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Guard cell photosynthesis is critical for stomatal turgor production, yet does not directly mediate CO_2- and ABA-induced stomatal closing

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SUMMARY

Stomata mediate gas exchange between the inter-cellular spaces of leaves and the atmosphere. CO_2 levels in leaves (*Ci*) are determined by respiration, photosynthesis, stomatal conductance and atmospheric [CO_2]. [CO_2] in leaves mediates stomatal movements. The role of guard cell photosynthesis in stomatal conductance responses is a matter of debate, and genetic approaches are needed. We have generated transgenic Arabidopsis plants that are chlorophyll-deficient in guard cells only, expressing a constitutively active chlorophyllase in a guard cell specific enhancer trap line. Our data show that more than 90% of guard cells were chlorophyll-deficient. Interestingly, approximately 45% of stomata had an unusual, previously not-described, morphology of thin-shaped chlorophyll-less stomata. Nevertheless, stomatal size, stomatal index, plant morphology, and whole-leaf photosynthetic parameters (PSII, qP, qN, $F_V'/F_{M'}$) were comparable with wild-type plants. Time-resolved intact leaf gas-exchange analyses showed a reduction in stomatal conductance and CO_2-assimilation rates of the transgenic plants. Normalization of CO_2 responses showed that stomata of transgenic plants respond to [CO_2] shifts. Detailed stomatal aperture measurements of normal kidney-shaped stomata, which lack chlorophyll, showed stomatal closing responses to [CO_2] elevation and abscisic acid (ABA), while thin-shaped stomata were continuously closed. Our present findings show that stomatal movement responses to [CO_2] and ABA are functional in guard cells that lack chlorophyll. These data suggest that guard cell CO_2 and ABA signal transduction are not directly modulated by guard cell photosynthesis/electron transport. Moreover, the finding that chlorophyll-less stomata cause a 'deflated' thin-shaped phenotype, suggests that photosynthesis in guard cells is critical for energization and guard cell turgor production.

Keywords: guard cell, chlorophyll, chlorophyllase, photosynthesis, CO_2, abscisic acid, stomata, turgor, *Arabidopsis thaliana*.

INTRODUCTION

Plants assimilate CO_2 during photosynthesis while simultaneously preventing excessive loss of water. Because the plant cuticle is impermeable to water and CO_2, transpirational water loss and CO_2 influx in plants are tightly regulated by the opening and closing of stomatal pores. Stomata are formed by two specialized guard cells, which are morphologically distinct from general epidermal cells (Franks and Farquhar, 2007). Stomatal aperture is regulated by both physiological and environmental factors, in particular CO_2, abscisic acid (ABA), humidity, drought, light, pathogens, and ozone (Vavasseur and Raghavendra, 2005; Kim *et al.*, 2010). The inter-cellular CO_2 concentration (*Ci*) is an important regulator of stomatal conductance. *Ci* levels increase rapidly daily during dark periods due to respiration (Hanstein and Felle, 2002). Moreover, photosynthetically

active radiation (PAR) causes a rapid reduction in *Ci* levels, which can reach ≈150 ppm (Hanstein and Felle, 2002; Roelfsema *et al.*, 2002). In addition, atmospheric CO_2 levels have been continuously rising since the beginning of the industrial era and are predicted to double within the present century (Keeling *et al.*, 2011). This [CO_2] rise, produces an increase in the inter-cellular [CO_2] levels (*Ci*) in leaves, and results in a reduction of stomatal apertures on a global scale (Morison, 1998; Hetherington and Woodward, 2003; Frommer, 2010) and therefore is predicted to have profound effects on global gas exchange, plant water use efficiency, leaf heat stress and plant yield (Sellers *et al.*, 1997; LaDeau and Clark, 2001; Medlyn *et al.*, 2001; Battisti and Naylor, 2009; Holden, 2009).

Characterization of Arabidopsis signaling mutants that show an impaired response to CO_2 in regulation of stomatal movements include the carbonic anhydrase mutant *ca1 ca4* (Hu *et al.*, 2010), the protein kinase mutants *ost1* (Xue *et al.*, 2011; Merilo *et al.*, 2013) and *ht1* (Hashimoto *et al.*, 2006), S-type anion channel mutants *slac1* (Negi *et al.*, 2008; Vahisalu *et al.*, 2008), *gca2* (Young *et al.*, 2006), and the dominant protein phosphatase mutants *abi1-1* and *abi2-1* (Webb and Hetherington, 1997; Leymarie *et al.*, 1998). These mutants have begun to reveal parts of the mechanisms that mediate CO_2 regulation of stomatal conductance. However, it is not entirely understood how photosynthesis, which assimilates CO_2 within mesophyll and guard cells, affects stomatal conductance regulation (Lawson, 2009).

CO_2 concentrations below ambient levels stimulate stomatal opening, while CO_2 concentrations above ambient levels induce stomatal closure (Mansfield *et al.*, 1990; Assmann, 1999). Stomatal conductance is regulated by inter-cellular [CO_2] (*Ci*) rather than ambient [CO_2] at the external leaf-contouring surface (Mott, 1988). However, it remains a matter of debate whether this signal is sensed directly by guard cells and/or by the mesophyll cells (Assmann, 1999; Lawson *et al.*, 2011) or whether a combination of the two contributes to the response. *Ci* in the plant is determined by several main parameters; atmospheric [CO_2], respiration, mesophyll photosynthesis together with mesophyll and stomatal conductance. Stomatal conductance is regulated by photosynthetic activity in the mesophyll (Roelfsema *et al.*, 2006; Fujita *et al.*, 2013), through reduction in *Ci* levels (Mott, 1988, 1990; Morison, 1998; Assmann, 1999). Furthermore, additional signals from the mesophyll have been implicated in mediating CO_2 responses (Hedrich *et al.*, 1994; Mott *et al.*, 2008, 2014). While other studies have provided evidence for a CO_2 sensory mechanism in guard cells (Gotow *et al.*, 1982; Fitzsimons and Weyers, 1986; Hu *et al.*, 2010).

The 'indirect' role of photosynthesis as a main driver of lowering *Ci* and thus in low CO_2-induced stomatal opening is well documented. Previous work has provided evidence that stomatal responses to *Ci* are dependent on the balance between the photosynthetic electron transport capacity and carbon reduction reactions (Messinger *et al.*, 2006). On the other hand, results obtained on stomatal function in transgenic plants with reduced RUBISCO suggest that stomatal conductance is not directly determined by the photosynthetic capacity of guard cells or the leaf mesophyll (von Caemmerer *et al.*, 2004; Baroli *et al.*, 2008). The role of photosynthesis in the direct response to CO_2-mediated stomatal conductance regulation is a subject of debate.

Photosynthesis takes place primarily in the mesophyll tissue, while epidermal cells lack chloroplasts in most species. Guard cells, which developed from protodermal cells, do contain photosynthetically active chloroplasts in most species (Outlaw *et al.*, 1981; Zeiger *et al.*, 1981; Shimazaki *et al.*, 1982; Zemel and Gepstein, 1985; Gotow *et al.*, 1988; Rother *et al.*, 1988). Some studies have proposed that guard cell chloroplasts are not a prerequisite for stomatal CO_2 responses (Nelson and Mayo, 1975; Roelfsema *et al.*, 2006), whereas others have proposed a role for guard cell chloroplasts in CO_2 regulation of stomatal conductance (Assmann and Zeiger, 1985). Whether guard cell photosynthesis contributes directly to stomatal regulation in response to CO_2 remains an open question (Lawson, 2009), demanding genetic analyses by guard cell-specific impairment of photosynthesis.

A role of guard cell photosynthetic electron transport in red light-induced stomatal opening has been proposed (Olsen *et al.*, 2002; Suetsugu *et al.*, 2014). Pharmacological evidence using norflurazon-treated plants, which lack functional photosynthetic activity in both mesophyll and guard cells, suggests that photosynthesis in mesophyll and guard cells is not required for ABA, blue light or CO_2 responses, but is required for stomatal responses to PAR (Roelfsema *et al.*, 2006).

The use of chemicals, as norflurazon, affects all plant tissues and may have additional pharmacological and physiological effects. Furthermore, norflurazon-treated leaves are unstable and not easily amenable to kinetic stomatal conductance analyses. Thus, development of a guard cell-targeted genetic approach was sought to specifically impair guard cell photosynthesis in the present study. Here, we pursued genetic modification of chlorophyll levels specifically in guard cells to determine whether guard cell photosynthesis is necessary for: (i) stomatal responses to [CO_2] changes; (ii) general stomatal function; and (iii) the stomatal ABA response.

