Alarm Photosynthesis: Calcium Oxalate Crystals as an Internal CO₂ Source in Plants

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Calcium oxalate crystals are widespread among animals and plants. In land plants, crystals often reach high amounts, up to 80% of dry biomass. They are formed within specific cells, and their accumulation constitutes a normal activity rather than a pathological symptom, as occurs in animals. Despite their ubiquity, our knowledge on the formation and the possible role(s) of these crystals remains limited. We show that the mesophyll crystals of pigweed (Amaranthus hybridus) exhibit diurnal volume changes with a gradual decrease during daytime and a total recovery during the night. Moreover, stable carbon isotope composition indicated that crystals are of nonatmospheric origin. Stomatal closure (under drought conditions or exogenous application of abscisic acid) was accompanied by crystal decomposition and by increased activity of oxalate oxidase that converts oxalate into CO₂. Similar results were also observed under drought stress in Dianthus chinensis, Pelargonium peltatum, and Portulacaria afra. Moreover, in A. hybridus, despite closed stomata, the leaf metabolic profiles combined with chlorophyll fluorescence measurements indicated active photosynthetic metabolism. In combination, calcium oxalate crystals in leaves can act as a biochemical reservoir that collects nonatmospheric carbon, mainly during the night. During the day, crystal degradation provides subsidiary carbon for photosynthetic assimilation, especially under drought conditions. This new photosynthetic path, with the suggested name “alarm photosynthesis,” seems to provide a number of adaptive advantages, such as water economy, limitation of carbon losses to the atmosphere, and a lower risk of photoinhibition, roles that justify its vast presence in plants.

In animals and humans, high levels of dietary oxalate act either as an antinutrient or as the main pathogenesis parameter of hyperoxaluria (Nakata, 2012; Aggarwal et al., 2013) or urolithiasis since 70% of all urinary calculi are composed mainly of calcium oxalate (Vijaya et al., 2013). On the other hand, in photosynthetic organisms, the accumulation of calcium oxalate crystals at high levels is a normal activity tightly integrated with metabolism (Franceschi and Nakata, 2005). The huge variation in distribution among organs, tissues, and cells among plant species suggests multiple independent origins of crystal formation and its functions (Horner and Wagner, 1995). The production of calcium oxalate crystals has a long evolutionary history and probably evolved independently in major clades of symbiotic fungi and several times in the Plantae, as part of the overall process of biomineralization (Horner and Wagner, 1995; Franceschi and Nakata, 2005). Although this independent evolution of the biomineralization process has led to the development of alternative strategies encompassing the formation of other biominerals aside from CaOx crystals, oxalic acid metabolism itself seems to remain significant in defense reactions, irrespective of CaOx presence. For example, the grasses of the Poaceae family, where CaOx crystals are normally absent (Prychid and Rudall, 1999), have adopted the silicon biomineralization strategy (providing protection

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G.K. conceived and coordinated the study; G.K., G.L., D.N., and M.I.K. contributed to the design of the experiments; G.T. and A.G. performed the plant experiments as part of their PhD dissertations supervised by G.K., D.N., and G.L.; P.B. contributed to the plant experiments and the statistical analysis of the CaOx size distribution; M.G.O. and C.G.K. recorded the Raman spectra of CaOx crystals; E.D. carried out the stable carbon isotope composition measurements and C.F. the microscopy measurements; M.I.K. coordinated the metabolomic analyses; G.T. performed the metabolomic data acquisition; M.I.K. and G.T. carried out the multivariate statistical analysis of the metabolomic data; G.T., M.I.K., G.L., and G.K. drafted and G.K. finalized the manuscript.

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against biotic and abiotic stress factors), while the enzymes of oxalate metabolism participate in the resistance against pathogen attack. It is also known that germins, which have oxalate oxidase activity, seem to be specific for the Poaceae family and they have an important role in apoplastic H₂O₂ production induced by abiotic stresses, wounding, and pathogen infection (Lane, 1994; Kärkönen and Kuchitsu, 2015).

