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Article Addendum

The contribution of carbohydrates including raffinose family oligosaccharides and sugar alcohols to protection of plant cells from oxidative damage

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Recently we have reported that high intracellular levels of galactinol and raffinose in Arabidopsis plants overexpressing the heat shock transcription factor A2 or galactinol synthase are correlated with increased tolerance to methylviologen treatment and salinity or chilling stress, and galactinol and raffinose also are found to effectively protect salicylate from attack by hydroxyl radicals in vitro.[1,2] These findings indicate that galactinol and raffinose act not only as osmoprotectants, but also as antioxidants in the leaves of Arabidopsis plants. At the same time, we found that the rate constant ($1.1 \pm 0.29 \times 10^{10}$ $M^{-1}s^{-1}$) for the reaction between stachyose and hydroxyl radicals was higher than those of galactinol and raffinose and typical antioxidants. The accumulation of stachyose was only observed in seeds. Furthermore, glucose, fructose and sucrose which are abundant in higher plants efficiently scavenge hydroxyl radicals. Judging from the radical scavenging activity and the intracellular level of each compound reported here and previously, we suggest that carbohydrates including raffinose family oligosaccharides and sugar alcohols are present at high levels under normal and/or stressfull conditions, and act as antioxidants to protect plant cells from oxidative damage and maintain redox homeostasis.

Plants, unlike animals, are unable to move and therefore potentially subject to various forms of environmental stress such as high-light, heat-shock, drought, chilling, salinity and air pollution. It is generally accepted that the imposition of environmental stress gives rise to excess concentrations of reactive oxygen species (ROS), and that much of the injury to plants caused by exposure to stress is associated with oxidative damage at the cellular level.[3] Therefore, antioxidants and antioxidant enzymes such as ascorbate (AsA), glutathione (GSH), superoxide dismutase, AsA peroxidase, GSH peroxidase, thioredoxin peroxidase and catalase function to interrupt the cascades of uncontrolled oxidation in some organelles.[4]

The raffinose family oligosaccharides (RFOs), such as raffinose, stachyose and verbascose, are synthesized from sucrose by the subsequent addition of activated galactose moieties donated by galactinol.[5] Recently, it has been reported that the expression of enzymes related to the biosynthesis of galactinol and RFOs and their intracellular accumulation in plant cells are closely associated with the responses to environmental stress.[6,7] In fact, the expression of most of Arabidopisis galactinol synthase and raffinose synthase isoenzymes was induced under oxidative stress caused by environmental changes, resulting in increased intracellular levels of galactinol and raffinose.[1,2] Furthermore, galactinol synthase-overexpressing Arabidopsis plants with increased levels of endogenous galactinol and raffinose showed increased tolerance to drought stress, suggesting that galactinol and raffinose act as osmoprotectants under salinity stress.[6] Recently, we have reported that high intracellular levels of galactinol and raffinose in Arabidopsis plants overexpressing the heat shock transcription factor A2 or galactinol synthase are correlated with increased tolerance to methylviologen treatment and salinity or chilling stress, and galactinol ($7.8 \pm 0.81 \times 10^9$ M^{-1} s^{-1}) and raffinose ($8.4 \pm 0.46 \times 10^9$ M^{-1} s^{-1}) effectively protect salicylate from attack by hydroxyl radicals in vitro.[1,2] These findings indicated that galactinol and raffinose act not only as osmoprotectants, but also as antioxidants in the leaves of Arabidopsis plants.

Concomitantly with the determination of the reactivity of galactinol and raffinose with hydroxyl radicals, we calculated the rate constant for the reaction between stachyose and hydroxyl radicals, which was estimated to be $1.1 \pm 0.29 \times 10^{10}$ M^{-1} s^{-1}. It was higher than those for galactinol, raffinose and typical antioxidants such as AsA ($1.5 \pm 0.11 \times 10^9$ M^{-1} s^{-1}), GSH ($8.1 \pm 0.58 \times 10^9$ M^{-1} s^{-1}), proline ($1.6 \pm 0.09 \times 10^9$ M^{-1} s^{-1}),[2] and citrulline (3.9×10^9 M^{-1} s^{-1}).[8]

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It has been reported that RFOs accumulate in the late stages of soybean seed maturation and desiccation, indicating that they play a role in the desiccation tolerance of seeds as osmoprotectants.[9] In addition, they provide a readily metabolizable carbohydrate source for energy generation during germination.[10] Stachyose does not accumulate in the leaves of Arabidopsis under normal conditions or in response to several types of stress, whereas in seeds, it accumulates at relatively high levels.[1,6] It has been reported that high levels of stachyose synthase mRNA were transiently accumulated midway through seed development in adzuki bean, and the enzyme was also present in mature seeds of adzuki bean and during germination.[11] In fact, the level of stachyose was approx. three-fold higher than that of raffinose in seeds of Arabidopsis plants.[2] Stachyose increased slightly during the first hours of imbibition and decreased to undetectable levels at 7 days after imbibition in adzuki bean.[11] These findings indicated that stachyose is restricted to seeds and is one of the major soluble carbohydrates in seeds of the majority of plant species.

