Relationship between Glutathione and Dark-induced Senescence of Brassica campestris

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The effects of compounds that influenced the redox condition on the senescence of detached Brassica campestris leaves were examined. Glutathione treatment promoted the senescence of detached Brassica leaves in the dark. Glutathione treatment also increased the rate of respiration and serine content in the dark. The bithionine sulfoximine (a potent and specific inhibitor of γ-glutamylkeystone synthetase (EC 6.3.2.2)) strongly suppressed leaf senescence. These findings suggest that the intracellular glutathione concentration may be involved in importance for leaf senescence.

Key words: glutathione; senescence; bithionine sulfoximine; chlorophyll

Leaves senesce by gradually losing their biological activity after they are fully developed, subsequently abscise, and then die. Not only the age of the organ but environmental conditions profoundly affect this process. The process of leaf senescence has been believed to be a cellular event programmed in the nucleus.11 Research about plant senescence is very important for the storage of vegetables, flowers, and fruits.

Evidence has accumulated that peroxidase-based reactions may play a role in senescence. Lauriere23 showed that peroxidase activity often increased during senescence. It has been reported that H₂O₂ is involved in regulating senescence of a variety of plants.3 - 5) Hurung and Kao9 reported that flooding-enhanced chlorophyll degradation of tobacco leaves was due to a phenolic-peroxidase-H₂O₂ system. Also, damage to plants arising from environmental stress often appears to be caused by oxidants. For example, increased oxygen toxicity can be caused by air pollutants7 such as ozone and sulfur dioxide,8,91 certain herbicides,101 chilling,111 and microbial infection.121

Glutathione (γ-glutamyl-t-cysteinyl-glycine) is a low molecular weight thiol used in a wide range of metabolic processes.13) The functions of glutathione in higher plants include storage and transport of reduced sulfur, maintaining protein thiol, protecting membranes against peroxidation, and detoxifying of xenobiotics.14 - 18) These observations suggest that glutathione acts by mediating the response of plant cells to biological stress. The glutathione level has been reported to be increased by flooding the tobacco leaf90 and leaf tissue of Arabidopsis thaliana during dark-induced senescence, but it is not clear how glutathione is related to leaf senescence.

In this paper, we tried to treat detached Brassica leaves with glutathione or a glutathione synthesis inhibitor. When detached leaves were treated with glutathione in the dark, chlorophyll contents rapidly decreased. The relationship between glutathione and leaf senescence in the dark is discussed.

Materials and Methods

Plant material. Brassica campestris (L. Okiyo) seeds were sterilized in 1% sodium hypochlorite solution for 20 min. After subsequent germination on 0.8% agar in the dark at 25 C for 3 days, seedlings were transferred in pots and grown in a greenhouse for 3 weeks.

To induce senescence, 3-week old Brassica leaves were detached from the stem and steeped in 20 ml of the test solution and left in the dark or light.

Measurement of glutathione and free amino acid. For GSH + GSSG measurement, leaves (1 g fresh weight) were ground using a chilled mortar and pestle with 4 ml of 20 mm EDTA and deproteinized by 1 ml of 50% metaphoric acid. The homogenate was centrifuged at 30,000 x g for 15 min at 4 C, and the supernatant was used to measure GSH + GSSG by the DTNB-GSSG reductase method.19)

For GSSG and free amino acid measurements, leaves (1 g fresh weight) were ground using a chilled mortar and pestle with 0.25 ml lithium citrate buffer, pH 2.2. The homogenate was centrifuged at 30,000 x g for 15 min at 4 C. The supernatant was analyzed using an amino acid analyzer (Hitachi L-8500).

Measurement of chlorophyll contents. Leaves (1 g fresh weight) were ground using a chilled mortar and pestle with 20 ml of 95% ethanol. The homogenate was centrifuged at 30,000 x g for 15 min at 4 C. The supernatant was used to measure chlorophyll contents using Highman's20 method.

Measurement of respiration rate. The respiration rate was measured for CO₂ generation. CO₂ generation was measured using leaves in sealed containers and drawing with 1.0 ml gas samples after 1 h. CO₂ was measured on a gas chromatography with a charcoal column and thermal conductivity detector.

Other method. All data points represent the mean of three replicate experiments.

Results

Chlorophyll degradation during storage

A prominent event during the senescence of green leaves is the decrease in the chlorophyll content, which is generally used as an indicator of senescence. Figure 1A show that 3 mm GSH was effective in promoting the degradation of chlorophyll under the dark conditions. On the other hand, bithionine sulfoximine (BS) could prevent degradation of chlorophyll in the dark. It is known that BS is an irreversible inhibitor of γ-glutamylkeystone synthetase and a glutathione depleting agent.21,22) The effect of glutathione on chlorophyll degradation in the dark was stronger than that in the light (Fig. 1B).

Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; GSH + GSSG, total glutathione; BS, bithionine sulfoximine.
Changes in redox condition on addition of exogenous GSH

In untreated detached leaves, GSH + GSSG contents decreased by about 30% within the first day, then increased, and reached about 150% after 5 days (Fig. 2A) in the dark. We think that the decrease in GSH + GSSG within 1 day after cutting is a response to elimination of an active oxidant. In untreated leaves, GSSG also increased in the dark (Fig. 2B). GSH + GSSG levels were monitored in detached *Brassica* leaves following the addition of GSH or BS to the steeping solution at a final concentration of 3 mM in the dark. With the addition of 3 mM GSH, intracellular GSH + GSSG levels increased 2.4-fold within 2 days compared to the initial day, and maintained this level after 4 days in the dark (Fig. 2A). With exposure of the detached *Brassica* leaves to 3 mM GSH in the dark, GSSG levels increased faster than GSH, resulting in a 6-fold change within 2 days compared to the initial day (Fig. 2B). We tried to change the GSH concentration in steeping solution (5 mM, 1 mM, or 0.5 mM), but incorporated GSH was the same as 3 mM GSH steeping solution (data not shown). We guess that some regulation may work on the incorporation of GSH. Exposure to BS decreased the internal thiol level in detached *Brassica* leaves in the dark and reached almost zero within 2 days (Fig. 2A). Under light, the level of GSH and GSSG in leaves treated with 3 mM GSH increased a little (Figs. 3A, B). These experiments suggest that chlorophyll loss in detached *Brassica* leaves in the dark is greatly influenced by intracellular glutathione concentrations.

**Change in respiration and free amino acids**

The respiration rate and free amino acid levels were monitored in detached *Brassica* leaves following the addition of GSH or BS to the steeping solution at a final concentration of 3 mM under the dark conditions. In detached leaves with the addition of GSH in the dark, a significant increase in the respiration rate was observed during senescence (Fig. 4). After 3 days, the respiration rate in GSH treated leaves decreased only slightly. On the other hand, when detached leaves were incubated with 3 mM BS, the respiration rate slowly decreased. Under light, the respiration rate did not change during storage (data not shown).

We also measured the free amino acid contents in detached leaves incubated in the dark (Fig. 5), because we suppose that the increase on GSH in the dark affected the
increase in free amino acids (substrate of GSH). During dark incubation, the total free amino acids gradually increased. The serine contents especially increased much faster than the other free amino acids. The serine increased more rapidly in GSH-treated leaves than the control.

Discussion
This investigation showed that glutathione in *Brassica* leaves increased during dark-induced senescence and glutathione treatment accelerated leaf senescence still more. These results are in agreement with an earlier report about tobacco leaves. We also demonstrated that BS prevented leaf senescence as judged by chlorophyll degradation. These results suggest that the intracellular glutathione concentration may be involved in importance for leaf senescence.

It has been proposed that active oxygen species are involved in the deterioration of cells in senescing leaf tissue. Activated oxygen species, such as superoxide, hydrogen peroxide, and their interaction products may be involved in the oxidative processes required during the initiation and the promotion of senescence. These may oxidize sulfhydryl groups and thereby enhance the onset of senescence. We think that active oxygen species induce the synthesis of glutathione (Fig. 2A) and its oxidation (Fig. 2B). In the dark, oxidized glutathione can’t be reduced by the glutathione reductase system because of the lack of NADPH.

On the other hand, a long dark incubation may induce proteolysis and transport free amino acids to new leaves. DeKok et al. observed an increase of cysteine in senescing leaves in *A. thaliana* during dark incubation. The increase in glutathione after 2 days of dark incubation may be for the greater part due to the enhanced oxidation and supply of free amino acids especially serine (precursor of cysteine) (Fig. 5). Our data support the suggestion of Feller and Keist that accumulation of amino acids in detached leaves during senescence is due to a missing or heavily reduced sink capacity of the detached tissue.

Finally, our results indicated that an increase in glutathione is not a primary event during the onset of senescence but promotes the next stage of senescence. Also, glutathione accelerates the respiration rate of dark incubated *Brassica* leaves, and may contribute to the rapid progress of senescence. More studies are required to ascertain whether glutathione selectively attacks specific metabolites or generally continues the oxidized condition.

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References