Even though the CaOx crystals have intrigued plant scientists for a long time, their functional roles are still poorly understood. Currently, it is considered (Nakata, 2012; Webb, 1999; Franceschi and Loewus, 1995; He et al., 2014) that CaOx crystals are formed for (1) high-capacity regulation or sequestration of calcium, (2) ion balance, (3) detoxification from heavy metals and/or oxalate, (4) light collection and reflection, and (5) plant protection against herbivores. Most of these hypotheses focus on calcium ions, whereas oxalate is considered as a counterion required for calcium binding. On the other hand, plants may not have adopted this relatively convoluted mechanism of calcium sequestration, if not to use the organic part of the crystals for other purposes. It may be reasonable to assume that the oxalate represents a rich source of carbon that could be utilized in the form of CO₂ in photosynthesis. This idea was formulated previously (Karpilov et al., 1978; Loewus, 1999) but has never been proven experimentally, probably because the fate of the CO₂ released from the crystals is difficult to trace. If this hypothesis is true, the crystals should exhibit dynamic behavior that, in turn, should be influenced by the adequacy of the atmospheric carbon supply to the photosynthetic cells. Indeed, complete or partial degradation of the CaOx crystals has been observed (Webb, 1999; Franceschi and Horner, 1980; Calmes and Piquemal, 1977; Ibarslan et al., 2001; Tilton and Horner, 1980), thus supporting their dynamic nature. However, in most cases, these fluctuations in the crystal volume were linked to calcium demands and not to carbon cycling.

*Amaranthus hybridus* (rough pigweed, slim amaranth) is a summer annual C₄ weed species. Its aggressiveness has been related to highly efficient use of water under drought conditions (Weaver and McWilliams, 1980). *A. hybridus* was selected as a model system for this study because of the occurrence of distinct large crystal cells localized within the mesophyll between the veins (Fig. 1A), reaching a high content (14–21% of dry weight).

**RESULTS AND DISCUSSION**

FT-Raman analysis (Kontoyannis et al., 1997) validated that isolated spherical crystal aggregates (drusses; Supplemental Fig. S1) are comprised almost exclusively of calcium oxalate monohydrate (Supplemental Fig. S2). Diurnal fluctuations in the volume of CaOx crystals are reported here (Fig. 2A), although seasonal fluctuation of crystal dimensions (Calmes and Piquemal, 1977) and diurnal fluctuation of oxalate leaf contents (Seal and Sen, 1970; Jones and Ford, 1972) have been previously reported. All the above confirm that CaOx crystals are a dynamic system. According to Figure 2A, after midday, when stomata gradually closed, the mean crystal volume decreased, whereas a complete recovery was attained during the night (Fig. 2A). These observations suggest that the carbon in the CaOx crystals is derived from an alternative source other than the photosynthetic CO₂ fixation, with dark respiration being a prominent possibility. This is further supported by the fact that the ¹³C composition of the isolated crystals (−7.06 ± 0.12, n = 3) was higher than leaf biomass (−16.98 ± 0.22, n = 3).
suggesting a lower $^{13}$C discrimination in the CaOx crystals compared to the leaf bulk organic carbon. This result agrees with previous findings in other species (Raven et al., 1982; Rivera and Smith, 1979). Metabolic profiling indicated the presence of oxalate in the xylem sap (Supplemental Fig. S3), suggesting the root as another possible source of the CaOx crystal carbon. As previously reported, roots not only contain CaOx crystals but they can also metabolize oxalate (Franceschi, 1989; Horner et al., 2000; Kausch and Horner, 1984, 1985).

