Plant Physiol. Biochem. 40 (2002) 537–548

Review

Ascorbate and glutathione: guardians of the cell cycle, partners in crime?

Geert Potters [a,*], Laura De Gara [b,c], Han Asard [a,d,1], Nele Horemans [a]

[a] *Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium*
[b] *Dipartimento di Biologia e Patologia Vegetale, Università degli Studi di Bari, Via E. Orabona 4, 70125 Bari, Italy*
[c] *Università Campus Biomedico, Via Longoni 83, 00155 Rome, Italy*
[d] *Department of Biochemistry, University of Nebraska at Lincoln, Lincoln, NE 68588, USA*

Received 31 January 2002; accepted 20 March 2002

Abstract

Besides the implication of ascorbate and glutathione in the defence against oxidative stress, these two compounds are involved in plant growth and cell cycle control. Ascorbate metabolism is closely linked to the development of embryos and seedlings. Furthermore, ascorbate stimulates cell cycle activity in competent cells, while the oxidised form, dehydroascorbate, blocks normal cell cycle progression. Several possible mechanisms have been proposed to explain the effect of these compounds. The links between glutathione and the cell cycle are less clear. It has long been assumed that both compounds are closely linked by way of the Halliwell–Asada cycle. Any hypothesis concerning the pathways by which ascorbate or glutathione influence cell division, should take this connection into account. However, other mechanisms have been proposed for ascorbate-mediated cell cycle control, e.g. via the thioredoxin pathway.

Keywords: Ascorbate; Cell growth; Cell cycle; Dehydroascorbate; Dehydroascorbate reductase; Glutathione; Redox status

1. Introduction

Several reviews have been published lately on different aspects of ascorbate, ranging from its biosynthesis [83], its role in stress defence [72], the uptake mechanisms in plants [43] to its involvement as an electron donor in various biosynthetic reactions [6]. The biosynthesis and the role of glutathione in plant metabolism have been discussed, in detail [65,71 72], as well as its transport [37]. The involvement of ascorbate and glutathione in growth and division of plant cells has generally occupied a secondary role in these reports. Nevertheless, there is a rapidly growing field of interest, yielding fresh and exciting insights into the general function of either of these molecules. In this review we will summarise the state-of-the-art knowledge on the influence of both compounds over the progression of the cell cycle in plants.

2. The protagonists

2.1. Ascorbate

The organic molecule L-*threo*-hexenon-1,4-lactone is commonly known as L-ascorbic acid (L-AA) or vitamin C. The compound is one of the predominant weak acids in the plant cell, dissociating into the ascorbate anion (ASC) the predominant form of the molecule at a physiological pH (5–7). Both the acidic properties of L-AA (pK_{a1} 4.2) and its redox behaviour are associated with the enediol structure at C-2 and C-3. ASC is utilised in the cell as an electron donor, and the first ASC oxidation product is the semiquinone-like

Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase; ASC, ascorbate; BY-2, *Nicotiana tabacum* L. cv. Bright Yellow-2; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; γ-ECS, γ-glutamylcysteine synthase; GL, L-galactono-γ-lactone; GLDH, L-galactono-γ-lactone dehydrogenase; GSH, reduced glutathione; GSSG, oxidised glutathione; L-AA, L-ascorbic acid; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; ODD, 2-oxoacid-dependent dioxygenases; QC, quiescent center

* Corresponding author.
E-mail address: potters@ruca.ua.ac.be (G. Potters).

[1] A contribution of the University of Nebraska Agricultural Research Division, Lincoln, NE 68583. Journal Series N°13692

PII: S0981-9428(02)01414-6

Rubber

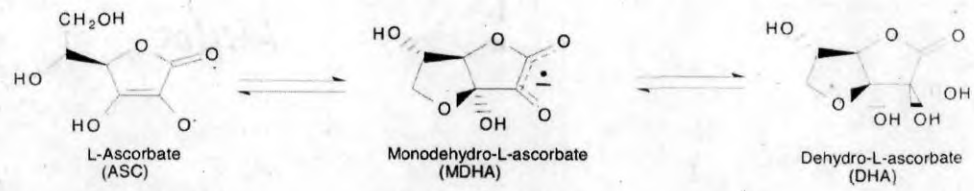


Fig. 1. The different redox forms of ascorbate.

free radical monodehydroascorbate (MDHA) (Fig. 1). Due to their highly delocalised free electron, MDHA radicals do not readily interact with other molecules (unlike the far more reactive superoxide or hydroxyl radicals), and are therefore generally considered to be less destructive [70].

