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Short Communication

Accumulation of lipid peroxide-derived, toxic α,β-unsaturated aldehydes (*E*)-2-pentenal, acrolein and (*E*)-2-hexenal in leaves under photoinhibitory illumination

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Abstract Lipid peroxide-derived α,β-unsaturated aldehydes (2-alkenals) are potent toxins that inhibit enzymes in the Calvin cycle and in the mitochondrial photorespiratory pathway. Production of 2-alkenals in illuminated leaves and their participation in cellular damages have been suggested from the observation that the transgenic tobaccos overexpressing 2-alkenal reductase (AER), a 2-alkenal-detoxifying enzyme, showed tolerance to strong light. In order to identify the involved 2-alkenal species, we here analyzed the leaf aldehyde compositions in the AER-overproducing tobaccos and wild type (SR1), and compared their changes under photoinhibitory light. Aldehydes were extracted from the leaves at an early stage of photoinhibition, derivatized with 2,4-dinitrophenylhydrazine and analyzed on reversed-phase HPLC. In dark-adapted leaves of these tobacco lines, more than 40 carbonyl species were found, of which 17 were identified. In SR1 leaves, 2-alkenals such as (*E*)-2-pentenal, acrolein, and (*E*)-2-hexenal were increased by 70–290% after 30 min-illumination. In the leaves of AER-tobaccos, light-dependent increase of these 2-alkenals was apparently lower. Thus the production of highly reactive 2-alkenals in leaves was enhanced under photooxidative stress. The tolerance to strong light due to the overexpression of AER can be explained by the scavenging of these species.

Key words: Acrolein, 2-alkenal reductase, At5g16970, (*E*)-2-hexenal, (*E*)-2-pentenal.

Lipid peroxide (LOOH)-derived aldehydes and ketones (hereafter collectively designated as carbonyls) are recently recognized as cell signals and toxic species in plant stress responses. Carbonyls at low concentrations can induce stress defense genes (Sattler et al. 2006; Weber et al. 2004), while at high concentrations, they can cause damages to cell components (O'Brien et al. 2005). Recent studies have shown that enhanced detoxification of carbonyls in transgenic plants improved their tolerance to various environmental stresses, as follows: Aldehyde reductase from *Medicago sativa* (alfalfa) overexpressed in *Nicotiana tabacum* (tobacco) improved tolerance to drought stress (Oberschall et al. 2000), methyl viologen and UV-B (Hideg et al. 2003). Aldehyde dehydrogenase isozymes from *Arabidopsis thaliana* improved the tolerance of transgenic *A. thaliana* plants to NaCl, heavy metals, methyl viologen, and H_2O_2 (Sunkar et al. 2003). Vice versa, the dehydrogenase-knockout mutants were more sensitive to dehydration and salt than wild type (Kotchoni et al. 2006). The observed correlation between the extent of damages and the content of aldehydes, as thiobarbituric acid-reactive substances, in these studies strongly supports that aldehydes are causes of stress-induced damages in vivo. It is known that a great variety of carbonyls are produced from LOOH (Grosch 1987; Kawai et al. 2007) and many of them occur in plant tissues (Schauenstein et al. 1977), but it has been scarcely investigated what carbonyl species are involved in stress-induced damage of plant cells.

Among LOOH-derived carbonyls, α,β-unsaturated aldehydes (2-alkenals) have potent cytotoxicity due to their high electrophilicity to form adducts with thiols, amines, and imidazoles in proteins and with nucleotide bases (Esterbauer et al. 1991). To photosynthesis, 2-alkenals showed greater toxicity than saturated aldehydes in vitro (Mano et al. 2009). In *A. thaliana*, we found a novel enzyme, 2-alkenal reductase (AER), to detoxify 2-alkenals (Mano et al. 2002). We have made transgenic tobaccos that overexpressed the AER gene At5g16970.

