to ambient air). 3-PGA contents slightly increased in 350 ppm CO2 during the 6 h of treatment, remained unchanged in wt in 2000 ppm CO2 and decreased in the mutants.

**Antioxidant protection**

Antioxidant enzymes and low molecular weight redox metabolites constitute the antioxidant defence system of the plants. Modifications of antioxidant enzyme activities often reflect general changes in the redox status and in ROS generation of the tissue, or compensatory responses to specific redox changes [26].

**Ascorbate contents and redox states**

At the beginning of the fumigation period, the ascorbate content was lowest in vtc1 with 1.44 ± 0.31 μmol Asc/g FW reflecting the mutational defect in ascorbate biosynthesis [17] (Fig. 1). The highest ascorbate levels were observed in wt, and intermediate contents in adg1 and pgr1. In wt the ascorbate content increased approximately 1.3-fold in ambient air, but hardly changed in low and high CO2. In adg1, starting with a lower content than wt, ascorbate levels increased to high values at 350 ppm and were unchanged in 0 and 2000 ppm CO2. In vtc1, with its low ascorbate contents due to the mutation in one of the main ascorbate biosynthetic enzymes, ascorbate increased 1.3-fold in CO2-free air and 2.1- and 1.8-fold in 350 and 2000 ppm CO2. It maximally reached 50% of the wt level in 350 ppm CO2. In pgr1, the ascorbate content increased to 4.5 ± 0.75 μmol Asc/g FW in 0 ppm and to similar levels around 6 μmol Asc/g FW in 350 ppm and 2000 ppm CO2. The CO2 availability affected the redox state of ascorbate to a minor extent. From an almost fully reduced level at the beginning of the fumigation period, the mean values after 6 h fumigation indicate slightly higher oxidation levels (72 – 91%) in 0 ppm and 350 ppm CO2 than in 2000 ppm CO2 (Figure 1).

**Glutathione contents and redox states**

Compensating the low ascorbate content, the glutathione content was highest in vtc1 at the beginning of the fumigation period. Lowest glutathione contents were observed in adg1, while the glutathione content was similar to wt in pgr1. During the 6 h fumigation the glutathione content increased in all samples to similar levels and redox states (Figure 1). Although not significantly different, the mean values indicate a trend towards slightly higher reduction at the end of the fumigation period (Figure 1).

**Activities of antioxidant enzymes**

Compared to wt, the three mutants revealed increased ascorbate peroxidase activities in high and low CO2. APx activity of adg1 was twice that observed in wt at ambient CO2 concentrations (Figure 1). In parallel, the SOD activity was 1.5-fold induced in adg1. Like for APx the CO2 availability scarcely affected SOD activity of vtc1. SOD and APx activities were only slightly higher in pgr1 than in wt, while that mutation increased glutathione reductase activity in 0 and 350 ppm CO2 (Figure 1). Surprisingly, SOD and GR activities were lower in 0 than 350 ppm CO2 (Figure 1).
Contents of soluble and hydrolysable sugars

In wt, pg1 and vt1 the CO2 availability barely changed the availability of soluble sugars, while in adg1, due to the limitation in chloroplast starch biosynthesis, the sugar concentration was higher in 350 ppm CO2 than in wt. In parallel, hydrolysable sugars were generally very low in adg1 and did not increase in high CO2. pg1 and vt1 had similar and slightly higher soluble sugar levels than wt at all CO2 concentrations applied. In contrast, the concentration of hydrolysable sugars was less in vt1 compared to pg1, which may reflect the effect of limited GDP-mannose pyrophosphorylase activity on cell wall biosynthesis [27]. Generally, in wt, vt1 and pg1 the sugar levels were only elevated at ambient CO2, but not in saturating CO2 demonstrating that in high CO2, in which Calvin-cycle activity should be stimulated, carbon assimilation was limited or carbohydrate consumption activated leading to similar carbohydrate pool sizes as in 0 ppm CO2 (Figure 2A and 2B).

ABA levels in ambient air

Consistent with previous observations [14], the ABA content was increased 2.1-fold in vt1 compared to wt. adg1 and pg1 showed insignificant increases in the range of 1.2- to 1.3-fold, respectively (Figure 2C).

