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Commentary

The presence of dehydroascorbate and dehydroascorbate reductase in plant tissues

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Ascorbate, an essential antioxidant in plant tissues, is oxidised to dehydroascorbate (DHA) via successive reversible single electron transfers with monodehydroascorbate (MDHA) as a free radical intermediate. Rapid regeneration of ascorbate from its oxidised forms is required to support antioxidant capacity. MDHA radicals have a relatively short lifetime and disproportionate at neutral pH values and above to DHA and ascorbate. MDHA radicals can be reduced directly to ascorbate by reduced ferredoxin and by NAD(P)H-dependent monodehydroascorbate reductases, which are found in several cellular locations. DHA can be reduced directly by reduced glutathione at alkaline pH values and also by enzymes which catalyse this conversion (dehydroascorbate reductases, DHAR).

It has recently been argued that DHA detected in chloroplast extracts is artefactual, that in vivo DHA levels are negligible and that this metabolite is formed purely artefactually during extraction of plant tissues [1]. This hypothesis was largely based on the observation that, at concentrations thought to exist in the chloroplast, DHA could cause oxidative inactivation of two enzymes known to be regulated by the thioredoxin system [1]. The authors therefore concluded that DHA accumulation must be avoided [1]. This conclusion is erroneous, however, since it does not take into account the dynamic nature of the regulation of the thiol-mediated enzymes of the chloroplasts. It is well established that the stromal enzymes regulated by the thioredoxin system require ongoing reduction to remain active (e.g. [2,3]). Data obtained by addition of oxidants to enzymes removed from a continuous system of thioredoxin reduction are not relevant to in vivo conditions in the chloroplast, where the activation state of thiol-regulated enzymes reflects differences between reductive and oxidative fluxes. Hence, it is important that DHA is reduced back to ascorbate. It does not, however, mean that DHA cannot exist in vivo. The authors' data show that DHA can oxidise thioredoxin and thioredoxin-activated enzymes; this simply means that, like ferredoxin, NADPH and reduced glutathione (GSH), thioredoxin can act as an intermediate in re-reducing the oxidised forms of ascorbate.

The argument that glutathione reductase (GR) is unimportant because DHAR may not exist is an unjustified extension of the hypothesis. The citation of the review of Allen [4] as stating that GR does not contribute to oxidative stress tolerance in transgenic plants is erroneous. Glutathione has many roles in the chloroplast, including participation in redox reactions other than those directly associated with the ascorbate pool, and in the control of gene expression [5]. Irrespective of the nature of the interactions between ascorbate and glutathione the latter pool can only be kept predominantly in the reduced state (as is found in all organisms) by the action of GR. Little, if any, oxidised glutathione (GSSG) is found in isolated chloroplasts [6] and the glutathione pool is well over 90% reduced in leaves [7,8]. In animal tissues such as kidney and red blood cells the GSH to GSSG ratio is higher than 500 [9]. Even in the system found to have the highest proportion of GSSG, blood plasma, the predominant form of glutathione is GSH [10].

While the AA/MDHA redox couple may function effectively without association to the GSH/GSSG redox couple in certain circumstances, the significance of GSH as a reductant in ascorbate regeneration is established in relation to stress tolerance [11–14]. A tropical fig mutant devoid of DHAR activity was found to be sensitive to high light [12]. Further evidence to support critical roles for DHAR, GSH and GR in maintaining the foliar ascorbate pool has been obtained in transformed plants overexpressing GR which have higher foliar ascorbate contents [8] and improved tolerance to oxidative stress [7,8,13,14]. Similarly, compensation of one antioxidant by the other can be observed, for example, decreases in the meristematic activity of *Arabidopsis* roots caused by GSH depletion were alleviated by adding ascorbic acid [15].

Ascorbate breakdown occurs via DHA which is catabolised to two- and four-carbon products such as oxalate and tartrate [16,17]. These can accumulate to relatively high levels in plant tissues. While the reactions through which ascorbate is catabolised in plants remain obscure, degradation is considered to occur by enzymic degradation of DHA. DHA is therefore a fundamental component of ascorbate turnover as well as the ascorbate redox system. While it is possible that, even under acidic extraction conditions, some over-estimation of tissue DHA contents is inevitable, the above considerations suggest that DHA is formed in vivo and is an important component of ascorbate metabolism. Further evidence to support this view is the existence of a high affinity DHA transporter on the plasma membrane [18].

DHARs that catalyse the reduction of DHA by GSH have been purified from rice, spinach and potato [19–22]. Several other proteins such as glutaredoxins (thiol transferases), protein disulphide isomerases, and even a Kunitz-type trypsin inhibitor have been shown to have DHAR activity [23,24]. Nevertheless, the amino acid sequence of the rice DHAR is quite distinct from these other enzymes [22]. Kato et al. [22] provided convincing proof that a specific DHAR enzyme does exist in a wide range of plant tissues. The argument put forward that this is artefactual because seed tissue contains many

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such DHAR-like proteins [1] is specious. The authors ignore the data by Kato et al. [22] showing that the protein is present in both photosynthetic and non-photosynthetic tissues, as well as being detectable in barley and rice.

Furthermore, we have found the presence of near perfect matches of the N-terminal sequence data from Kato et al. [22] with sequences from the *Arabidopsis* EST cDNA database using TFASTA from the GCG suite of programs [25]. The best of a group of very similar matches is as follows:

Arabidopsis EST at 3017	MALEICVKAAVGAPDHLGDCPFSQRA
	::*::****** ** ******
rice DHAR sequence	GVEVXVKAAVGHPDTLGDCPF

All significant sequences matches were near N-termini of derived EST protein sequences.

These matching *Arabidopsis* EST cDNAs were translated and in stretches of derived amino acid sequence of up to 120 residues no matches to trypsin inhibitors, glutaredoxin, peroxiredoxin, thioredoxin or protein disulphide reductase were detected, despite these sequences being well represented from plants in the EMBL and GenBank data bases. This rules out the authors' arguments that Kato et al. [20] had purified an artefactual DHAR. So it is likely that DHAR does exist, although the chloroplast isoform may prove elusive for some time.

The absence of detectable DHAR enzyme on non-denaturing polyacrylamide gels stained for DHAR activity and not ascribed to artefacts cannot be taken as definitive proof that a bona fide DHAR does not exist. The enzyme may simply not withstand this type of extraction and assay protocol or may be too dilute to detect in this assay system. Similarly, the argument that DHAR activity is often found not to increase in stressed plants is evidence that only incidental activities from other proteins are being measured [26–29].

These considerations support the view that DHA and DHAR exist in leaves and other plant tissues. The oxidising ability of DHA does not preclude its presence in chloroplasts where redox regulation of photosynthetic enzymes requires the dynamic interaction of reducing and oxidising components.

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