Abbreviations: AER, 2-alkenal reductase; DNPH, 2,4-dinitrophenylhydrazine; FW, fresh weight; HHE, 4-hydroxy-(*E*)-2-hexenal; HNE, 4-hydroxy-(*E*)-2-nonenal; PI, photoinhibitory illumination; PRK, phosphoribulokinase; PSII, photosystem II; rRT, relative retention time
This article can be found at http://www.jspcmb.jp/

As compared with the basal AER activity in leaves of SR1 plants, three independent transgenic lines showed 100–200 fold higher AER activity in leaves, and 4–6 fold higher in roots, and showed tolerance to photooxidative treatments with strong light or methyl viologen (Mano et al. 2005) and to aluminum treatment on roots, which induced the formation of reactive oxygen species (Yin et al. 2010). In order to investigate the carbonyl species that are relevant to plant stresses, we have recently developed an analysis method (Matsui et al. 2009) and found that, on aluminum treatment, various 2-alkenals such as acrolein and 4-hydroxy-(*E*)-2-nonenal (HNE) were increased in the roots of sensitive SR1 line in association with the cell injury, while their increase was suppressed in the tolerant transgenic lines (Yin et al. 2010). This demonstrated that 2-alkenals are produced in oxidative stress and cause cellular damage (Figure 1). In this study, aiming at finding the carbonyl species that are relevant to photooxidative stress in leaves, we analyzed the changes of distinct carbonyls in leaves on photoinhibitory illumination. By comparing the results obtained for the phototolerant AER-overproducers and for the photosensitive wild type, we found that 2-alkenals such as acrolein, (*E*)-2-pentenal and (*E*)-2-hexenal correlated with the photoinhibition.

Tobacco (*N. tabacum*) cv Petit Havana SR1 and its transgenic lines P1#14 and P1#18, both overproducing *A. thaliana* AER (Mano et al. 2005), were cultured on soil supplemented with Hyponex in a growth chamber with day (16 h; 100 μmol photons $m^{-2} s^{-1}$ with white-fluorescent lamps) and night (8 h) temperatures of 25°C and 15°C, respectively. Extraction of carbonyls, their derivatization with 2,4-dinitophenylhydrazine (DNPH) and their separation with a reverse-phase HPLC are described elsewhere (Matsui et al. 2009). Briefly, we collected one leaf per plant, and central parts (0.5 g fresh weight) except for midrib were incubated in acetonitrile (5 ml) containing 2-ethylhexanal (12.5 nmol; as the internal standard (I. S.)) and 0.005% (w/v) butylhydroxytoluene at 60°C for 30 min. Carbonyls were derivatized with 0.5 mM DNPH and 0.5 M formic acid in the extract (25°C, 60 min), thereafter mixed with saturated NaCl solution (5 ml) and $NaHCO_3$ (0.5 g), and centrifuged. The derivatives collected in the acetonitrile phase were dried in vacuo, and the residual was dissolved in acetonitrile (0.2 ml), and passed through a BondElute C18 cartridge (sorbent mass 200 mg, Varian), which trapped pigments. The pass-through and an additional wash with 0.3 ml acetonitrile were collected and a 10 μl aliquot was injected into a Wakosil DNPH-II column (4.6×150 mm, Wako Pure Chemical) with the following elution condition (1 ml min^{-1} flow rate): 0–5 min, 100% Eluent A (Wako); 5–20 min, a linear gradient from 100% A to 100% Eluent B (Wako); 20–25 min, 100% B and 25–45 min, a linear gradient from 100% B to acetonitrile. Detection wavelength was 340 nm. Carbonyls corresponding to the peaks were identified based on their retention time as compared with dinitrophenylhydrazones of authentic carbonyls (Matsui et al. 2009). (*Z*)-3-Hexenal was purchased from Bedoukian, care of Shouei-Kagaku Co., Ltd. (Tokyo, Japan). HNE was from Alexis Japan, care of Biolinks K. K. (Tokyo). 4-Hydroxy-(*E*)-2-hexenal (HHE) was prepared from acid-hydrolysis of HHE acetal, which was synthesized according to Rees et al. (1995). Peaks that did not match any known standard carbonyls were represented by their retention time relative to that of I. S. (relative retention time; rRT).