Crystal decomposition during the day (Fig. 2A) might be related to carbon demands for photosynthesis, the latter being, in turn, influenced by the stomatal function. In order to examine these dependencies, abscisic acid (ABA), a hormonal regulator of plant responses to water stress (Cutler et al., 2010), was applied exogenously on cut leaves. Cut leaves were used to exclude potential interference from root-borne oxalate. We opted for a 6-h-long time-series experiment, measuring the metabolic profile of leaves harvested every hour from both the control and the ABA-treated leaves. Both control and ABA-treated leaves did not demonstrate any macroscopic deterioration during the experiment. In the latter, stomata remained closed throughout the 6-h period (Fig. 2B), whereas a gradual reduction of the crystal volume was recorded (Fig. 2C; also compare Fig. 1, B and C). Despite stomatal closure, the ABA-treated leaves did not exhibit any substantial metabolic decline compared to the control leaves (Fig. 2B). The metabolic stability of ABA-treated leaves was also indicated by the relatively small number of metabolites significantly different between the ABA-treated and the control leaves, i.e. the ‘significant’ metabolites of this comparison (Fig. 4). In addition, the variation in the ABA-treated leaf metabolic profile during the experiment was clearly different, but smaller, than that observed in the control leaves (Fig. 3A). The observed significant decrease in the concentration of disaccharides, e.g. Suc, in the ABA-treated compared to the control leaves (Fig. 4) is consistent with a reduction in the photosynthetic activity expected due to the CO$_2$ starvation. In addition, the significant increase in the concentration of Val agrees with previous observations in ABA-treated or water-stressed plants (Dugas et al., 2011). Finally, Glu plays a signaling role in the activated ionotropic receptors that act as amino acid-gated Ca$^{2+}$ channels (Forde, 2014), and these receptors interact with ABA (Kang et al., 2004). Thus, the observed increase in the concentration of this amino acid may also be related with the regulation of the massive Ca$^{2+}$ flow, which follows crystal decomposition.

The composition of crystals should be accompanied by the release of considerable amounts of oxalate in ABA-treated leaves. However, oxalate was not detected, suggesting its rapid breakdown into CO$_2$ and H$_2$O$_2$ by oxalate oxidase (Lane, 1994). Histochemical detection indicated that this enzyme is mainly localized in the spongy mesophyll cells and, indeed, its activity increased due to the ABA treatment (Fig. 1, D–F). Moreover, the histochemical localization of catalase, the enzyme that cleaves H$_2$O$_2$ to H$_2$O and O$_2$, resembled that of oxalate oxidase, but it exhibited strong staining in both the control and the ABA-treated leaves (Supplemental Fig. S4). In combination, these observations further support the hypothesis that crystal decomposition is correlated with oxalate oxidase and catalase activities. The produced CO$_2$ could be photosynthetically assimilated while the toxic H$_2$O$_2$ could be scavenged by catalase. Then, the photosynthetic metabolism in the ABA-treated leaves would remain active, despite their closed stomata and the accompanied CO$_2$ starvation (Fig. 2B). However, the closed stomata did not permit direct photosynthetic activity measurements in the
ABA-treated leaves. Chlorophyll fluorescence measurements showed that the operational efficiency of the PSII photochemistry ($\Delta F/Fm'$) in the ABA-treated leaves remained substantial despite the CO$_2$ starvation conditions (Fig. 2D). However, the lower $\Delta F/Fm'$ values compared to the control leaves along with the metabolic profiling measurements suggested that the CO$_2$ supply from the breakdown of the oxalate that could be derived from the
Figure 4. Profiling data in significant metabolites of control and ABA-treated leaves. A, Hierarchical clustering of metabolic profiles (Pearson distance metric) based on eleven metabolites with significantly increased (POS, positive) or decreased (NEG, negative) concentration in ABA-treated compared to control leaves, according to significance analysis for microarrays (SAM). B, The time profile of 11 significant metabolites in control and ABA-treated leaves. Numbering (e.g. POS1 and NEG3) indicates hierarchy in significance among metabolites of each group, with 1 depicting most statistically significant (see Supplemental Methods). Color codes, value definitions, and symbols are same as in Figure 3.
crystal decomposition is lower than the CO₂ supply from the open stomata. Indeed, considering the mean photosynthetic activity of the plants used in our experiments (i.e. 13 µmol m⁻² s⁻¹) and the mean leaf CaOx content (i.e. 18% of dry weight), we estimated that under stomatal closure, the amount of CO₂ that can be released from complete crystal decomposition could support the above photosynthetic rate for 70 min or maintain a low photosynthetic rate of 3 µmol CO₂ m⁻² s⁻¹ for 4 to 5 h. These estimates may be conservative because a considerable amount of crystals (located in the stem and in the bundle sheaths) and any root-borne oxalate were not included in the calculations. In addition to oxalate released during crystal decomposition, high amounts of Ca²⁺ ions are also released. A possible mechanism for their quenching could be based on Ca-binding proteins given that the occurrence of such proteins in crystal cells has already been reported (Franceschi et al., 1993; Webb, 1999). Calcium ion management during crystal recycling could be a subject of further studies.