Figure 1
Redox stabilization and antioxidant defense in response to differing CO2 availability: Content of total antioxidant amount and reduction status (ascorbate and glutathione) and activities of selected antioxidative enzymes: total soluble ascorbate peroxidases (APX), total soluble superoxide dismutases (SOD), glutathione reductase (GR) and catalases (CAT) in wt, adg1, pg1 and vt1 under different CO2 regimes (0 ppm, 350 ppm and 2000 ppm CO2) measured in aqueous extracts. The data are means of n = 4–6, ± SE from at least 4 different experiments.
Figure 2  
Content of soluble (A) and hydrolyzable sugars (B) in wt, adg1, pgr1 and vtc1 under different CO2 regimes (0 ppm, 350 ppm and 2000 ppm CO2). The data are means of n = 4–6, ± SE from at least 4 different experiments. Abscisic acid (ABA) content (C) in wt, adg1, pgr1 and vtc1 after fumigation with 350 ppm CO2. The data are means of n = 3 ± SE.
Photosynthetic performance of wild-type and mutants

To test wt and mutants for limitations in photosynthetic electron transport, in the final 2 hours of the fumigation period the photosynthetic response of the plants to a 3.6-fold increase in the light intensity from 80 to 285 μmol quanta m⁻² s⁻¹ was tested by monitoring chlorophyll-a-fluorescence parameters (Table 2). In ambient air, the quantum yield of photosystem II (ΦPSII, (Fm'−F0)/Fm') was similar at standard light conditions in wt, adg1 and vtc1 and slightly higher in pgr1. In response to increased light, it decreased most in pgr1 with a steady-state level of 0.31. In vtc1, the steady-state ΦPSII was highest, while in wt and adg1 it was slightly lower.

In 0 ppm CO₂, ΦPSII strongly decreased in all plants prior to the increase in light intensity (Table 2). In response to the 3-fold increase in light intensity ΦPSII further decreased to levels between 0.032 (adg1) and 0.093 (wt) indicating severe reduction of photosynthetic electron transport efficiency.

At 2000 ppm CO₂ (Table 2), ΦPSII of all lines was highest at standard light conditions (F₀/Fm between 0.663 and 0.734) and dropped in response to increased light to levels around 0.5 in wt, adg1 and vtc1 and as low as 0.35 in pgr1.

Transcript level regulation in response to high and low CO₂

Semi-quantitative RT-PCR analyses at least in triplicates were performed to assess the transcript regulation of selected genes in leaves 6 h after fumigation beginning with the respective CO₂ concentration (Fig. 3). Compared to wt, in 350 ppm CO₂ the transcript levels of the plastidic genes psaA and psbA, which encode core subunits of photosystem reaction center I and II, respectively, and that of the nuclear encoded small subunit of RuBisCO were not significantly changed in the mutants adg1, pgr1 and vtc1, reflecting acclimation. The transcript level for ferritin-1 which has been suggested as marker gene for hydrogen peroxide [28] decreased in all mutants. In contrast, transcripts for Bap1, which is a marker gene for singlet oxygen signalling [28], were specifically increased in the ascorbate deficient mutant vtc1.

The transcript levels of the three tested nuclear encoded chloroplast antioxidant enzymes Csd2, sAPx and 2CPA showed mutant specific patterns. Csd2 and sAPx transcript levels were wt-like in vtc1, increased in adg1 and decreased in pgr1, while the 2CPA transcript levels were specifically decreased in adg1. Stp1 transcript amounts, which are suppressed by the cellular carbohydrate availability [29], were doubled in pgr1 and increased 3.5-fold in vtc1, but were unchanged in adg1.

In adg1 and pgr1, like in wt, the transcript levels of the two plastome encoded genes psaA and psbA and the nuclear encoded RbcS, Stp1, Bap1 and Ferritin-1 increased in response to 0 and 2000 ppm. Fig. 3 gives the transcript modifications in response to depleting or saturating CO₂ of wt and mutants each normalized to the respective level in 350 ppm. In vtc1, the response of Bap1, which was already strongly increased at 350 ppm, was weakest. In 2000 ppm CO₂ treated vtc1 psbA transcripts were hardly increased and psaA transcripts were even slightly decreased indicating a specific response pattern of vtc1 to high CO₂.