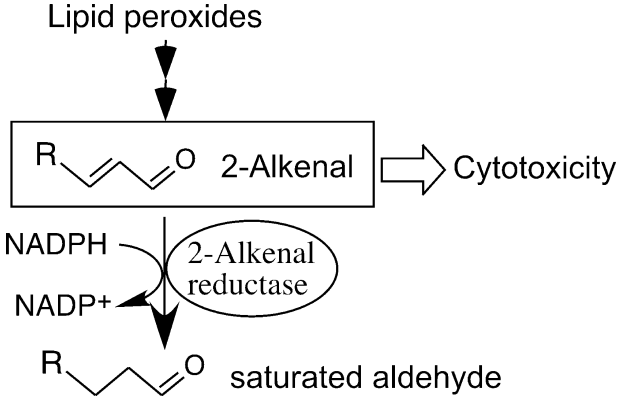

Figure 1. Action of 2-alkenals and the catalytic activity of AER.

In the leaves of plants that had been adapted in darkness for 2 h, we could distinguish more than 40 species of the dinitrophenylhydrazone-carbonyls (Supplemental data), of which 17 identified carbonyls are listed in Table 1. From the peak area relative to that of the I. S., the content of each carbonyl species were determined. It was found that several C1–C9 carbonyls were contained in tobacco leaves at the level of nmol (g fresh weight (FW))$^{-1}$, even without photooxidative production of reactive oxygen species. Some carbonyls such as formaldehyde, acetaldehydes and acetone were contained at more than 10 nmol (g FW)$^{-1}$. Their abundance in leaves fits the fact that these are major oxygenated volatile organic compounds emitted from leaves (Fall 1999). Interestingly, highly reactive 2-alkenals such as acrolein, crotonaldehyde, (*E*)-2-pentenal, (*E*)-2-hexenal, HHE and HNE were contained at the level of 2-5 nmol (g FW)$^{-1}$. These 2-alkenals in dark-adapted leaves probably represent the constitutive lipid peroxidation due to the reactive oxygen species produced in association with mitochondrial respiration. The occurrence of HNE in mitochondria from non-stressed *Arabidopsis* leaves has been recently shown as immunological detection of the HNE-modified proteins (Winger et al. 2007). Also in the roots of non-stressed tobaccos, acrolein, HHE, HNE and (*E*)-2-heptanal were contained at nmol (g FW)$^{-1}$ level (Yin et al. 2010). Thus

Table 1. Contents of carbonyls identified in tobacco leaves.

Carbonyl	Leaf content/nmol (g fresh weight)$^{-1}$		
	SR1	P1#14	P1#18
formaldehyde	21.62 ± 0.59	24.18 ± 1.07	25.94 ± 1.35
acetaldehyde	8.32 ± 1.05	11.80 ± 1.18	16.48 ± 0.86
HHE	3.52 ± 0.26	3.61 ± 0.13	3.86 ± 0.42
acetone	7.79 ± 0.57	10.63 ± 0.42	12.19 ± 1.12
acrolein	3.38 ± 0.36	2.30 ± 0.26	4.24 ± 1.08
propionaldehyde	2.92 ± 0.23	3.38 ± 0.40	4.39 ± 0.44
crotonaldehyde	0.98 ± 0.12	2.04 ± 0.54	1.42 ± 0.19
butyraldehyde	1.06 ± 0.18	1.18 ± 0.05	1.92 ± 0.29
phenylacetaldehyde	1.04 ± 0.02	1.21 ± 0.26	3.62 ± 0.32
(*E*)-2-pentenal	1.49 ± 0.26	1.21 ± 0.24	2.49 ± 0.68
HNE	2.70 ± 0.24	2.89 ± 0.33	3.69 ± 0.30
n-pentanal	10.79 ± 0.96	9.17 ± 0.69	13.04 ± 0.73
(*Z*)-3-hexenal	26.77 ± 13.14	3.11 ± 2.18	4.63 ± 1.97
(*E*)-2-hexenal	4.11 ± 1.10	1.13 ± 0.91	2.73 ± 0.75
n-hexanal	0.92 ± 0.04	1.13 ± 0.08	1.14 ± 0.11
(*E*)-2-heptenal	0.96 ± 0.26	0.33 ± 0.06	0.99 ± 0.24
n-heptanal	0.38 ± 0.12	0.22 ± 0.03	0.66 ± 0.11

Fully expanded leaves of 8–10 week-old plants were harvested after a 2 h-dark adaptation. Carbonyls were extracted, derivatized with DNPH, analyzed with HPLC as described in the text, and listed in the order of elution (see Supplemental data). Average ± standard deviation of 3 individual plants. Contents of identified aldehydes were corrected for the DNP-derivatization efficiency and for the absorbance at 340 nm (Matsui et al. 2009).