The correlation between the CO₂ starvation conditions and the CaOx crystal decomposition was reinforced by a whole plant experiment, in which four plant species possessing CaOx crystals and representing different functional groups (C₃, C₄, and Crassulacean acid metabolism [CAM] plants; see “Materials and Methods”) were exposed to drought under controlled conditions (A. hybridus, Dianthus chinensis, Pelargonium peltatum, and Portulacaria afra; Table I). Water deficit caused a reduction in leaf water potential and a decrease in stomatal conductance. Importantly, in accordance with all previous observations, this decrease was accompanied by a significant reduction in the crystal volume in all plants (Table I). Oxalate oxidase activity increased in all cases, except for D. chinensis. In this plant, the activity of the enzyme was very high irrespective of the treatment (Table I). The observed differences in plant responses may be explained by the different tolerance to water stress, as it was indicated by the water potential values.

According to our results, the formation of the CaOx crystals within specialized cells can act as a biochemical mechanism that stores carbon in the form of calcium oxalate, mainly during nighttime, when stomata are closed and photosynthetic reactions are inoperative. During the day, the degradation of the crystals provides subsidiary carbon for photosynthetic assimilation, especially under conditions of carbon starvation. The signal is probably given by the hormone ABA. Hence, we suggest this new photosynthetic path to be called “alarm photosynthesis.” This pathway for photosynthetic carbon assimilation could be added to the already known C₄ and CAM photosynthetic metabolic pathways and can be operational in numerous plant species, irrespective of their functional (i.e. C₃, C₄ or CAM) group. Alarm photosynthesis as a syndrome could have evolved multiple times independently in various plant lineages, as in the case of CAM and C₄ syndromes, which are both considered as convergent adaptations to stressful environments (Edwards and Ogburn, 2012). The data of this study support the idea that this is also the case for the alarm photosynthesis, indicating water stress and the consequent carbon starvation within mesophyll tissues as the ultimate selective factor.

The function of alarm photosynthesis may provide a number of competitive advantages: (1) It contributes to carbon and water economy, since carbon acquisition from the atmosphere is associated with water losses. For this reason, we predict that at the interspecific level the frequency of occurrence and the intensity of alarm photosynthesis will tend to be higher in xerophytes (e.g. cacti that are known to contain large quantities of CaOx crystals; see Garvie, 2006). Recent data support that this trend may also exist at the intraspecific level. According to Brown et al. (2013), the number of the CaOx crystals in the phylloides of the Acacia species increases with

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**Table I.** Means comparison of leaf water potential, stomatal conductance, oxalate oxidase activity, and crystal volume in leaves of well-watered and water-stressed plants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A. hybridus (C₃)</th>
<th>D. chinensis (C₄)</th>
<th>P. peltatum (C₄)</th>
<th>P. afra (Succulent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>WW</td>
<td>WS</td>
<td>WW</td>
<td>WS</td>
</tr>
<tr>
<td>Parameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water potential (Ψw) MPa</td>
<td>-0.976</td>
<td>-1.416 **</td>
<td>-0.66</td>
<td>-1.1 **</td>
</tr>
<tr>
<td>±0.077</td>
<td>±0.097 ***</td>
<td>±0.014</td>
<td>±0.152</td>
<td>±0.062</td>
</tr>
<tr>
<td>Stomatal conductance (gₛ)</td>
<td>0.330</td>
<td>0.008 ***</td>
<td>0.236</td>
<td>0.008 **</td>
</tr>
<tr>
<td>mol H₂O m⁻² s⁻¹</td>
<td>±0.019</td>
<td>±0.001</td>
<td>±0.038</td>
<td>±0.001</td>
</tr>
<tr>
<td>Oxalate oxidase activity</td>
<td>39.2</td>
<td>120.9</td>
<td>8,622.1</td>
<td>6,038.7</td>
</tr>
<tr>
<td>mmol H₂O₂ min⁻¹ g⁻¹ FW</td>
<td>±1.8</td>
<td>±26.9</td>
<td>±709.4</td>
<td>±718.9</td>
</tr>
<tr>
<td>Crystal volume µm³</td>
<td>11,580</td>
<td>560 ***</td>
<td>12,383</td>
<td>7,729 **</td>
</tr>
<tr>
<td>±499</td>
<td>±19</td>
<td>±904</td>
<td>±885</td>
<td>±1120</td>
</tr>
</tbody>
</table>
CaOx crystals can maintain a basal level of activity during partial or full stomatal closure. Low CO₂ levels in combination with high light intensities could increase the photooxidative risk and lead to instability of the PSII (Murata et al., 2007; van Rensen, 2002). Thus, alarm photosynthesis could act as a quenching valve for the energy excess accumulated in the electron transport chain, when the light reactions are not in pace with photosynthetic CO₂ assimilation from the atmosphere. (3) Storage of carbon in the form of CaOx crystals confers some metabolic advantages, considering that these deposits are metabolically and osmotically inactive. Only if needed, CO₂ and calcium ions can be released.