In this study, the role of guard cell photosynthesis in stomatal CO_2 and ABA responses was investigated through a comparison of wild-type Arabidopsis plants and transgenic plants that lack chlorophyll specifically in guard cells. Guard cell chlorophyll-deficient plants were generated using expression of a truncated form of chlorophyllase

(chlorophyll degrading) enzyme that is constitutively active (Harpaz-Saad *et al.*, 2007), using a guard cell-specific expression strategy (Gardner *et al.*, 2009). Interestingly, we show here a unique not previously reported, morphological phenotype of thin-shaped stomata in the transgenic lines. Data presented here suggest a key function of guard cell photosynthesis for intact turgor development and energization of stomatal guard cells. Furthermore, analyses of gas-exchange and stomatal movements showed that intact kidney-shaped stomata with chlorophyll-lacking guard cells respond to [CO_2] changes. Similarly, ABA-induced stomatal movement analysis showed that guard cell photosynthesis is not a direct mediator of ABA-induced stomatal closure. These data provide direct genetic-based evidence, that CO_2 and ABA guard cell signal transduction is not directly transduced by guard cell photosynthesis/electron transport and reveal a crucial function of guard cell photosynthesis in turgor production.

RESULTS

Guard cell-targeted chlorophyll degradation in transgenic Arabidopsis plants

To repress the photosynthetic activity in guard cells, we generated transgenic Arabidopsis plants, that are deficient in chlorophyll specifically in their guard cells. The chlorophyllase (Chlase) enzyme is a rate-limiting enzyme in chlorophyll catabolism (Harpaz-Saad *et al.*, 2007). Chlorophyllase catalyzes de-phytilation of chlorophyll; the major light-harvesting pigment in plants (Harpaz-Saad *et al.*, 2007). Over-expression of an N-terminal truncated form of *Citrus sinensis* chlorophyllase (ChlaseΔN) in diverse heterologous systems was previously shown to be highly active, resulting in robust chlorophyll breakdown within photosynthetic membranes (Harpaz-Saad *et al.*, 2007). Therefore, the ChlaseΔN cDNA was cloned into the stable (pHUASGW7) expression vector and transformed into a guard cell-specific-expression (E1728) enhancer trap line (Gardner *et al.*, 2009) (Figure 1a). This line expresses GFP constitutively in mature guard cells (Ohashi-Ito and Bergmann, 2006; Gardner *et al.*, 2009) (Figure 1b). Screening of hygromycin-resistant T2 plants using confocal microscopy revealed a reduction in guard cell chlorophyll levels.

Three independent Guard Cell-ChlaseΔN expressing lines (GC-ChlaseΔN) #4, #5 and #8 were selected for detailed characterization. These independent transgenic (T3 generation) GC-ChlaseΔN Arabidopsis lines were grown on soil at ambient [CO_2], and showed normal morphology (Figure 1c). Confocal microscopy analyses showed that guard cell chlorophyll auto-fluorescence levels were dramatically reduced in GC-ChlaseΔN expressing plants (Figure 2b,d,f) compared with wild-type guard cells (Figure 2a,c,e). Chlorophyll auto-fluorescence levels of the mesophyll appeared normal in the GC-ChlaseΔN

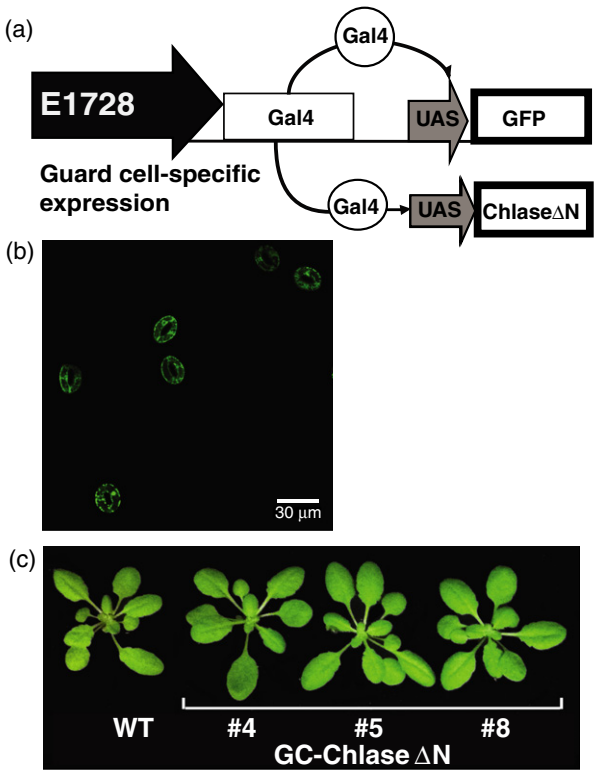

Figure 1. Generation and phenotype of guard cell-targeted chlorophyll-deficient plants.
Arabidopsis plants with reduced chlorophyll levels in guard cells were generated using constitutive guard cell-specific expression of the highly active version of *Citrus sinensis* chlorophyllase protein (Harpaz-Saad *et al.*, 2007).
(a) The constitutively active N-terminally truncated chlorophyllase, (ChlaseΔN) (Harpaz-Saad *et al.*, 2007) was sub-cloned to the pHUASGW7 expression vector (pHUAS-ChlaseΔN) and then (b) transformed to an E1728, guard cell-specific-expressing enhancer trap line (Gardner *et al.*, 2009) (GC-ChlaseΔN), which constitutively expresses GFP in guard cells.
(c) Representative images of wild-type (WT) and three GC-ChlaseΔN lines (T3 generation) (#4,#5 and #8) grown under ambient CO_2 conditions for 5 weeks.

lines (compare Figure 2g,h). This observation was confirmed by quantification of whole-leaf chlorophyll levels, which showed that total chlorophyll levels in the GC-ChlaseΔN lines were comparable to wild-type levels (Figure 3a). Together, these results indicate that the expression of the constitutively active form of the chlorophyllase enzyme (ChlaseΔN) in guard cells induced chlorophyll degradation solely in guard cells and had no effect on mesophyll chlorophyll levels.

Substantially reduced levels of chlorophyll in guard cells and high percentage of deflated stomata in transgenic plants

Interestingly, confocal (Figures 2b,d and 3c) and DIC (Figure 3e) microscopic analyses revealed two morphological states of stomata in the GC-ChlaseΔN lines: (i) normal kidney-shaped stomata; and (ii) thin-shaped stomata.