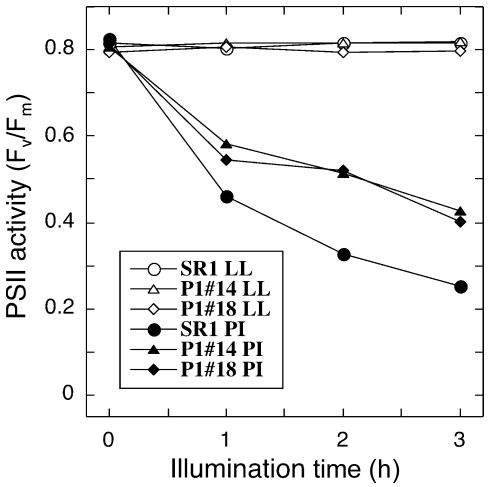

Figure 2. Photoinhibition time course of AER-overproducing lines and SR1. Leaves attaching to plants of SR1 and two AER-overproducing lines (P1#14 and P1#18) were illuminated at 2,000 μmol m^{-2} s^{-1} (PI) and 10 μmol m^{-2} s^{-1} (low light; LL). For measurement of chlorophyll fluorescence, illumination was interrupted at the indicated time, and the leaf was kept in darkness for 15 min in order to obtain the maximal photochemical yield of PSII (F_v/F_m). Average of 2 runs.

the observed levels of these aldehydes in non-stressed cells can be regarded as physiologically basal levels. The value 1 nmol (g FW)$^{-1}$ corresponds with the tissue concentration of 1 μM if these species are distributed homogenously in the leaf tissue. The real concentrations in certain cellular compartments would be higher by 1 or 2 orders of magnitude, and under stress conditions they will be increased, as described below.

For identifying the carbonyls that are correlated with photooxidative injury, we first optimized the illumination condition. A fully expanded mature leaf attaching to an 8-10 week-old plant was illuminated at 2,000 μmol PPFD m^{-2} s^{-1} with a metal halide lamp (LA-180Me, Hayashi Watch-Works, Co., Ltd., Tokyo, Japan). The maximal photochemical yield of photosystem II (PSII), as determined by the parameter F_v/F_m of chlorophyll fluorescence (van Kooten and Snel 1990), using a MINI-PAM chlorophyll fluorometer (Walz, Dordrechet, Germany), decreased in both SR1 and AER-overproducing lines, and the decrease was significantly smaller in the latter (Figure 2). This loss of PSII activity correlated with the increase in ion leakage from leaf disks, an indicator of global cell death (Mano et al. 2005). We expected that the carbonyls causing cell injury should have been produced and accumulated at early stages of photoinhibitory illumination (PI) before the damage become apparent. Also, the content of such species, most probably 2-alkenals, should be lower in the phototolerant AER-overproducing lines than in the sensitive SR1 line. Thus, leaves were collected at 30 min PI, and the carbonyl contents in them were compared with those in the dark-adapted leaves.

Typical chromatograms of the carbonyls obtained from dark-adapted and PI-treated leaves are shown in Figure 3. In the SR1 leaves (top panel), some peaks of DNP-derivatives of carbonyls were increased by PI, but others did not change. These PI-induced carbonyls are most probably produced in chloroplasts because the plastid constitutes a major fraction of lipids in the leaf cell and they are rich in polyunsaturated fatty acids. Noticeably, in P1#14 and P1#18 (middle and bottom panels), increase of some PI-induced carbonyls was apparently smaller (indicated by arrows). We picked up these peaks and examined the difference of PI-induced changes.