Again the three transcripts for nuclear encoded chloroplast antioxidant enzymes Csd2, sAPx and 2CPA showed specific response patterns. Csd2 hardly responded to changes in the CO₂-availability. Only in pgr1, where the transcript levels were decreased at 350 ppm CO₂, an increase in 0 and 2000 ppm CO₂ was observed. sAPx transcripts, which were decreased in wt Arabidopsis in response to increased and also decreased CO₂-availability, strongly increased in adg1 treated with 2000 ppm CO₂ and increased in pgr1 at 0 ppm CO₂. In vtc1, the transcript level responded wt-like in 0 ppm CO₂ and atypically increased in 2000 ppm CO₂. 2CPA transcripts showed the previously reported CO₂ dependency [30]. The regulation amplitude increased in adg1. The high CO₂-response was

| Table 2: Effective quantum yield of PSII at different CO₂ regimes (0 ppm, 350 ppm and 2000 ppm CO₂) in response to an increase in the light intensity from 80 to 285 μmol quanta m⁻² s⁻¹. |
| --- |
| **CO₂** | **wt** | **adg1** | **pgr1** | **vtc1** |
| 0 ppm CO₂ | | | | |
| 0 min | 0.318 ± 0.071 | 0.167 ± 0.050 | 0.192 ± 0.090 | 0.278 ± 0.063 |
| 4.5 min | 0.093 ± 0.040 | 0.032 ± 0.008 | 0.071 ± 0.043 | 0.051 ± 0.039 |
| 350 ppm CO₂ | | | | |
| 0 min | 0.580 ± 0.039 | 0.596 ± 0.029 | 0.661 ± 0.033 | 0.599 ± 0.041 |
| 4.5 min | 0.388 ± 0.036 | 0.395 ± 0.029 | 0.314 ± 0.037 | 0.437 ± 0.032 |
| 2000 ppm CO₂ | | | | |
| 0 min | 0.663 ± 0.025 | 0.704 ± 0.022 | 0.734 ± 0.012 | 0.710 ± 0.019 |
| 4.5 min | 0.518 ± 0.033 | 0.482 ± 0.046 | 0.345 ± 0.026 | 0.519 ± 0.037 |

The data are means of 6 measurements.
abolished in pgr1 and reversed to an increased transcript level in 2000 ppm CO2 in vtc1.

**Discussion**

Photosynthetic activity and metabolism depend on the stoichiometrical assembly and regulated interaction of nuclear and chloroplast encoded proteins [31]. In addition, changing environmental conditions are continuously sensed and used to adjust the photosynthetic apparatus for balanced supply of energy, reductive power and assimilates. The basic mechanism of regulation involves coordination of gene expression. Although various studies on this topic have identified candidate signals, the complexity of interaction and the multiplicity of signals are far from being understood. Here, a set of biochemical and physiological data and the transcripts were analyzed upon variation of the CO2-availability in order to tentatively identify potential signalling dependencies (Table 3). Approaching CO2 saturation releases the electron pressure within the photosynthetic electron transport chain, increases the acceptor availability at PSI and decreases photorespiration intensity. High quantum yields of PSII (Table 2) indicated efficient electron consumption. Despite metabolic activation, the cellular reduction state of NADP(H) increased in wt under saturating CO2 (Table 1). In parallel, the ATP availability decreased (Table 1) [6].

**Regulation of ROS-responsive genes**

Six hours of illumination in low, ambient or high CO2 elicited significant changes in transcript abundance. The increase of ferritin-1 and Bap1 transcript amounts in 0 and 2000 ppm CO2 indicates redox imbalances and stimulation of ROS signalling in high as well as low CO2 [28]. This is surprising since relaxation of electron pressure should be maximal at saturating CO2 with concomitantly low rates of ROS generation. However, a high activation state of the Calvin cycle needed for efficient carbon fixation at saturating CO2 depends on a highly reduced thioredoxin system that in turn activates the redox regulated enzymes [32]. The increase of ferritin-1 and Bap-1 transcript levels suggests that regulated electron drainage maintains sufficient electron pressure and is involved in the up-regulation of the ROS-related marker genes.