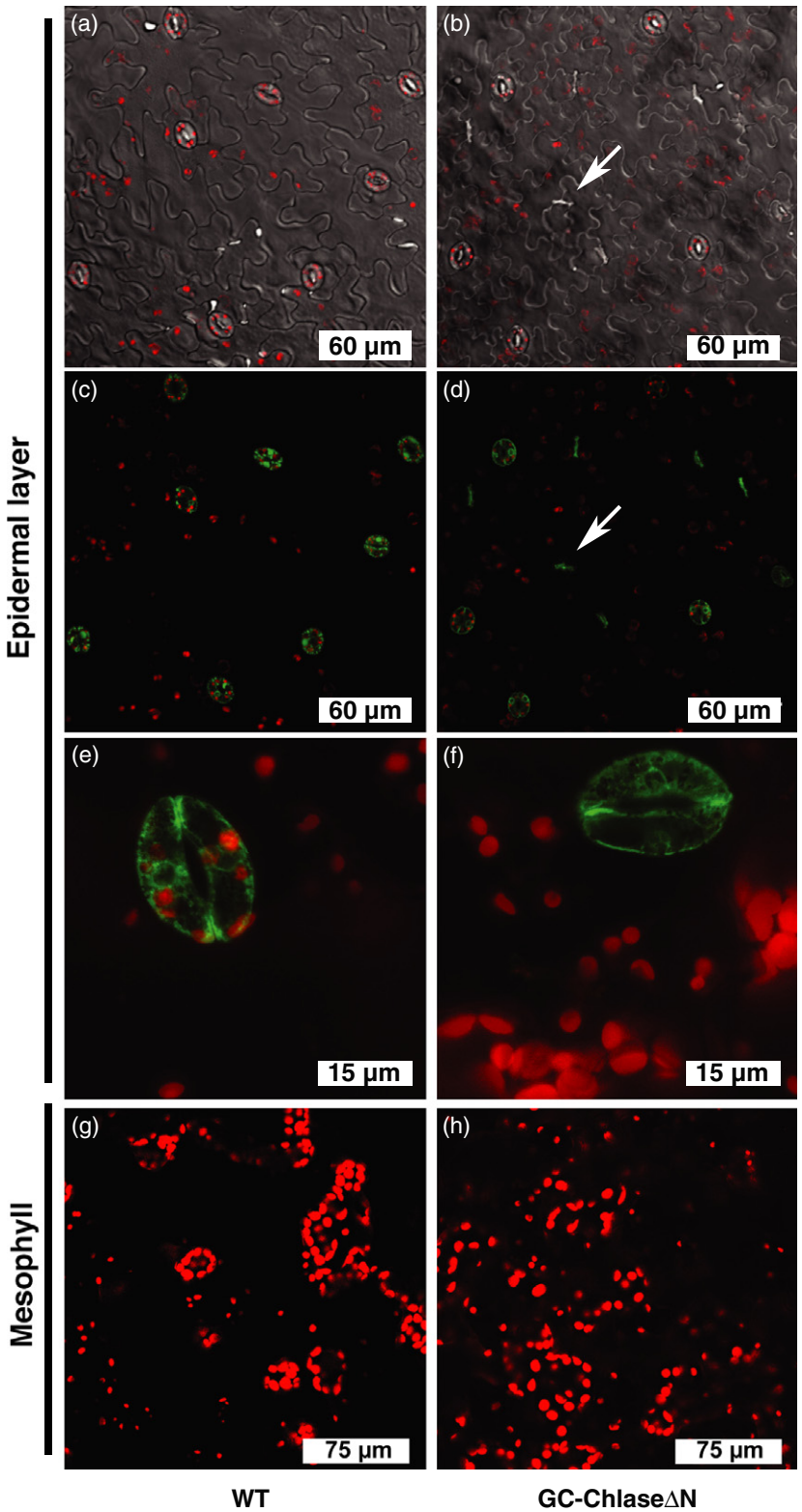

Figure 2. Chlorophyll fluorescence levels were reduced exclusively in GC-ChlaseΔN guard cells.
(a–f) The abaxial epidermis layer of the fifth true rosette leaf was imaged using confocal microscopy. Representative images for wild-type (WT) control E1728 enhancer trap plants (a, c, e) and GC-ChlaseΔN (T3 generation) transgenic plants (b, d, f) are shown. Merged confocal laser microscopic images show Arabidopsis leaf guard cells among epidermal cells. (b, d) Thin-shaped stomata detected in GC-ChlaseΔN lines. An example is marked by the white arrow. Panels (a, b): Confocal images of chlorophyll auto-fluorescence (red) overlaid on the corresponding bright-field images. Panels (c–f): confocal images recorded showing chlorophyll auto-fluorescence (red) and GFP fluorescence (green) corresponding to GFP expression specifically in guard cells of the E1728 enhancer trap plants.
(g, h) mesophyll layer of the fifth rosette true leaf was imaged using confocal microscopy. Representative confocal images show chlorophyll auto-fluorescence (red) in mesophyll of the E1728 enhancer trap plants (g) and GC-ChlaseΔN (T3 generation) transgenic plants (h).

Confocal microscopy analyses detected reduced chlorophyll auto-fluorescence levels in stomata of the GC-ChlaseΔN lines. Guard cell chlorophyll levels in kidney-shaped stomata of the transgenic lines varied from significant reduction to complete lack of measurable chlorophyll. Thin-shaped stomata of the transgenic lines did not show

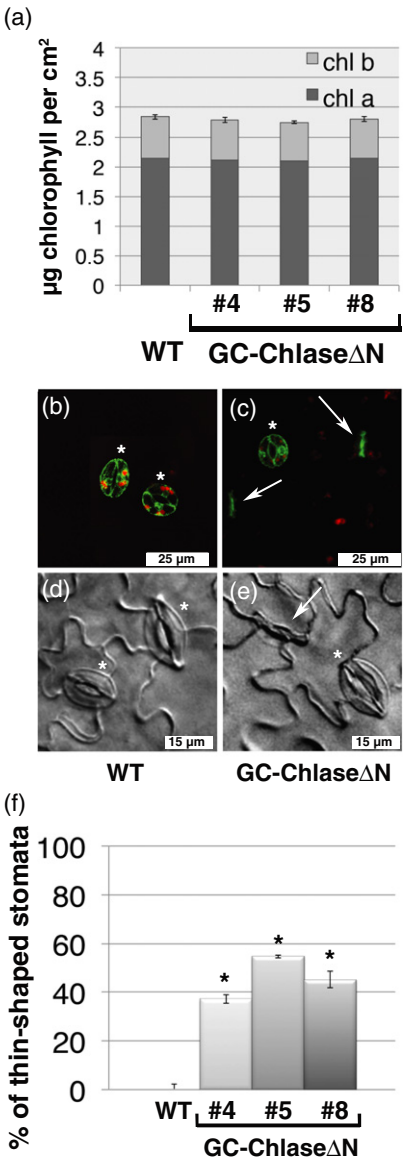

Figure 3. Expression of chlorophyllase in guard cells did not alter whole-leaf chlorophyll levels in GC-ChlaseΔN plants, but produced a large fraction of chlorophyll-less stomata that are thin-shaped.

(a) Total chlorophyll per leaf area (leaf disc) was quantified from the fifth rosette leaf of 4-week-old transgenic GC-ChlaseΔN (T3 generation) lines (#4, #5, and #8) and the wild-type (WT) control E1728 enhancer trap line. Data are means ± standard error of the mean (s.e.m.) (n = 6; four discs from the fifth leaf from six different plants).

(b–e) The abaxial epidermis layer of the fifth rosette leaf was imaged using confocal (b, c) and DIC (d, e) microscopy. Representative images for wild-type (WT) control E1728 enhancer trap plants (b, d) and GC-ChlaseΔN (T3 generation) transgenic plants (c, e) are shown. Two morphological types of stomata are detected: (1) normal kidney-shaped stomata (asterisk) in WT (b, d) and GC-ChlaseΔN plants (c, e); and (2) thin-shaped stomata (arrow) exclusively in GC-ChlaseΔN plants (c, e).

(f) Percentage of thin-shaped stomata from the total number of stomata (kidney-shaped and thin-shaped stomata in true leaf). Data are the mean ± s.e.m. (n = 4 leaves from independent plants; in each leaf four images [×40 magnification] were taken and data were averaged). Unpaired Student's t-test between wild-type and GC-ChlaseΔN presented as $^*P < 0.01$. Note that the same data set used for panel (f) was used in Figure S2(a).

any measurable chlorophyll auto-fluorescence signals (e.g. Figure 3c). Wild-type control plants had kidney-shaped stomata (Figure 3b,d) and normal chlorophyll levels (Figure 3b). In addition, the stomata of transgenic lines, including kidney-shaped and thin-shaped deflated stomata, showed intact GFP expression in guard cells (Figures 2d,f and 3c), implying functional protein biosynthesis and viability of guard cells. Interestingly, quantification of the thin-shaped and kidney-shaped stomata in true leaves revealed that approximately 45% of GC-ChlaseΔN plants had a thin-shaped morphology, while no thin-shaped stomata were observed in wild-type controls (Figure 3f); 37.1% [line #4], 54.6% [line #5] and 45.1% [line #8].

To quantify chlorophyll levels in guard cells of the transgenic lines, confocal microscopy imaging and chlorophyll auto-fluorescence signal measurements of individual stomatal guard cells were conducted. Figure 4(a,b) displays the relative total chlorophyll auto-fluorescence levels per stoma in each line. Average chlorophyll levels of all kidney and thin-shaped stomata showed extensive average

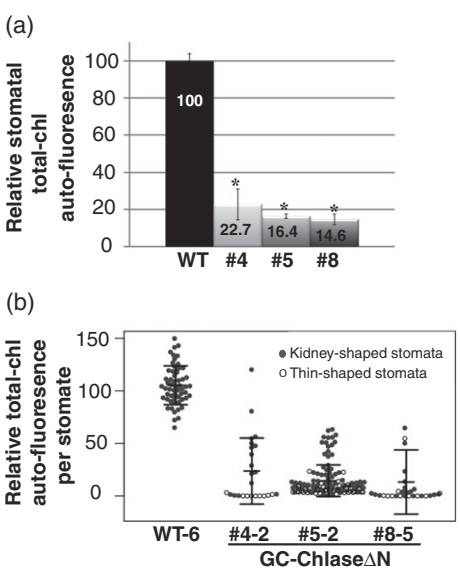

Figure 4. More than 90% of GC-ChlaseΔN guard cells show reduced chlorophyll levels.

Total chlorophyll auto-fluorescence of individual guard cells was analyzed from confocal guard cell Z-stack images.

(a) The average chlorophyll auto-fluorescence measured for the wild-type (WT) control E1728 plants was designated as 100%. Average chlorophyll auto-fluorescence of GC-ChlaseΔN stomata is depicted as percentages relative to wild-type (WT) controls. Data are the mean ± standard error of the mean (s.e.m.). WT [E1728] n = 8 plants, total approximately 400 stomata; line #4 n = 5 plants, total 122 stomata; line #5 n = 5 plants, total 400 stomata; line #8 n = 5 plants, total 168 stomata. Unpaired Student's t-test between wild-type and GC-ChlaseΔN presented as $^*P < 0.01$.