Of the separated peaks, six species of carbonyls were found to increase by PI more in SR1 leaves than in the two AER-overproducing lines (Figure 4). Three were identified as (*E*)-2-pentenal, acrolein and (*E*)-2-hexenal. The rest (rRT0.556, rRT0.527 and rRT0.758) did not correspond with any known carbonyls. In contrast to these 6 carbonyls, malondialdehyde, a typical aldehyde that is correlated with various environmental stresses, was not detectable in the tobacco leaves in this experiment. (*E*)-2-Pentenal showed the largest increase in SR1 by 11.7 nmol (g FW)$^{-1}$, a 290% of the value in dark-adapted leaves. In the leaves of AER-overproducers P1#14 and P1#18, its increase was 4.7 and 3.8 nmol (g FW)$^{-1}$, respectively. Increases of acrolein and (*E*)-2-hexenal in SR1 leaves were both 6.7 nmol (g FW)$^{-1}$, 150% and 70% of the respective values in dark-adapted leaves. In the AER-overproducing lines, their increases were apparently smaller than those in SR1, and in most

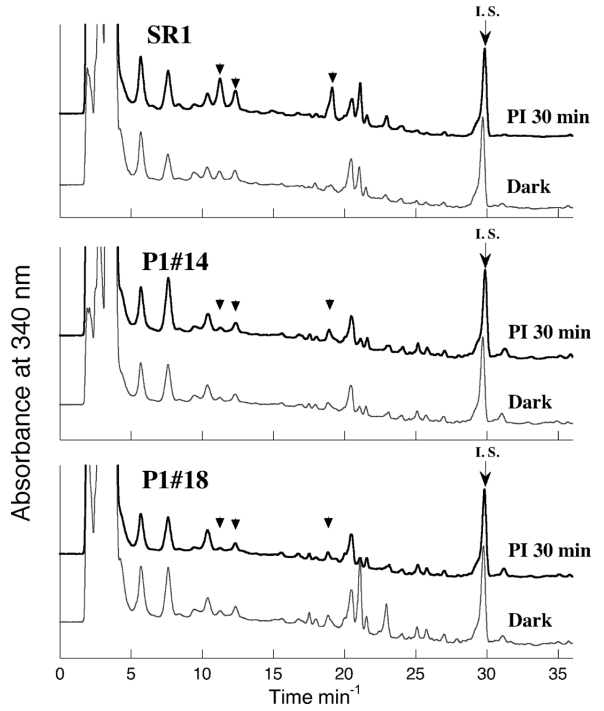

Figure 3. Typical chromatograms of the dinitrophenylhydrazone-derivatives of carbonyls extracted from dark-adapted (lower trace) and PI-treated (upper trace) leaves of SR1, P1#14 and P1#18 lines. The peak just before 30 min represents 2-ethylhexanal added as I. S. Arrows indicate the peaks that showed apparently larger increase by PI in SR1 than in the two transgenic lines.

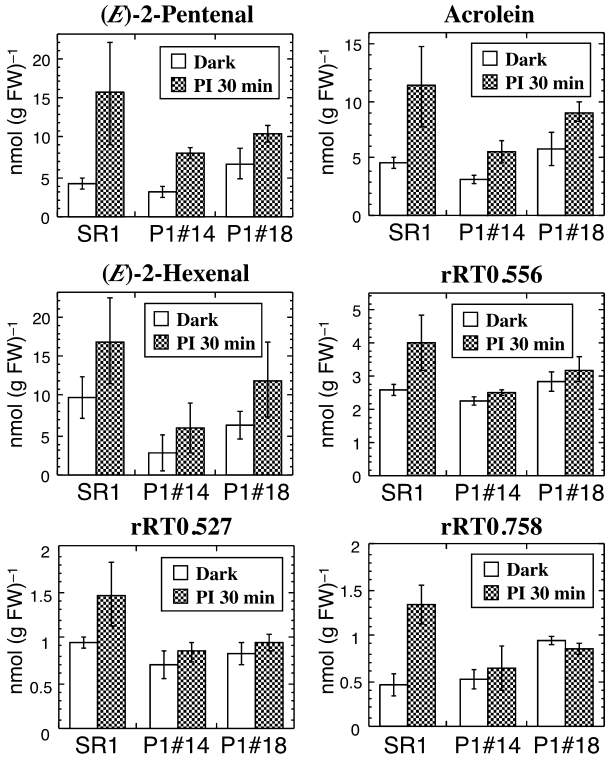

Figure 4. PI-inducible carbonyls that were suppressed in AER-overproducing tobaccos. FW, fresh weight.

cases they were less than half. The other three unidentified carbonyls behaved similarly, i.e., increased to higher levels in SR1 than in the AER-overproducing lines, upon PI (Figure 4). Such correlation of these 2-alkenals with PI-induced damages suggests that they were involved in the cell injury, as demonstrated for aluminum-stressed roots (Yin et al. 2010).