The CO2 dependent response was altered in the mutant genetic backgrounds. In adg1, the Bap1 transcript levels were less induced in 0 ppm and more in 2000 ppm (Figure 3) demonstrating that limitations in chloroplast carbohydrate storage affect the responsiveness of singlet oxygen-signalling. It is tempting to assume that in adg1 increased APx and SOD activities (Figure 1) antagonized Bap1 induction in low CO2 (Figure 3) due to higher antioxidant protection, while the high transcript accumulation in 2000 ppm CO2 results from carbohydrate
inhibition of photosynthetic electron transport due to insufficient capacities for starch biosynthesis. Transcript
amount co-regulation analysis shows among all genes tested the strongest correlation of Bap1 with the ascorbate
content (K = −0.74; Table 3). Consistent with the hypo-
thesis by op den Camp et al. [28] that Bap1 is regulated by
specific regulation of the glutathione pool and,
although to different extends, negatively correlated with
the high correlation with glutathione data results from the
adg1-specific regulation of the glutathione pool and,
therefore, may also result from disturbed chloroplast ca-
bohydrate metabolism.

Ascorbate-dependent regulation
In a genome-wide transcript analysis of vtc1 plants, Pas-
tori et al. [14] identified 171 genes with altered expres-
sion, among which defence genes constituted a significant
subgroup. Here, in the steady state, in 350 ppm CO2, to
which the plants were adapted to, the transcript levels of
2CPA, Csd2 and sAPx were balanced. The transcript level
of ferritin-1 was even decreased. The transcript level of
Bap-1 was 3.73-fold increased and constitutively high
upon variation of the CO2-availability, demonstrating
that the transcript abundance is dominantly regulated by
the mutational defect in ascorbate biosynthesis.

The table shows the correlation coefficient K calculated between metabolite, enzyme activities and every single transcript detected by semi-
quantitative RT-PCR. Bold printed numbers with * stand for 0.3<|K|<0.6 and bold printed numbers with ** for strong negative and positive
correlations |K|>0.6.
analysis the correlation between ascorbate and defence gene expression was stronger when the data set of the \textit{vtc1}-mutant was not included (K = -0.67) demonstrating that in \textit{vtc1} hardening responses may mask signalling induced by the variation of CO$_2$-availability.

The strongest relationship between the reduction state of ascorbate and transcript level regulation was observed for 2CPA. In previous experiments strong suppression of 2CPA transcription was observed upon ascorbate application \cite{29,34,35}. Here, responses upon internal variation of the ascorbate content were analyzed. A strong negative correlation (-86\%; Table 3) excludes sensing the ascorbate content, but absence of correlation with the ascorbate content (K = 0.23; Table 3) excludes sensing the ascorbate availability. It is postulated that either specifically dehydroascorbate or, more likely, the electron consumption in dehydroascorbate reduction regulates 2CPA transcript abundance.

**Coupling of transcript abundance regulation**

A set of four genes, i.e. Bap1, psaA, psbA and Stp1, showed a similar transcript pattern in response to the seven metabolic parameters, i.e. glutathione contents and reduction state, 3-PGA and DHAP contents, the calculated NADPH reduction state and assimilatory force, and the hydrolysable sugars (Table 3). It should be noted that transcript levels of psaA and psbA changed in parallel in response to the imposed metabolic and mutational strains. This contrasts the anti-parallel responses of psaAB and psbB observed upon transfer to photosystem I and II-specific light regime previously described by Pfannschmidt et al. \cite{11}. It is concluded that a variation between 0 and 2000 ppm CO$_2$ in the mutant background elicits more severe changes in metabolism and signalling than altering the light quality from red to far red at very low photon flux density \cite{11}. However, the photosystem I transcript psaA decreased relative to the photosystem II transcript psbA when the electron pressure was reduced by increasing the CO$_2$-availability (Figure 3), e.g. from 1.07 (0 ppm) to 1 (350 ppm) to 0.88 (2000 ppm) in \textit{wt} indicating a stronger transcription of genes for the PS-I reaction centre protein in 0 ppm CO$_2$ and those encoding PS-II reaction centre proteins in 2000 ppm CO$_2$. In \textit{adg1}, the gradual response was unaffected, however generally the transcript abundance of psbA was higher than that of psaA (Figure 3). In \textit{pgr1} the relative transcript abundance normalized to \textit{wt} at 350 ppm was also higher under all three CO$_2$ conditions, suggesting that the higher psbA transcript levels were caused by photoinhibitory high carbohydrate availability or a limitation in the Rieske activity.