(b) Scatter plot of the relative chlorophyll auto-fluorescence per stoma of representative plants, where each circle represents the chlorophyll fluorescence of a guard cell relative to the average of all WT guard cells measured. Grey circles represent kidney-shaped stomata and white circles represent thin-shaped stomata. Horizontal lines depict the mean and standard deviation for each plant. Note that the same data shown in (b) are included in Figure S1(b).

chlorophyll fluorescence reductions in GC-ChlaseΔN lines, by 78.1% (line #4), 84.4% (line #5) and 73.7% (line #8) compared with wild-type levels (Figure 4a) ($P < 0.001$; Unpaired Student's *t*-test). A detailed scatter plot of the data reveals that >90% of GC-ChlaseΔN guard cells had abnormally-reduced chlorophyll levels (Figure 4b). Guard cell chlorophyll levels varied from partial reduction to many guard cells exhibiting a complete lack of chlorophyll auto-fluorescence. Furthermore, almost all the deflated, thin-shaped stomata were chlorophyll-less, based on lack of quantifiable chlorophyll auto-fluorescence (thin-shaped stomata; Figure 4b white circles). Note that Figure 4(b) display data from one representative plant from each independent line. For complete data-set see Figure S1(b).

As a large fraction (approximately 45%) of stomata in the GC-ChlaseΔN lines had a thin-shaped morphology (Figures 3f and S1b), chlorophyll auto-fluorescence levels were re-evaluated solely in kidney-shaped stomata. The average chlorophyll auto-fluorescence, calculated solely from kidney-shaped stomata of the GC-ChlaseΔN lines, was significantly reduced, by 69.6 ± 8.4% (line #4), 74.6 ± 2.8% (line #5) and 68.7 ± 9.9% (line #8) of wild-type guard cell levels (Figure S1a) ($P < 0.001$; unpaired Student's *t*-test).

Guard cell chlorophyll-deficient lines retain stomatal conductance responses to [CO_2] shifts

Stomatal conductance responses to [CO_2] shifts were investigated in intact leaves of the guard cell chlorophyll-deficient lines. Time-resolved stomatal conductance responses and photosynthetic parameters were recorded using an intact leaf gas-exchange analyzer (LI-COR 6400). CO_2-induced stomatal conductance responses were analyzed in two sets of sequential CO_2 shifts (Figure 5a,b,f,g). The steady-state stomatal conductances of all three GC-ChlaseΔN lines were significantly smaller than wild-type controls, under both 450 ppm and 360 ppm CO_2 (Figure 5a,f). When steady-state stomatal conductance was achieved at 450 ppm [CO_2], transgenic lines showed significant reduction in average stomatal conductance to 64% (#4), 59.5% (#5) and 47.5% (#8) of wild-type levels ($P < 0.05$; $n = 3$ leaves in three distinct plants analyzed per genotype, unpaired Student's *t*-test, between wild-type [WT] and GC-ChlaseΔN lines, at the 30 min data point). Similarly, when steady-state stomatal conductance was achieved at 360 ppm [CO_2], transgenic lines showed a comparable reduction in average stomatal conductance; to 67% (#4), 60% (#5) and 59.5% (#8) of wild-type levels ($P < 0.05$; $n = 3$). Normalization of gas-exchange data, relative to the last data point at 360/450 [CO_2] prior to [CO_2] transitions, revealed similar time-resolved stomatal conductance responses to CO_2 in GC-ChlaseΔN and wild-type plants, which were not statistically different (Figure 5b,g) ($P > 0.1$; $n = 3$).

Net CO_2-assimilation rates were found to be slightly lower than wild-type levels in all three GC-ChlaseΔN lines under high and ambient [CO_2] levels (Figure 5c). Following low [CO_2] (100 ppm) exposure, average net CO_2-assimilation rates of GC-ChlaseΔN lines decreased to 82.8% (#4, $P = 0.5$), 61.8% (#5, $P = 0.007$) and 56.8% (#8, $P = 0.01$) of wild-type levels (Figure 5c, $n = 3$ leaves in three distinct plants analyzed per genotype, unpaired Student's *t*-test, between WT and GC-ChlaseΔN, 50 min after transition to 100 ppm CO_2). Figure 5(h) shows a similar and significant reduction in net CO_2-assimilation rate to 54.5% (line #4), 55% (line #5) and 55.7% (line #8) of wild-type levels ($P < 0.05$; $n = 3$ leaves in three distinct plants analyzed per genotype, unpaired Student's *t*-test, between WT and GC-ChlaseΔN, 50 min after transition to 100 ppm CO_2). These results correlate with the decrease in stomatal conductance of GC-ChlaseΔN lines under these conditions (Figure 5a,f).

Interestingly, although net assimilation rates dropped instantaneously following the transition from 450 to 100 ppm CO_2 (Figure 5c), net assimilation rates in wild-type and the transgenic lines increased with time. After 40 min at a CO_2 concentration of 100 ppm, assimilation rates reached similar values obtained at 450 ppm (Figure 5c). As low CO_2 induces stomatal opening, stomatal conductance (Figure 5a) and CO_2 influx increases with time. The added CO_2 supply from stomatal opening (Figure 5a) is quickly consumed by the Calvin-Benson cycle, as judged by the increasing net CO_2 assimilation rate (Figure. 5c). Together, the combined data may explain the low and relatively stable *Ci* concentrations we observed during low CO_2 treatments (Figure 5e).

Analysis of the intrinsic water use efficiency (iWUE), defined as the ratio between *A* and stomatal conductance, showed an increase in iWUE of the GC-ChlaseΔN lines (Figure 5d,i). This effect is likely due to the reduction in the total stomatal conductance in these lines (Figure 5a).

To determine whether chlorophyll reduction in guard cells affects leaf photosynthesis, photochemical efficiency parameters were investigated. Transgenic and wild-type plants showed insignificant differences in the maximum efficiency of photosystem II (PSII) photochemistry at 150 μE (F_v'/F_m'; Figure 5k), PSII operating efficiency (ΦPSII; Figure 5l), non-photochemical quenching (qN; Figure 5m) and similar photochemical quenching (qP; Figure 5n), (21°C, 150 μmol m^{-2} sec^{-1}, ambient [CO_2]; $P > 0.05$, $n = 3$ leaves in three distinct plants analyzed per genotype). Together, these data showed that the GC-ChlaseΔN lines have normal photosynthetic activity parameters in whole leaves compared with those in wild-type (Figure 5k–n) and continue to respond to [CO_2] shifts (Figure 5b,g). Nevertheless, steady-state stomatal conductances of the GC-ChlaseΔN lines were significantly smaller by approximately 40% compared with wild-type conductances