The increased levels of the above-identified 2-alkenals correspond to the order of 10 μM in the leaf cell. Because the PI-induced production of the 2-alkenal should be primarily localized in chloroplasts as discussed above, their intra-plastidic concentrations could reach sub-mM levels. We have previously found that acrolein and (*E*)-2-hexenal at 2 mM inactivated CO_2-photoreduction in isolated chloroplasts by 100% and 45%, respectively (Mano et al. 2009). Also in *A. thaliana* leaves, acrolein caused greater damage to photosynthesis than (*E*)-2-hexenal, when added exogenously (Alméras et al. 2003). These results support that the 2-alkenals found in this study, especially acrolein, can exert toxicity to leaf cells.

In order to compare the toxicity of the three PI-relevant 2-alkenals found here, we determined their effects on the activity of phosphoribulokinase (PRK), the most sensitive enzyme to acrolein (Mano et al. 2009). Stroma fraction was prepared from spinach chloroplasts after hypotonic rupture and desalting, and was treated with 0.5 mM acrolein, (*E*)-2-pentenal or (*E*)-2-hexenal. After 15 min-incubation at 25°C, aldehydes were quenched with 1.5 mM cysteine, and then the mixture was passed through a Sephadex G25 column. PRK activity was determined as previously reported (Mano et al. 2009). Acrolein inactivated PRK by 98.2 ± 0.4%, (*E*)-2-pentenal 21.1 ± 5.9% and (*E*)-2-hexenal 8.8 ± 6.4%, respectively (PRK activity in untreated control was 720 μmol NADPH (mg chlorophyll)$^{-1}$ h^{-1}). Thus acrolein was the most toxic. Because these 2-alkenals were increased on PI by comparable amounts (5–10 nmol (g FW)$^{-1}$ in SR1; Figure 4), acrolein probably had the largest effect in leaves. (*E*)-2-Hexenal is one of the typical 'green leaf volatiles' and has been known to associate with biotic stress responses (Matsui 2006), but its emission from PI-treated *Phragmites* and *Arabidopsis* leaves has also been reported (Loreto et al. 2006). When it is increased to a high level as observed in the present study, (*E*)-2-hexenal would also contribute to the damage considerably.

Because the thylakoid electron transport chain is rather insensitive to 2-alkenals (Mano et al. 2009), primary targets of acrolein produced during PI would be Calvin cycle enzymes. The tolerance of PSII against PI in the AER-lines (Figure 2) was therefore an indirect effect of AER, as follows: In SR1, when the CO_2-fixation rate is lowered by the 2-alkenals photoproduced in chloroplasts, the electron sink capacity is reduced, and a

light-excess status is resulted, causing inactivation of PSII and simultaneously enhancing the production of reactive oxygen species. In the AER-overproducing plants, the 2-alkenal levels in chloroplasts are not increased, and thereby the electron sink capacity is maintained even under strong light. Thus the leaves can avoid a 'light excess' status. We are now testing this hypothesis by determining acrolein-modification of chloroplast proteins in PI-treated leaves.

In conclusion, we found that acrolein, (*E*)-2-pentenal and (*E*)-2-hexenal were increased in leaves on photoinhibitory illumination. These 2-alkenals are very likely involved in the photooxidative damage of leaf cells because (i) they were increased at early stages of the stress treatment, (ii) their increases were smaller in the phototolerant lines, and (iii) they have strong in vitro toxicity to photosynthesis. These results provide direct evidence for the production of such toxic molecules in vivo and support their toxic effects in photooxidative stress. Thus 2-alkenals are, like reactive oxygen species, critical stress factors in leaves. The insight obtained here will be useful for creating molecular strategies to establish environmental stress-tolerant crops.

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