CONCLUSION

Drought, in combination with coincident high temperature and light intensity, represents the most important environmental stress factor that limits plant survival and crop productivity (Chaves et al., 2003; Flexas et al., 2006). Research into drought resistance mechanisms has become of great significance, as climate change scenarios predict intensified drought conditions in many parts of the world and concomitant yield reduction (Long and Ort, 2010). Plants in arid regions or seasonally dry environments face a constant trade-off between acquiring atmospheric carbon dioxide for photosynthesis while losing water vapor or saving water with a subsequent reduction of photosynthesis and thus growth. Alarm photosynthesis allows the utilization of large CaOx crystals as dynamic internal carbon pools, irrespective of the availability of the atmospheric CO₂ and thus preventing water losses. This function probably acts harmoniously with the other already known stress adapted CAM and C₄ syndromes and may play a crucial role in the surviving of plants under drought conditions, further justifying the extensive occurrence of CaOx crystals in the Plant Kingdom.

Future investigations on the contribution of alarm photosynthesis in drought tolerance and the mechanisms controlling crystal size will lead to a better understanding of the responses of wild and cultivated plants in a warmer and water limiting planet. Additionally, a systematic quantification of calcium oxalate crystals and an evaluation of the intensity of alarm photosynthesis at the interspecific level in different climatic regions of the planet are needed. Taking into account the estimations that a large tree may produce annually up to several hundred kilograms of calcium oxalate in its aboveground tissues (Horner and Wagner, 1995; Cailleau et al., 2011; see also Garvie 2006), calcium oxalate crystals as part of the phytomineralization process could represent a considerable carbon sink with a long residence time at the ecosystem as well as at the global level. The discovery of alarm photosynthesis gives rise to the investigation of its potential role in global carbon cycle and the effect of climate change on this process.

MATERIALS AND METHODS

Plant Material

Pigweed plants (Amaranthus hypochondriacus, Amaranthaceae [C₄ plant]) were grown from seeds in a growth chamber. China pink plants (Dianthus chinensis, Caryophyllaceae [C₄ plant]), ivy-leaf geranium plants (Pelargonium peltatum, Geraniaceae [C₃ plant]), and elephant bush plants (Portulacaria afra, Portulacaceae [succulent, probably facultative CAM plant]) were obtained from a commercial nursery. All plants were transplanted (A. hypochondriacus plants 10 days after showing) in pots filled with a commercial soil mixture (fertilized peat mixture, pH 5.5–6.5, organic matter 50%, clay kg/m³, perlite 5–10%) and grown under a 12/12-h photoperiod, radiation 400 μmol quanta m⁻² s⁻¹ (Powerstar HQI-BT-400W/D ORSAM), day/night temperature 30/19°C, and air relative humidity 75%.

Microscopy

For scanning electron microscopy, small pieces of leaves were fixed in 3% glutaraldehyde in 0.1 mM phosphate buffer, pH 7.2, for 24 to 36 h at 4°C, dehydrated with a 30 to 100% acetone gradient, critical point dried, mounted on stubs with self-adhesive double-sided carbon discs (Agar Scientific), and sputter coated with gold. Observations and digital micrographs were taken with a JEOL JSM-6360 scanning electron microscope at 20 kV.