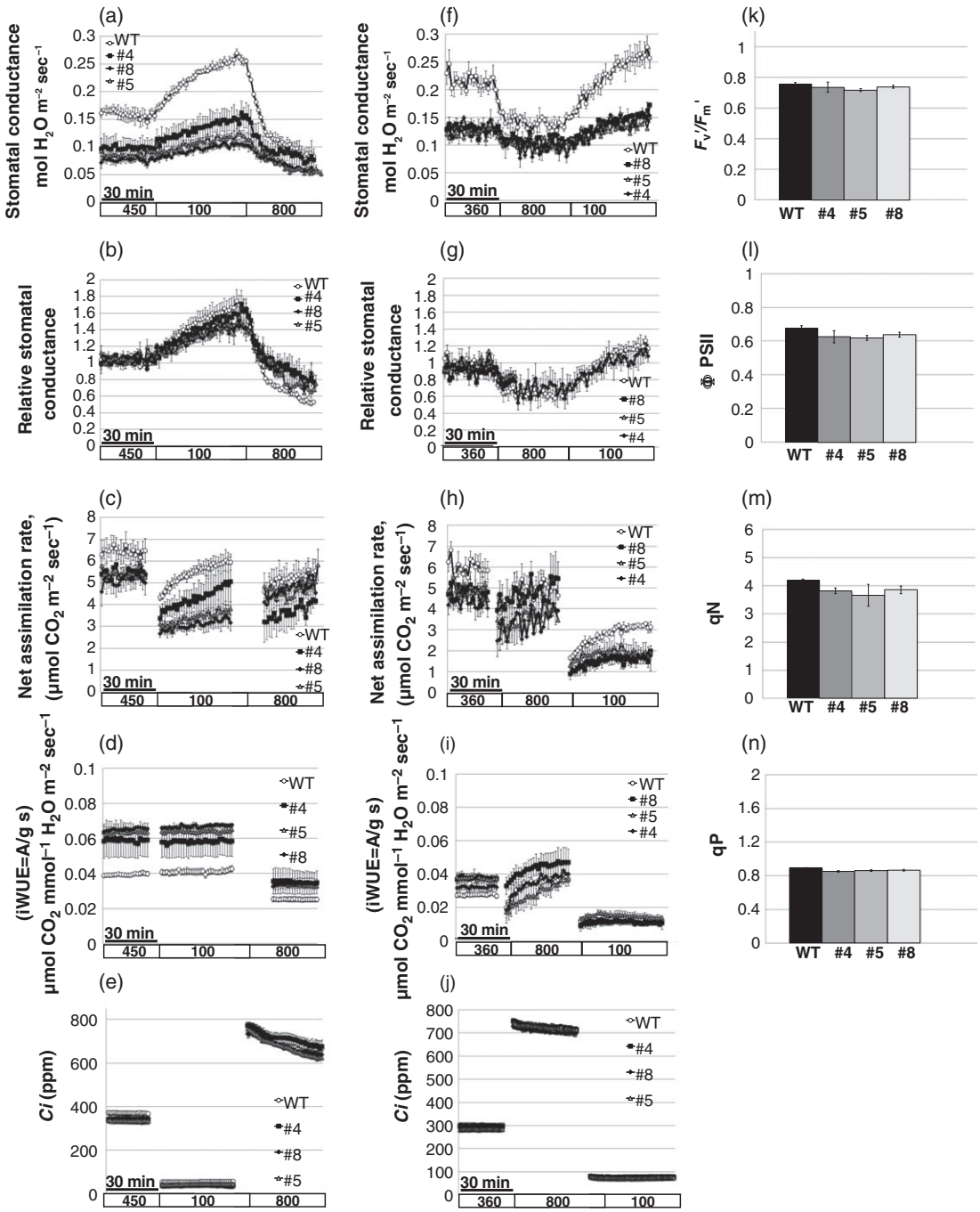

Figure 5. Guard cell chlorophyll-deficient plants display reduced stomatal conductance and respond to [CO_2] shifts.

Time-resolved stomatal conductance responses and net CO_2-assimilation rates at the imposed [CO_2] shifts (bottom in ppm) in wild-type (WT) and in three independent GC-ChlaseΔN (T3 generation) lines (#4, #5, and #8) were analyzed using intact whole-leaf gas exchange.

(a, f) Stomatal conductance in mol H_2O m^{-2} sec^{-1}.

(b, g) Data shown in (a, f) were normalized to the last 30 sec time-point of 450 ppm [CO_2] exposure.

(c, h) Net CO_2-assimilation rates (μmol CO_2 m^{-2} sec^{-1}).

(d, i) Intrinsic water use efficiency (iWUE).

No difference was observed between wild-type and GC-ChlaseΔN transgenic plants with respect to chlorophyll fluorescent parameters, in leaves that were pre-adapted at 150 μmol m^{-2} sec^{-1} photosynthetically active radiation (PAR), under ambient CO_2 levels.

(e, j) The corresponding inter-cellular [CO_2] (*Ci*) levels are shown.

(k) Maximum efficiency of photosystem II (PSII) photochemistry at 150 μE, F_v'/F_m'.

(l) PSII operating efficiency (ΦPSII).

(m) Non-photochemical quenching (qN).

(n) Photochemical quenching (qP).

Data in (a–n) are the mean of $n = 3$ plants ± s.e.m. (k–n). Unpaired Student's *t*-test between wild-type and GC-ChlaseΔN shows no significant differences.

(Figure 5a: reduction by 36% [#4], 40.5% [#5], 52.5% [#8]). Transgenic lines had a large fraction of approximately 45% of thin-shaped stomata, which may account for the reduced stomatal conductance (Figure 3f; 37% [#4], 54.6% [#5], 45% [#8]). The detailed experimental procedures used for gas-exchange measurements can be found in Supporting Information.

Thin-shaped stomata of guard cell chlorophyll-deficient lines and low stomatal conductance

We investigated whether the reduced stomatal conductance level in GC-ChlaseΔN plants leaves was in part attributable to differences in stomatal density & development (Engineer *et al.*, 2014) or completely attributable to the thin-shaped stomata in these leaves. Stomatal indices of the fifth true leaf showed that GC-ChlaseΔN plants have normal stomatal indices, similar to the parallel grown wild-type controls (Figure S2a; $P > 0.4$, $n = 4$ leaves in four distinct plants analyzed per genotype, unpaired Student's *t*-test, between WT and GC-ChlaseΔN lines). Similar observations were made in cotyledons, where stomatal indices were not significantly different from wild-type (Figure S2b), although a large fraction of GC-ChlaseΔN stomata were thin-shaped (Figure S2c,d). These results imply that the reduction in steady-state stomatal conductance of the GC-ChlaseΔN lines is a result of the thin-shaped stomata formed in these leaves.

Guard cell chlorophyll-deficient plants show reduction in guard cell-starch levels

Reduction of chlorophyll levels is predicted to affect photosynthetic efficiency and CO_2 assimilation. Iodine staining of WT and GC-ChlaseΔN plants showed that starch synthesis in whole leaves was not disrupted in the GC-ChlaseΔN plants (Figure S3a,b). Further microscopic analysis of theses leaves by bright-field Z-stack imaging, showed that guard cell starch content was clearly reduced in GC-ChlaseΔN lines (Figure S3d,f) compared with wild-type guard cells (Figure S3c,e). Mesophyll starch levels appeared normal in the GC-ChlaseΔN lines (Figure S3h) and were comparable to those in wild-type leaves (Figure S3g). These results are in line with the observation of restricted guard cell chlorophyll reduction in GC-ChlaseΔN lines (Figure 2). To quantify starch content levels in guard cells of the transgenic lines, light microscopy imaging and starch signal measurements of individual stomata were conducted. No starch-stain signals were found in thin-shaped stomata. Therefore, starch levels were solely quantified in kidney-shaped stomata. Figure S3(i,j) display the relative total starch levels per stoma in each line. Data show a relative reduction in GC-ChlaseΔN lines, by $24.1 \pm 5.0\%$ (line #4), $24.8 \pm 5.2\%$ (line #5) and $28.5 \pm 5.6\%$ (line #8) compared with wild-type levels (Figure S3i,j) ($P < 0.03$; unpaired Student's *t*-test).

Chlorophyll-deficient kidney-shaped stomata respond to $[CO_2]$

To study stomatal responses at the single stoma level, wild-type and the GC-ChlaseΔN plants were analyzed for their stomatal responses (Figure 6a–c). Plants were grown for 4 weeks in a growth chamber under ambient CO_2 levels. Intact plants were than exposed to 200 ppm CO_2, in a CO_2 controlled growth chamber for 2 h and then stomatal apertures in the fifth leaf were directly imaged. Plants that were pre-incubated for 2 h in 200 ppm CO_2 were subsequently transferred to an 800 ppm-CO_2 chamber for an additional 45 min and then stomatal apertures in the fifth leaf were directly imaged and analyzed. As transgenic lines were easily identified by their thin-shaped stomata, all experiments conducted were $[CO_2]$ treatment-blind experiments. As approximately 37–54% of the stomata in transgenic lines were thin-shaped (Figure 3f), stomatal apertures of GC-ChlaseΔN lines were separated into two groups: (1) kidney-shaped stomata; and (2) thin-shaped stomata. Stomatal responses of GC-ChlaseΔN lines were then compared with those of wild-type controls. Data analyses revealed that stomatal lengths of GC-ChlaseΔN and wild-type plants were not significantly different ($P > 0.2$; unpaired Student's *t*-test). Therefore, stomatal apertures are presented as pore width (μm). Stomatal image analyses showed that kidney-shaped stomata of the GC-ChlaseΔN lines responded to $[CO_2]$ shifts (Figure 6). These data indicate that wild-type levels of chlorophyll in guard cells are not needed for CO_2-induced stomatal closing. Thin-shaped stomata did not respond to $[CO_2]$ shifts and were constitutively closed, suggesting an impaired guard cell turgor production and/or dysfunctional turgor production.