It is tempting to suggest that the signal is transmitted by PQ-dependent redox signals. However, in \textit{vtc1} more \textit{psaA} than \textit{psbA} was observed at 350 ppm CO$_2$, a balanced psaA/psbA ratio in 0 ppm CO$_2$ and an inverted ratio in 2000 ppm, demonstrating that the ascorbate availability affects the stoichiometry of transcripts for the photoreaction centres upon variation of the CO$_2$-availability. After 6 h fumigation with 350 ppm CO$_2$, the steady state quantum yield of PS-II \((F_{M’}-F_{S’})/F_{M’}\) was \textit{wt}-like (Table 2), while the quantum yield was adjusted to 1.23-fold higher levels within 4.5 min of illumination upon doubling of the light intensity (Table 2). On the background of decreased ascorbate availability (Figure 2A) this demonstrates that the photosynthetic electron transport chain was otherwise protected. Chlorophyll fluorescence analysis showed that the acclimation was insufficient to protect the photosynthetic membrane in low CO$_2$. The low quantum yield of PS-II (Table 2) demonstrates even stronger photosynthetic impairment than in \textit{wt}. That the regulation of the quantum yield of PS-II does not correlate with the psaA/psbA-ratios, supports the hypothesis that the regulation of the transcript abundance of the photoreaction centre proteins is more dependent on ascorbate-specific signals than on the redox state of the PQ-pool. The PQ-dependent long term acclimation response postulated by Pfannschmidt et al. \cite{11} appears further subordinate to signals linked to the metabolic state of the PGA and DHAP concentrations, the NADPH/NADP$^+$ ratio, the assimilatory force and hydrolysable sugars (Table 3). In \textit{adg1} a perfect positive correlation (K = 1) was observed between the concentration of soluble sugars and the psbA transcript levels, whereas the other lines showed a high negative correlation (\textit{wt}: K = -1; \textit{pgr1}: K = -0.96; \textit{vtc1}: K = -1), highlighting the importance of carbohydrate-dependent signals in psaA and psbA regulation.

**Correlation with the adenylate status**

In animals, the energy status sensed for instance via insulin-like growth factor 1 or by AMP-dependent kinases plays an important role in regulation of gene expression \cite{36}. However, here except an only weak (K = 0.41) correlation for 2CPA, no correlations between the adenylate status and transcript levels were indicated. Due to the photautotrophic nature of plants, plants rarely encountered energy deprivation. Further on, the chloroplast and cellular adenylate status directly coordinates metabolic pathways via feed-back and feed-forward mechanisms \cite{37}. The lack of strong energy-linked regulation demonstrates that the adenylate phosphorylation state may not be a major signal, which is directly linked to the regulation of nuclear gene expression in context of photosynthesis.

However, the analysis of \textit{pgr1} showed a mutant specific regulation of Csd2, sAPx and 2CPA upon altered CO$_2$-availability (Figure 3). Because \textit{pgr1} is unable to acidify the thylakoid lumen below pH 6 due to a mutation in the Rieske protein \cite{18}, the ATP/ADP ratio was very low in the
morning and generally decreased irrespective of the CO₂ concentration (Table 1). The concentrations of most metabolites were not significantly changed in response to altered CO₂ availability compared to wt indicating efficient metabolic compensation (Figure 3). The transcript levels of Csd2, sAPx and 2CPA were regulated in a mutant specific manner, while RbcS, Bap1, Fer1 and Stp1 responded wt-like demonstrating that ROS- and carbohydrate signalling pathways were unaffected [28,29,38]. Upon the different treatments of pgr1 the transcript abundance of sAPx highly correlated with the calculated reduction state of NADPH (K = 0.99), while no correlation was observed in the other plant lines. It is therefore concluded that the mutation in the Rieske protein limits the fine-control of sAPx expression and makes it more dependent on stromal redox signals. The difference in sAPx regulation between pgr1 and wt also demonstrates that in wt the Rieske protein influences sAPx expression. Excluding ROS-signalling, because the Bap1 control was wt-like demonstrating that ROS- and carbohydrate signalling pathways were unaffected [28,29,38]. Upon the different treatments of pgr1 the transcript abundance of sAPx highly correlated with the calculated reduction state of NADPH (K = 0.99), while no correlation was observed in the other plant lines. It is therefore concluded that the mutation in the Rieske protein limits the fine-control of sAPx expression and makes it more dependent on stromal redox signals. The difference in sAPx regulation between pgr1 and wt also demonstrates that in wt the Rieske protein influences sAPx expression. Excluding ROS-signalling, because the Bap1 control was wt-like, either the redox state of the PQ pool or thylakoid acidification/the adenylate status may modulate sAPx transcript abundance.