MDHA disproportionates spontaneously to ASC and dehydroascorbate (DHA) with a rate constant between 10^5 and 2.8×10^6 M^{-1} s^{-1} (at pH 7 [41]). Takahama ([88], and references quoted herein) reports an equilibrium constant of 10^{-8}.

$$2\ \text{MDHA} \leftrightarrows \text{ASC} + \text{DHA}$$

The standard redox potential (E_0') of the ASC/MDHA couple at pH 7.0 is +330 mV. However, due to the low MDHA concentration (lowered by spontaneous disproportionation) the effective redox potential of this couple is around +60 mV [70]. The oxidised form, DHA (Fig. 1), which exists as a bicyclic monomeric hydrate in aqueous solution, does not have acid characteristics. It is a rather unstable molecule, which undergoes spontaneous and irreversible hydrolysis to 2,3-diketogulonic acid [32], which breaks down further to yield approximately 50 different compounds, including oxalate and tartrate [56].

ASC is present in the cytosol, chloroplast, vacuoles, mitochondria and extracellular matrix. An overview of concentrations in different compartments, as well as the different transport pathways from and to the cytosol, has been presented in Horemans et al. [43]. Chloroplastic and cytosolic ASC concentrations can be high (10 and 20 mM, respectively) [8]. This situation probably reflects the high need in these compartments for scavenging of active oxygen species (AOS).

ASC is involved in the protection of different cell compartments against oxidative damage. For example, different isozymes of ASC peroxidase (APX) are found in the cell wall, in the cytosol, the mitochondria and in the peroxisomes [93], to assist in the detoxification of AOS at these locations. ASC is probably one of the most important factors in the scavenging of ozone and ozone-derived AOS in the apoplast, either through direct (chemical) interaction, or by detoxifying ozone breakdown products [14,60]. AOS are generated during aerobic metabolism in the chloroplast and during exposure to stress conditions, and are bound to inflict serious damage upon the cell, if not readily detoxified [33]. ASC reacts rapidly with superoxide and singlet oxygen, ozone (chemically), and hydrogen peroxide (enzymatically, through APX), and will thus assist in neutralising these AOS. Additionally, ASC regenerates the chloroplastic lipophilic antioxidant α-tocopherol (vitamin E) from the α-chromanoxyl radical [7]. Carotenoid pigments (carotenes and xanthophylls) also depend on ASC for their regeneration.

The requirement for ASC in the activity of 2-oxoacid-dependent dioxygenases (ODD), is generally less acknowledged than its role as an antioxidant. Among the reactions, catalysed by ODDs, we find hydroxyproline synthesis, providing animals with functional collagen, and plants with cell wall constituents like extensins [26,30]. But also certain plant growth regulators like ethylene or gibberellins require ASC for an ODD at some point in their synthesis, as well as anthocyanins, or the animal neurotransmitter noradrenaline [6].

2.2. Glutathione

GSH is an abundant and ubiquitous thiol in fungal, plant and animal tissues and occurs in two distinct redox forms. The reduced form GSH, is a tripeptide (γ-Glu–Cys–Gly). The chemical reactivity of its thiol group (standard redox potential –230 mV, at equimolar concentrations), its relative stability and high solubility in water make GSH a particularly adequate electron acceptor or donor in physiological reactions. Oxidation causes two reduced molecules to be linked by way of a cysteine disulfide bond, designated GSSG. Chloroplastic GSH concentrations are estimated to be 1–4.5 mM, but information about concentrations in other compartments is still lacking [72].