Kidney-shaped stomata that are chlorophyll-less respond to $[CO_2]$ and abscisic acid

The above gas-exchange and stomatal movement analyses showed that plants with reduced levels of chlorophyll specifically in their guard cells respond to CO_2 shifts. However, it remained unclear whether complete elimination of guard cell photosynthesis, by chlorophyll degradation, affects stomatal CO_2 responses. Furthermore, the question, whether guard cell photosynthesis contributes directly to ABA-induced stomatal closing, remains unanswered. Therefore, stomatal responses to CO_2 or ABA were investigated in the three independent transgenic lines, solely in kidney-shaped stomata that had no detectable chlorophyll auto-fluorescence signals ($[CO_2]$ Figure 7a–c; [ABA] Figure 8). The experiments showed that chlorophyll-less (kidney-shaped) stomata responded to $[CO_2]$ shifts, with confidence levels between 90–97% ($P = 0.03$ (lines #4), $P = 0.07$ (lines #5), and $P = 0.1$ (lines #8), pairwise Student's *t*-test, $n = 3$–4 leaves in 3–4 distinct plants analyzed

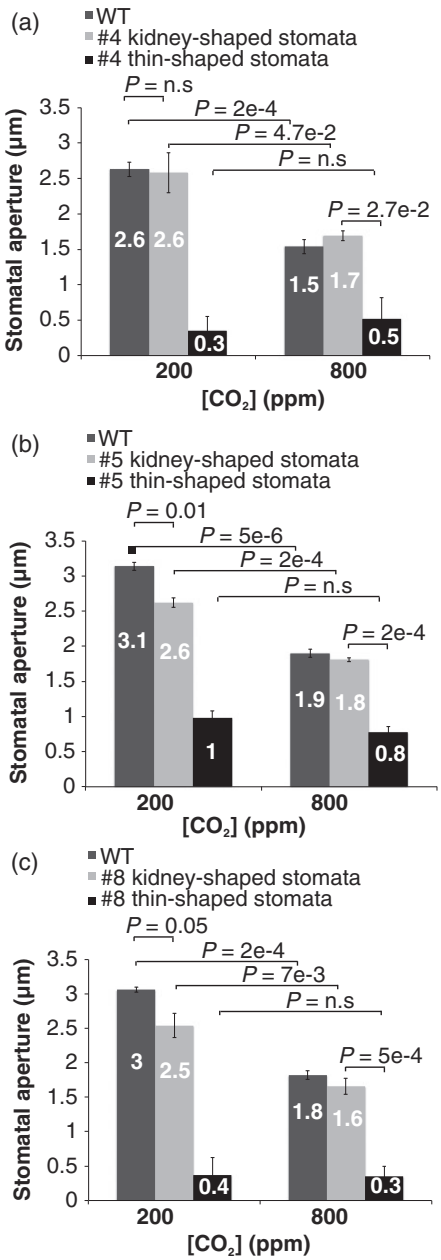

Figure 6. Kidney-shaped stomata of the GC-ChlaseΔN lines respond to [CO_2] shifts, while thin-shaped stomata are continuously closed.

Stomatal apertures in response to [CO_2] changes were measured in wild-type (WT) and GC-ChlaseΔN plants. Intact plants were grown for 4 weeks in a growth chamber under ambient CO_2 levels. Plants were than exposed to 200 ppm CO_2, in a CO_2-regulated growth chamber, for 2 h and then stomatal apertures in the fifth leaf were directly imaged and analyzed. Plants that were pre-incubated for 2 h in 200 ppm CO_2 were subsequently transferred to an 800-ppm CO_2 chamber for an additional 45 min and then stomatal apertures of the fifth leaf were directly analyzed.

(a–c) Stomatal aperture measurements of WT and GC-ChlaseΔN lines (a) #4, (b) #5 and (c) #8 in response to CO_2 changes. In each experiment (a–c) $n = 4$ plants, total approximately 120 stomata were measured for each genotype and treatment (200/800 ppm CO_2). Data represent means ± standard error of the mean (s.e.m.) ([CO_2] treatment-blind experiments). Pairwise Student's *t*-test between the different CO_2 treatments is presented above columns in each graph (n.s. = non-significant).

per genotype, approximately 45 stomata were measured for each treatment). Corresponding scatter-plot presentations of these data show stomatal apertures measured in individual experiments (Figure 7a–c). Examination of the GC-ChlaseΔN lines #4, #5 and #8 showed that in eight out of 10 experiments, chlorophyll-less stomata responded significantly to [CO_2] shifts, with $P < 0.05$. In one of four experiments with line #4, chlorophyll-less stomata responded to [CO_2] shifts with a confidence level of 91% (*P*-value = 0.09), while in the other three experiments chlorophyll-less stomata responded to [CO_2] shifts with a confidence level >99%, (*P*-values < 8e–05). In two out of three experiments using line #8, chlorophyll-less stomata responded to [CO_2] shifts with a high confidence level of >96%, while in the third experiment (Figure 7c: #8-1) chlorophyll-less stomata showed no clear response (*P*-value = 0.45). This experimental result can explain the somewhat smaller confidence level of 90% ($P = 0.1$) calculated for line #8 when data were averaged (Figure 7c). Furthermore, investigation of stomatal responses to ABA also showed significant stomatal closing in chlorophyll-less (kidney-shaped) stomata (Figure 8a,c) Line #8 $P = 7e–04$, pairwise Student's *t*-test, $n = 8$ plants, total approximately 240 stomata; (Figure 8b,d) Line #4 $P = 0.02$, pairwise Student's *t*-test, $n = 3$ plants, total approximately 110 stomata were measured for each treatment and genotype). Together these data suggest that complete removal of guard cell chlorophyll does not disrupt the primary stomatal conductance response to [CO_2] elevation at a 90% statistical confidence level and to ABA.

To further test, whether chlorophyll degradation in guard cells induces developmental changes in the stomata of the transgenic lines, stomatal length data was extracted from stomatal CO_2 response analyses in Figure 7. The data showed that kidney-shaped stomata of GC-ChlaseΔN plants, that are chlorophyll-less, have similar stomatal lengths as wild-type stomata (Figure S4; $P \geq 0.2$ (lines #4), $P \geq 0.6$ (lines #5), and $P \geq 0.2$ (lines #8), unpaired Student's *t*-test, between WT and GC-ChlaseΔN lines, $n = 3$–4 leaves in 3–4 distinct plants analyzed per genotype, approximately 45 stomata were measured for each treatment). Together, stomatal indices (Figure S2) and stomatal length (Figure S4) analyses suggest that stomata of GC-ChlaseΔN lines developed similar to those in wild-type plants.

DISCUSSION

Linear electron transport (Hipkins *et al.*, 1983; Shimazaki and Zeiger, 1985; Cardon and Berry, 1992; Tsionsky *et al.*, 1997; Lawson *et al.*, 2002, 2003) and carbon fixation (Lawson, 2009) take place in guard cell chloroplasts. Previous studies have examined the roles of photosynthesis in stomatal responses, however most of these studies have focused on effects of mesophyll photosynthesis (Lee and Bowling, 1992; Baroli *et al.*, 2008), or could not distinguish