Imbibition of dry seeds is associated with a rapid increase in oxygen uptake and mitochondrial respiration supporting ATP synthesis.[12] It is estimated that up to 2% of mitochondrial O_2 consumption in seeds is involved in the generation of H_2O_2.[13] In addition, during germination and early post-germinative growth in oilseeds, fatty acids derived from storage triacylglycerol are activated to acyl-CoA and then converted to sucrose by the sequential actions of the β-oxidation pathway and glyoxylate cycle in the peroxisome, and the gluconeogenic pathway in the cytosol. Accompanying the provision of metabolic energy and carbon skeletons for germination and early post-germinative seedling growth, it seems likely that the generation of ROS increases during germination of seeds. However, seeds of pea and bean contain low concentrations of AsA and dehydro AsA.[14,15] The embryonic axes have very weak activities of AsA peroxidase isoenzymes which play an important role in the metabolism of H_2O_2 in higher plants, during the initial stage of germination.[15] Thereafter, these enzymes have a coordinated increase in activity in parallel with AsA content. These findings suggest that the scavenging activity of H_2O_2 in seeds is low at the initial stage of germination. On the other hand, ROS promotes seed germination of cereal plants such as barley, wheat and rice, and several mechanisms have been proposed for its action.[16] Thus, various ROS-mediated responses protect cells against oxidative stress and maintain redox homeostasis.[17]

As Arabidopsis seeds contained galactinol, raffinose and stachyose at 9.3, 0.92 and 4.5 μmol/g FW, respectively, judging from the ratio of volume to weight of seeds, the concentrations of galactinol, raffinose and stachyose in seeds are estimated to be 7.4, 0.74 and 3.6 mM. The concentrations that reduce hydroxylation of salicylate by 50% (ID_{50}) of galactinol, raffinose and stachyose were 3.1 ± 0.3, 2.9 ± 0.2 and 2.2 ± 0.1 mM, respectively.[2] Accrodingly, these findings suggest that in the Arabidopsis seeds, the initial intracellular levels of galactinol and stachyose as antioxidants are sufficient to achieve a positive effect on the protection of cellular components from oxidative damage.

Santarius and Milde (1977)[18] have reported that raffinose is significantly accumulated in chloroplasts of frost-hardy leaves of *Brassica oleracea* (green cabbage). Furthermore, a data-base analysis (TargetP: http://www.cbs.dtu.dk/services/TargetP/) predicts that the raffinose synthase-6 protein is distributed in chloroplasts. If 10 to 50% of the intracellular amount of raffinose is accumulated in chloroplasts which occupy 9.5% of the total cellular volume in a mature leaf mesophyll cell,[19] the raffinose concentration in chloroplasts is estimated to be 0.27 to 1.35 mM in Arabidopsis leaves which contain raffinose at approx. 200 nmol g^{-1} FW under stressful conditions; thus, the level of raffinose in chloroplasts is comparable to levels of AsA and GSH, abundant antioxidants. These findings suggest that raffinose also acts as an antioxidant together with well-known ROS-scavenging enzymes and non-enzymatic antioxidants, such as α-tocopherol (vitamin E), β-carotene, AsA and GSH in chloroplasts of higher plants which are potentially a powerful source of oxidants.

Previously, it was reported that sucrose, which is the most abundant carbohydrate in plant leaves, can scavenge hydroxyl radicals in vitro.[20] Mannitol, which functions as an osmoprotectant, is a widely distributed sugar alcohol in organisms from bacteria to algae, fungi and higher plants, including many crops such as celery, olive and carrot.[21] Shen et al., (1998)[22] have reported that the accumulation of mannitol in chloroplasts can supplement endogenous radical scavenging mechanisms and reduce oxidative damage to cells by hydroxyl radicals in transgenic tobacco plants. We have confirmed that glucose (rate constant: 4.0 ± 0.28 x 10^9 M^{-1} s^{-1}, ID_{50}: 6.0 ± 0.4 mM), fructose (3.8 ± 0.23 x 10^9 M^{-1} s^{-1}, 6.2 ± 0.4 mM), sucrose (8.9 ± 0.68 x 10^9 M^{-1} s^{-1}, 2.7 ± 0.2 mM) and mannitol (5.0 ± 0.22 x 10^9 M^{-1} s^{-1}, 4.8 ± 0.2 mM) can scavenge hydroxyl radicals.[2]

As described above, ROS are inevitably generated in higher plants and pose a hazard when present in high concentrations by irreversibly damaging different macromolecules, like protein, lipid and DNA.[23] The scavenging systems for ROS are micro-compartmented in the vicinity of where the ROS are produced, suppressing the interaction of ROS with target cellular components. On the other hand, at moderate concentrations the ROS play an important role in signaling processes as regulatory mediators.[24,25] Thus, the balance between the detrimental and beneficial roles of ROS could be determined by the local level of ROS related to the function of each organelle and the state of cellular scavengers. Judging from the radical scavenging activity and the intracellular level of each compound reported here and previously, we suggest that carbohydrates including raffinose family oligosaccharides and sugar alcohols are present at high levels under normal and/or stressful conditions, and act as antioxidants to protect plant cells from oxidative damage and maintain redox homeostasis.

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