Light microscopy, fresh leaves were cut with a cryotome (Leica CM 1850) and observed directly or used for histochemical staining. Sections were observed and photographed with an Olympus BX40 light microscope equipped with an Olympus DP70 digital camera. Images of histochemical staining were enhanced with image processing software (Adobe Photoshop CS5) by correcting for brightness and contrast. These corrections were equally applied to all images (control and ABA-treated leaves).

Measurements of Crystal Density and Size

Crystal density and size were measured in leaves bleached in sodium hypochlorite solution (2%) for 24 h. Images of isolated crystals or crystals in chlorinel-bleached leaves were obtained with a Axioslab microscope (Carl Zeiss) equipped with a digital camera (DSC-S75; Sony), and dimensions were measured by digital image analysis (Image Pro Plus v. 5.1.0.20; MediaCybernetics).

Crystal Isolation

CaOx crystals were isolated by leaf tissue rupture in a homogenizer with isopropanol. After crystal precipitation, pellet was cleared by sequential washings with pure isopropanol and isolation after precipitation until microscopical observation revealed the absence of cell debris (Supplemental Fig. S1).

Histochemical Detection and Enzyme Assays

Cryosections were stained for oxalate oxidase activity using 0.6 mg mL⁻¹ 4-chloro-1-naphthol solution in sucinate buffer (25 mM sucinate and 3.5 mM Fe-Na EDTA, pH adjusted to 4.0 with NaOH) containing 1 unit mL⁻¹ peroxidase (horseradish peroxidase; MP Biomedicals) and 2.5 mM oxalate. Blank staining was done using the same solution omitting oxalate (Dumas et al., 1995).

Staining for catalase activity was done using 3,3-diaminobenzidine (Frederick and Newcomb, 1969). Briefly, sections were incubated with 1 mg mL⁻¹ 3,3-diaminobenzidine solution in Tris-HCl buffer (200 mM Tris adjusted to pH 9.0 with 100 mM HCl) and 0.06% H₂O₂ for 60 min at 30°C. Sections were washed with Tris-HCl buffer and observed directly. Blank staining was done by preincubating sections in 50 mM 3-amino-1,2,4-triazole solution in Tris-HCl buffer for 30 min and incubating in the staining solution containing 50 mM 3-amino-1,2,4-triazole. Oxalate oxidase activity in leaf extracts was measured using 3-methyl-2-benzothiazolinone hydrazone and N,N-dimethylaniline (Laker et al., 1980; Goyal et al., 1999). Extracts were prepared by homogenizing 1 g of leaf tissue in 6 mL phosphate buffer (100 mM phosphate and 500 mM Suc) at pH 7.0 at 4°C and centrifuging at 15,000 × g for 15 min. Activity was tested in both the supernatant and the pellet (suspended in 6 mL of extraction buffer) by adding 225 μL of extract, 20 μL of 300 mM oxalate solution, and 2.5 units of horseradish peroxidase in aqueous solution (5 μL), in 2.7 mL reaction buffer (25 mM succinate and 2.5 mM FeNa EDTA, pH 4.0 adjusted with NaOH). Reaction mixture was incubated at 35°C for 30 min under dim light and stirring. Absorbance was measured during incubation in a double-beam spectrophotometer (UV-160A; Shimadzu).
Shimadzu) at 600 nm and at 5-min intervals using a nonextract photometric blank. Due to the membrane localization of oxalate oxidase in *A. hybrida* (Goyal et al., 1999), activity was only detected in pellet suspensions and not in supernatants.

**FT-Raman Spectra**

Raman spectroscopy of crystals was done as described elsewhere (Kontoyannis et al., 1997). Spectra of isolated crystals were recorded using a FRA-106/S FT-Raman (Bruker) with the following characteristics: The laser excitation line used was the 1064 nm of a Nd:YAG laser. A secondary filter was used to remove the Rayleigh line. The scattered light was collected at an angle of 180° (back-scattering). The system was equipped with a LN2-cooled Ge detector (D1 481). The power of the incident laser was 370 mW, and the focused laser spot had a diameter of 100 μm. The recorded spectra were the sum of 300 scans, and the typical spectral resolution was 4 cm⁻¹. To verify Raman instrument calibration, the cyclohexane bands at 1029 and 1158 cm⁻¹ were used.