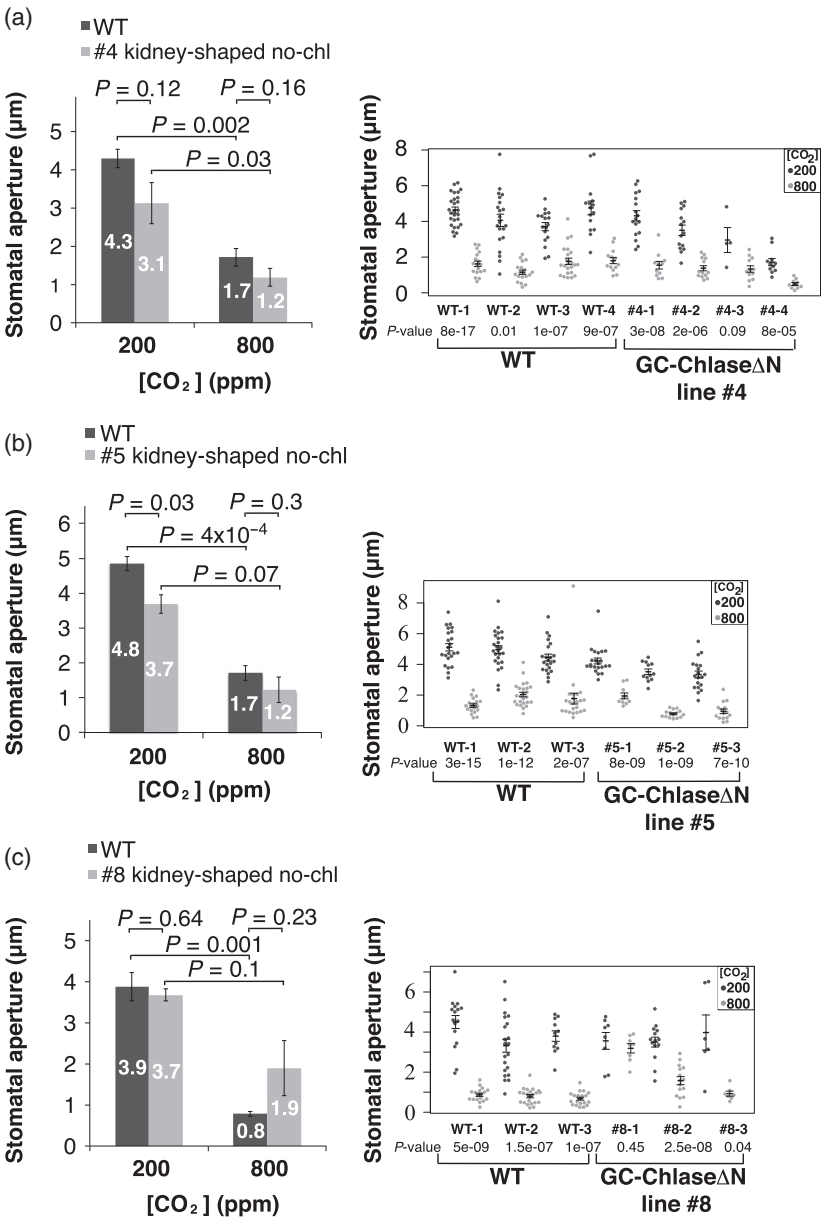

Figure 7. Kidney-shaped chlorophyll-less stomata respond to CO_2 shifts.
Stomatal apertures in response to [CO_2] changes were measured in WT from kidney-shaped stomata, while in the transgenic (GC-ChlaseΔN) plants stomatal apertures were measured solely from kidney-shaped stomata that did not have a measurable chlorophyll fluorescence signal. Plants were exposed to 200 ppm CO_2, in a CO_2-regulated growth chamber, for 2 h and then stomatal apertures in the fifth leaf were sampled and analyzed. Plants that were pre-incubated for 2 h in 200 ppm CO_2 were subsequently transferred to an 800 ppm CO_2 chamber for an additional 90 min and then stomatal apertures in the fifth leaf were analyzed. Stomata in epidermal peels were imaged using both bright-field and a red chlorophyll fluorescence filter, in order to detect the chlorophyll-less stomata in the transgenic lines.
(a–c) The average and the corresponding scatter plot of the data are presented for each line (a) #4, (b) #5, and (c) #8, where each circle represents one stomatal aperture measured. Dark grey circles represent stomatal apertures after 200 ppm and light grey circles after 800 ppm CO_2 induction. In each experiment (a–c) n = 3–4 plants, total approximately 45 stomata were measured for each treatment (200/800 ppm CO_2). Column and scatter plot horizontal lines represent means ± standard error of the mean (s.e.m.) (CO_2 treatment-blind analyses). Pairwise Student's t-tests between the same line under different CO_2 treatments and between the different lines under the same treatment are presented in each graph. Note that the same data sets used for panels (a–c) were used in Figure S4 (a–c).

between the effects of photosynthesis in mesophyll cells versus guard cells (von Caemmerer *et al.*, 2004; Messinger *et al.*, 2006; Lawson *et al.*, 2008).

A few studies have addressed the role of guard cell chloroplasts in stomatal conductance regulation. Studies of the distinctive orchid species, *Paphiopedilum*, showed that guard cells, which do not have chloroplasts still maintain functional stomatal responses to CO_2 (Nelson and Mayo, 1975). However, a later analysis between *Paphiopedilum* and the guard cell-chloroplast-containing genera *Phragmipedium*, revealed a weaker stomatal conductance response to *Ci* change (Assmann and Zeiger, 1985). The lack of chloroplasts in the orchid species *Paphiopedilum*, was suggested to be an ecophysiological adaptation to water deficit in limestone habitats (Assmann and Zeiger, 1985; Zhang *et al.*, 2011). Although these data suggest the involvement of guard cell chloroplasts in the regulation of stomatal conductance to CO_2, whether guard cell photosynthesis or other chloroplast functions were the underlying reason remains to be determined.

Other studies have investigated the role of photosynthesis in stomatal conductance regulation using norflurazon, which inhibits chlorophyll synthesis in all plant tissues, including guard cells and mesophyll cells. Stomata of norflurazon-treated albino leaves showed functional stomatal responses to CO_2 (Roelfsema *et al.*, 2006; Hu *et al.*, 2010). However, norflurazon may have additional pharmacological

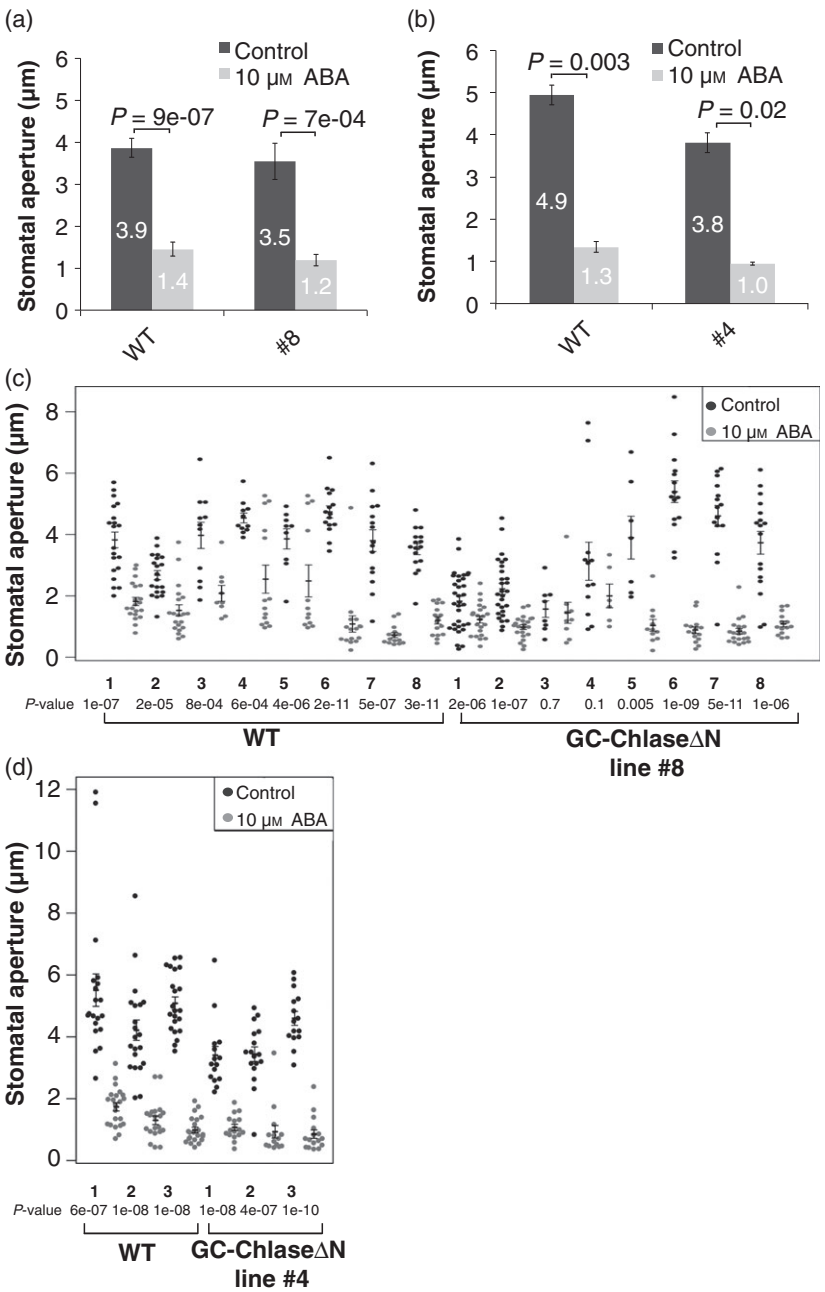

Figure 8. Kidney-shaped chlorophyll-less stomata respond to ABA.
Stomatal apertures in response to ABA were measured in WT from kidney-shaped stomata, while in the transgenic (GC-ChlaseΔN) plants stomatal apertures were measured solely from kidney-shaped stomata that did not have any measurable chlorophyll fluorescence signal.
(a, b) Average stomatal apertures and (c, d) the corresponding scatter plot of the data are presented for each line, where each circle represents one stomatal aperture measured. Circles represent stomatal apertures measured after 1 h exposure to 0.1% ETOH (dark grey circles) or 10 μM ABA (light grey circles). (a, c) Line 8, $n = 8$ plants, total approximately 240 stomata were measured for each treatment and genotype. (b, d) Line 4, $n = 3$ plants, total approximately 110 stomata were measured for each treatment and genotype. Error bars represent means ± standard error of the mean (s.e.m.) (ABA treatment-blind analyses). Pairwise Student's *t*-tests between the same line under different treatments are presented in each graph. Fifth true leaves were floated on opening buffer for 2 h under 120 μE white light to pre-open stomata. Leaves were then exposed to 10 μM ABA or 0.1% ETOH for 1 h. Stomata in epidermal peels were imaged using both bright-field and a red chlorophyll fluorescence filter, in order to detect the chlorophyll-less stomata in the transgenic lines.

effects, norflurazon-treated leaves of Arabidopsis are very thin and labile, and therefore are less suitable for intact leaf gas-exchange analyses. The long-standing question, whether guard cell photosynthesis plays a role in CO_2 regulation of stomatal conductance, was addressed here using a guard cell targeted transgenic approach.