The Csd2 transcript levels positively correlated with APx activity in all lines under all treatments (Table 3). Concomitantly, in pgr1, also an almost perfect correlation was observed with the redox state of NADPH. Compared to sAPx the Csd2 transcript level was regulated with higher amplitudes. sAPx and Csd2 transcripts encode two promiscuous enzymes that the mutation in the Rieske protein limits the fine-control of sAPx expression and makes it more dependent on stromal redox signals. The difference in sAPx regulation between pgr1 and wt also demonstrates that in wt the Rieske protein influences sAPx expression. Excluding ROS-signalling, because the Bap1 control was wt-like, either the redox state of the PQ pool or thylakoid acidification/the adenylate status may modulate sAPx transcript abundance.

Conclusion
In the cell, metabolite, redox and energy signals are tightly linked, which makes differentiation of signalling cascades difficult. This study demonstrates that comparison of mutants with specific limitations in the coordination of plant acclimation at least transiently uncouples signalling branches. Comparison of adg1, pgr1 and vtc1 with Arabidopsis wild-type plants showed coordinated expression of Bap1, psaA, psaA and Stp1, which have been discussed previously to respond specifically to singlet oxygen [28], the redox state of the PQ pool [11] and monosaccharide availability [29], respectively. Like Ferritin-1, Bap1 and Stp1 correlated strongest with the ascorbate contents, while for psaA and psbA a stronger link to the assimilatory force and NADPH/NADP⁺ ratio was indicated. Correlation of 2CPA expression, whose transcription is controlled by the acceptor availability at photosystem I in wt [30], with the redox state of the ascorbate pool further strengthens the links between the antioxidant system and the photosynthetic electron transport and more specifically chloroplast-to-nucleus signalling. It is postulated that during evolution a stabilized network has evolved which links photosynthetic metabolism to nuclear gene expression. Mutants might be well balanced under standard conditions, but application of environmental changes leads to an altered acclimation in comparison to wt, which allows to tentatively differentiate parallel induced signalling cascades.

Methods
Plant material and growth conditions
Arabidopsis thaliana wt (Col-0), the mutants adg1 [16], pgr1 [15] and vtc1 [17] grew in controlled environment (10 h of light, 100 μmol quanta m⁻² s⁻¹, 23°C and 14 h darkness at 18°C; 50% relative humidity) on a 1:1:1 mixture of Frühsdorfer Erde Klocke P, perlite and vermiculite for 5 weeks. Beginning 2 h after onset of light, sets of plants (wt, adg1, pgr1 and vtc1) were fumigated for 6 h with synthetic air in 28 l Perspex chambers or 5-1 Sekuroka* glove bags with 0 and 2000 ppm CO₂, respectively, or maintained in ambient air. A Millipore Tylan RO 7030 system controlled the gas flow at 2 l/min.

Metabolite analysis
Total and reduced ascorbate was quantified spectrophotometrically according to [39] from plant material frozen in liquid nitrogen by recording the decrease of absorption at 265 nm following addition of ascorbate oxidase. Glutathione contents were determined fluorimetrically after derivatization with monobromobimane and HPLC separation on a "reverse phase" Hypersil BDS-C15 5 μm column from tissue extracted in 0.1 M HCl and 5 mM diethylenetriamine pentaaetic acid [40]. 3-PGA and DHAP contents of perchloric acid extracts were quantified according to Dietz & Heber [41], ATP and ADP with firefly enzyme according to the luminometric method described by Kaiser & Urbach [42]. Assimilatory force and NADPH-reduction state were calculated as described in Dietz & Heber [24]. Reducible hydrolysable and soluble sugars were determined according to Yemm & Willis [43] with anthrone reagent. ABA contents were quantified according to Weiler [44] from freeze-dried leaf material.

Chlorophyll-a-fluorescence measurements
Between 4–6 h after onset of the CO₂ fumigation, the response of the mutants to an increase in light intensity to 285 μmol quanta m⁻² s⁻¹ was monitored for 4 min using a Mini-PAM Fluorometer (Walz, Effeltrich, Germany). 30 s before and during the illumination with 285 μmol quanta m⁻² s⁻¹, the fluorescence parameters Fₛ and Fₘ [45] were determined every 30 s with saturating light pulses (1s; >3000 μmol quanta m⁻² s⁻¹). The quantum yield of PS-II (ΦPSII) was calculated as (Fₘ - Fₛ)/Fₘ.
**Determination of protein content and biochemical analyses of enzyme activities**

Enzyme activities were determined according to [34] and standardized on protein contents of the samples determined with the BIORAD protein assay (BioRad Laboratories, München, Germany).