**Stable Carbon Isotope Composition of Isolated Crystals and Whole Leaves**

Samples from control leaves or isolated crystals were analyzed with a ThermoScientific Delta Plus mass spectrometer. The samples were introduced into a Thermo-Flash elemental analyzer where CO₂ and N₂ gas were produced by combustion at 1020°C. The gases, moved along in a continuous flow of helium, were separated by a GC column and introduced into a continuous flow gas source mass spectrometer for carbon and nitrogen isotopic ratio determination. The isotopic ratios are expressed for carbon as δ¹³C versus PDB (a marine carbonate). Analytical precision was 0.1‰ for δ¹³C.

**Photosynthetic and Transpiration Parameters**

Stomatal conductance (gₛ) was measured with a portable porometer (AP4; Delta-T Devices) calibrated on the same day. Operational efficiency of PSII photochemistry (Genty et al., 1989; ΔF/ Fm) was measured with a portable chlorophyll fluorometer (PAM 2100; Walz) using red LED measuring light (650 nm, PFD < 0.15 μmol quanta m⁻² s⁻¹). Saturation pulse (white halogen; PFD approximately 15,000 μmol quanta m⁻² s⁻¹) lasted for 0.8 s. Net photosynthetic activity was measured using a portable photosynthesis system (ADC Pro Plus; ADC BioScientific).

**ABA Treatment**

Leaves from 20 plants (three leaves per plant) were cut under water and placed in petri dishes filled with artificial xylem sap (30 leaves) or with artificial xylem sap containing 200 μM ABA (Sigma-Aldrich; 30 leaves). All leaves were placed under light (400 μmol quanta m⁻² s⁻¹; Powerstar HQI-BT-400W/D; OSRAM) at 30°C for 6 h. Measurements of gₛ and chlorophyll fluorescence parameters were undertaken every hour using five leaves from each treatment. Immediately after, leaves were removed for microscopic examination and measurement of crystal size.

**Water Stress Treatment and Water Potential Measurements**

Ten plants were grown as described previously. After 30 d of growth, water stress was imposed on five plants by retarding irrigation for 7 d, whereas the rest of the plants received normal irrigation. Leaf water potential was measured using a Scholander pressure chamber.

**Metabolomic Data Acquisition and Analysis**

The gas chromatography-mass spectrometry metabolic profile acquisition of the control and ABA-treated leaves was carried out after appropriate adaptation of previously published protocol (Dutta et al., 2009; Kanani et al., 2010). The metabolomic profile of each physiological state/time point was estimated from the mean profile of two separate leaves harvested at the particular state, and the profile of each leaf was measured at least three times. Specific details are provided in Supplemental Methods and Supplemental Data Set S1. After appropriate data correction, normalization, and filtering (Kanani et al., 2010; Kanani and Klapa, 2007), profiles of 55 metabolite derivatives were considered in the multivariate statistical analysis using the hierarchical clustering analysis, principal component analysis, and significance analysis for microarrays algorithms implemented in the ‘omic’ data analysis software TM4 MeV (v4.8.1) software (Saeed et al., 2003, 2006). Further details on the metabolomic data normalization, filtering, and statistical analysis can be found in Supplemental Methods.

**Supplemental Data**

The following supplemental materials are available.

Supplemental Figure S1. Isolated crystals from control leaves of pigweed plants using polarizing microscopy.

Supplemental Figure S2. Typical Raman spectrum of isolated crystals of pigweed leaves.

Supplemental Figure S3. MS-reconstructed chromatogram of xylem sap, indicating presence of oxalate peak.

Supplemental Figure S4. Histochemical detection of catalase activity.

Supplemental Figure S5. Paired SAM curve normalized with C0 metabolic profiles of ABA treated with respect to control leaves considered in metabolomic analysis.

**Supplemental Data File S1.** Raw data set of metabolomics analysis (as Excel file).

**Supplemental Methods.**

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