CO_2 regulation of stomatal conductance does not directly require WT chlorophyll levels

In the present study, photosynthesis was targeted specifically in guard cells, by generating transgenic Arabidopsis plants, which degrade chlorophyll in guard cells. Data reported here show that extensive diminution of guard cell chlorophyll, which impairs photosynthesis in guard cells and consequently reduced starch levels in guard cells, did not affect the development of aerial tissue. Furthermore, plant growth, whole-leaf chlorophyll *a*/*b* content and photosynthetic parameters of whole leaves (PSII, qp qN, F_V'/F_M') remained intact. Time-resolved stomatal conductance responses to [CO_2] in intact leaves showed a reduction in steady-state stomatal conductance and net assimilation rates of the transgenic

lines when compared with WT plants. Normalization of whole-leaf gas-exchange data showed that stomata of the transgenic plants respond like wild-type plants to [CO_2] shifts. Interestingly, a large fraction of the guard cells in transgenic plants showed a not previously described thin-shaped morphology both in cotyledons and in mature leaves. Confocal analysis and stomatal aperture measurements revealed that the thin-shaped stomata of the transgenic plants lack chlorophyll and are continuously closed. Therefore, we suggest that the reduction in the steady-state stomatal conductance, and as a consequence reduction in net assimilation and increase intrinsic water use efficiency (iWUE), are attributable to the thin-shaped closed stomata.

Approximately 55% of the guard cells in the GC-ChlaseΔN plants are kidney-shaped, and approximately 90% of these kidney-shaped guard cells showed extensively reduced levels of chlorophyll (exhibiting an average of approximately 26% chlorophyll levels compared with WT guard cells, Figure S1a). Stomatal aperture measurements revealed that these kidney-shaped stomata, which were severely reduced in their chlorophyll content, retain the ability to respond to [CO_2] shifts. Furthermore, when solely kidney-shaped stomata that had no chlorophyll signal were analyzed, stomatal responses to [CO_2] changes were still observed at a 90% statistical confidence level. Furthermore, similar analysis showed that chlorophyll-less kidney-shaped stomata respond significantly to ABA. Together, our findings suggest that photosynthesis in guard cells does not directly mediate CO_2- and ABA-induced stomatal closure.

The chloroplast is an organelle of substantial importance to plants, which harbors multiple biological processes. Although our data indicate that photosynthesis in guard cells does not play a role in CO_2 and ABA regulation of stomatal conductance, guard cell chloroplasts may still play a role via non-photosynthetic pathways. For example, starch and malate metabolism are involved in the regulation of stomatal conductance (Outlaw and Lowry, 1977; Willmer and Beattie, 1978; Schnabl, 1980; Tallman and Zeiger, 1988; Reckmann *et al.*, 1990; Asai *et al.*, 2000; Roelfsema *et al.*, 2006). These processes do not directly require photosynthesis in guard cells, as carbohydrates could be imported into the guard cells from other cells (Gotow *et al.*, 1988; Tallman and Zeiger, 1988; Poffenroth *et al.*, 1992; Talbott and Zeiger, 1993, 1996; Stadler *et al.*, 2003; Kang *et al.*, 2007).

Previous research has shown slowed CO_2 control of stomatal conductance in the β-carbonic anhydrase double mutant *ca1 ca4*, while ABA-induced stomatal closure remained intact (Hu *et al.*, 2010). β-Carbonic anhydrase 1 (CA1) is mainly targeted to the chloroplast stroma (Fabre *et al.*, 2007; Hu *et al.*, 2010) and is highly expressed in guard cells (Hu *et al.*, 2010). This raises the question of whether these carbonic anhydrases affect photosynthesis in whole leaves. Previous research showed no effect of *ca1 ca4* mutation on several photosynthesis parameters. However, assimilation was not analyzed at a wide range of *Ci*. Experiments show that whole-leaf assimilation rates as a function of inter-cellular [CO_2] (*A*/*Ci*) were not altered in the *ca1 ca4* double mutant compared with wild-type plants (Figure S5). These results and previous findings further highlight the need in the present study to investigate specifically the role of guard cell photosynthesis in stomatal CO_2 and ABA responses.

Photosynthesis in guard cells is essential for guard cell turgor production

Previous studies have shown that guard cells have low chlorophyll content and, low activity levels of RUBISCO and Calvin-Benson cycle enzymes (Outlaw, 1982; Reckmann *et al.*, 1990) and limited capacity to perform photosynthesis compared with mesophyll cells (Lawson *et al.*, 2002; Vavasseur and Raghavendra, 2005). These cumulative data may imply a small or insignificant role of photosynthesis in guard cells. On the other hand, guard cells were previously suggested to fix CO_2 by both RUBISCO and PEPCase, and to be capable of producing all the ATP that is required for light-induced stomatal opening (Daloso *et al.*, 2015). An interesting and unexpected result obtained in the present study was that approximately 45% of the guard cells in the transgenic plants displayed a thin-shaped morphology, which has not been previously reported. All of these thin-shaped stomata exhibited cytoplasmic GFP signals, which suggests that these cells exhibit normal protein synthesis and cell viability. Nevertheless, these guard cells had no detectable chlorophyll and the stomata were continuously closed. We therefore propose that photosynthesis in guard cells is an indispensable process for stomatal physiology. It is probable that the lack of photosynthesis in guard cells impairs energy (ATP/NADPH) production, which is required for development and maintenance of turgor in guard cells.

Interestingly, the stomata, that showed a kidney-shaped phenotype, did maintain guard cell turgor and responded to CO_2 shifts. Their chlorophyll level distribution varied from partial reduction to no detected chlorophyll. These data suggest a range of kinetic states of chlorophyll degradation in individual guard cells. It is conceivable that loss of turgor in the thin-shaped stomata results from a prolonged lack of guard cell photosynthesis. Whereas kidney-shaped stomata, which had no chlorophyll signal, resulted from a recent, complete, chlorophyll removal. The presence of kidney-shaped chlorophyll-less stomata allowed us to measure direct CO_2 and ABA responses in non-photosynthetic-active stomatal guard cells.

Starch which is stored in guard cell chloroplasts, is considered to function in stomatal conductance regulation by:

(i) the interconversion of starch to sugars, altering guard cell turgor (osmotic changes) (Tallman and Zeiger, 1988); and by (ii) synthesis of malate, which acts as a counter-ion to K^+, from carbon skeletons derived from starch (Outlaw and Lowry, 1977; Willmer and Beattie, 1978; Schnabl, 1980; Hedrich *et al.*, 1985; Reckmann *et al.*, 1990; Asai *et al.*, 2000). The sources of carbohydrates for starch in guard cells can be a product of guard cell photosynthesis or imported from the mesophyll cells (Gotow *et al.*, 1988; Tallman and Zeiger, 1988; Poffenroth *et al.*, 1992; Talbott and Zeiger, 1993, 1996; Kang *et al.*, 2007). The large fraction of thin-shaped stomata implies that guard cell photosynthesis is required for intact stomatal guard cell morphology and physiology. Furthermore, we show that following a reduction in guard cell chlorophyll levels, starch levels reduced significantly by approximately 30%, which supports a model in which guard cell photosynthetic carbon fixation provides a carbon source for starch within guard cells (Lawson, 2009). However, the relative average reduction in (kidney-shaped) guard cell chlorophyll levels by approximately 74% did not correspond to the relative drop in guard cell starch levels. These results suggest the need for additional carbon sources for guard cell starch metabolism, which most likely are imported from the mesophyll or are produced in guard cells prior to chlorophyll removal in the present study. These data are in line with the hypothesis that apoplastic sucrose is a source for guard cell symplastic sucrose and acts as an osmoticum for stomatal opening or replacing guard cell carbon stores (Lu *et al.*, 1997; Ewert *et al.*, 2000; Outlaw and De Vlieghere-He, 2001; Stadler *et al.*, 2003). Nevertheless, we have found that guard cell photosynthesis is critical for intact guard cell turgor production.

In conclusion, using genetic approaches the present study investigates the function of guard cell photosynthesis in stomatal conductance regulation in response to [CO_2] changes, ABA, and in guard cell osmoregulation. CO_2- and ABA-induced stomatal movements are not directly mediated by guard cell photosynthesis. Furthermore, guard cell photosynthesis is found to be critical and indispensable for guard cell energization, osmoregulation, and turgor production.

EXPERIMENTAL PROCEDURES

Experimental procedures can be found in Supporting Information Methods S1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Kidney-shaped stomata of the GC-ChlaseΔN lines show reduced chlorophyll levels.

Figure S2. Guard cell chlorophyll-deficient plants have normal stomatal indices including a large fraction of thin-shaped stomata.

Figure S3. Guard cell chlorophyll-deficient plants show reduction in guard cell starch levels.

Figure S4. Kidney-shaped chlorophyll-less stomata have normal stomatal length.

Figure S5. Impaired carbonic anhydrase activity in *ca1 ca4* does not influence whole leaf CO_2-assimilation responses.

Methods S1. Experimental procedures.

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