**RNA isolation, cDNA synthesis and RT-PCR analysis**

Approximately 100 mg plant material pulverized in liquid nitrogen was extracted in 500 μl 100 mM Tris-HCl, pH 8.5, 25 mM EDTA, 25 mM EGTA, 100 mM β-mercaptoethanol and 2% SDS with 500 μl phenol and 300 μl chloroform. The aqueous phase was re-extracted first with 1 ml phenol/chloroform (1:1), then with 1 ml chloroform prior to precipitation of the RNA at 4°C by addition of 1 ml isopropanol. For further purification the precipitate was dissolved in 10 mM Tris, 1 mM EDTA, re-precipitated with 3 volumes 8 M LiCl and washed with 70 % ethanol. The RNA dissolved in DEPC-treated water was quantified spectrophotometrically. Residual DNA was removed by treatment with RNase-free DNase (1 U; Promega). Prior to cDNA synthesis, the DNase was inactivated by adding EDTA to a final concentration of 2.5 mM and by incubation at 70°C for 20 min. 5 μg DNase treated RNA was used for cDNA synthesis using oligo(dT) and a primer mix containing a mixture of oligonucleotides matching all plastid-encoded genes as primers [46] for 1 h at 42°C. The samples were standardized on actin-2 transcript amount. Genes of interest were analysed by semi-quantitative RT-PCR (PCR settings: 1 cycle at 94°C for 3 min, for the optimized number of cycles: 45 s 94°C, 30 s 50°C, and 15 s 72°C). Following separation of the PCR products on ethidiumbromide stained agarose gels, the bands were quantified densitometrically [34]. Each band was normalized against the intensity obtained with the same cDNA template. The RNA dissolved in DEPC-treated water was quantified spectrophotometrically. Residual DNA was removed by treatment with RNase-free DNase (1 U; Promega). Prior to cDNA synthesis, the DNase was inactivated by adding EDTA to a final concentration of 2.5 mM and by incubation at 70°C for 20 min. 5 μg DNase treated RNA was used for cDNA synthesis using oligo(dT) and a primer mix containing a mixture of oligonucleotides matching all plastid-encoded genes as primers [46] for 1 h at 42°C. The samples were standardized on actin-2 transcript amount. Genes of interest were analysed by semi-quantitative RT-PCR (PCR settings: 1 cycle at 94°C for 3 min, for the optimized number of cycles: 45 s 94°C, 30 s 50°C, and 15 s 72°C). Following separation of the PCR products on ethidiumbromide stained agarose gels, the bands were quantified densitometrically [34]. Each band was normalized against the intensity obtained with the same cDNA template.

**Statistical analysis**

From the calculated means and standard deviations the significance of differences was determined by Student’s t test. Data sets were designated significantly different, if the P value was below 0.05. Coefficiency analysis was performed by Pearson correlation analysis. Coefficients higher than r = | 0.6 | was defined as strong correlation, and between | 0.3 | and | 0.6 | as weak correlations.

**Abbreviations**

2CP: 2-cysteine peroxiredoxin; 3-PGA: 3-phosphoglycerate; APX: ascorbate peroxidase; cDNA: copy DNA; Csd: copper/zinc superoxide dismutase; DEPC: diethylpyrocarbonate; DHAP: dihydroxyacetone phosphate; Fv: assimilatory force; FW: fresh weight; P: plastoquinone; Psx: peroxiredoxin; PS: photosystem; ROS: reactive oxygen species; RT-PCR: amplification of transcripts by polymerase chain reaction after reverse transcription into cDNA; RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase; SOD: superoxide dismutase; wt: wild-type

**Authors’ contributions**

DW carried out the metabolite analyses, most of the gene expression analyses, performed the statistical analysis and contributed to writing the manuscript. MB performed preparatory work for the gene expression analysis, carried out the chlorophyll-a fluorescence analysis, was involved in designing and validation of the project and contributed to writing of the manuscript. AK was involved in transcript analysis and project development, and helped with the manuscript, RS provided technical support and discussion, WH determined the ABA-contents and K-JD was the principal investigator who coordinated the project and was involved in data interpretation and writing of the manuscript. All authors agreed on the final manuscript.

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