The different functions fulfilled by GSH in plants have recently been reviewed [65,71]. A short summary will therefore be sufficient here. In general, the physiological significance of glutathione in plant cells covers three categories: sulphur metabolism, defence against oxidative stress and detoxification of xenobiotic compounds. Its role in sulphur metabolism is evident from the fact that GSH is the predominant non-protein thiol. Furthermore, GSH regulates sulphur uptake at root level [42] and the pathways for sulphur assimilation and GSH synthesis are intertwined at a molecular level [65].

The fact that GSH is involved in defence reactions against oxidative stress, as an antioxidant, is widely acknowledged. Its importance is illustrated by the fact that GSH biosynthesis is stimulated when the cell encounters stress conditions, as if the cell builds up its defence capability. GSH accumulates in response to increased AOS generation, or to compensate for decreases in the defence

capacity of other antioxidants, and GSH levels are constitutively higher in plants adapted to stress conditions [61,64,82]. Supplementary evidence for the role of GSH in cell defence was provided by transgenic *Arabidopsis* plants with a limited biosynthetic capacity for GSH. The decreased GSH pool apparently renders them substantially more sensitive to different stresses (such as Cu, Cd, photooxidative or ozone stress) [97]. Furthermore, the reactive cystein residue enables GSH to keep thiol group-containing proteins in their native state during stress conditions. For example, under water stress conditions, GSH protects specific protein thiol groups from irreversible formation of intramolecular disulfide bonds.

GSH metabolism is also tied into other defence pathways. For example, different sources have described a role for GSH in ASC regeneration, both in the chloroplast and in the cytosol ([72]; see further in this text). GSSG reduction itself requires NADPH as a source of electrons for its reduction. The GSH–GSSG redox pair can therefore only function with an adequate supply of NADPH. Recent experiments indicate that GSH may function as a cellular sensor to ensure maintenance of the NADPH pool [65], further illustrating the intertwining of the metabolism of different redox compounds. These connections undoubtedly increase the chances of the plant cell for displaying an optimal defence capacity against stressful conditions.

A third series of processes involving GSH, concerns the detoxification of heavy metals and xenobiotic compounds. Polymerisation of GSH yields phytochelatins, which are crucial in controlling cellular heavy metal concentrations. These phytochelatins have a particularly high affinity for copper and cadmium ions and are able to form complexes with these heavy metal ions. These complexes will be transported into the vacuole, leaving cytoplasmic enzymes unharmed [76]. GSH is also used by the GSH S-transferases in the detoxification of organic compounds [61]. Xenobiotics, but also metabolites such as anthocyanins, are covalently linked to GSH and then transported into the vacuole [37].

3. Evidence for the involvement of ASC in cell cycle control

3.1. ASC metabolism is connected to growth and division

Its free radical scavenging properties make ASC pivotal in cell defence. However, confining the molecule to having only this role in plant physiology would do injustice to the collected data. A fair amount of evidence has been provided for its involvement in a series of other processes. We have already mentioned the role of ASC as cofactor in different dioxygenase reactions, or in the biosynthesis of the plant hormones gibberellic acid and ethylene [6]. The hypothesis, first raised by Reid in 1941 [78], that ASC is somehow involved in growth control of plant cells, has gained new supportive evidence. Mainly during the last 20 years, data have been collected, supporting a role for ASC in growth regulation.

A first set of data describes the correlation between ASC content and the growth capacity in different plant tissues. Furthermore, plant ascorbate levels depend on the age of the organism or the tissue. For example, in the apoplastic fluid of *Pinus pinaster* Aiton hypocotyls, ASC content and redox status seem to be decreasing in parallel with the growth activity along the hypocotyl axis, as well as with hypocotyl age [80].

A decrease in ASC content has also been observed in *Pisum sativum* seedlings from meristematic to differentiated tissues. Germinating pollen grains of *Dasypyrum villosum* actively synthesise ASC [24]. Also, growing plants (and cell cultures) exhibit continuous biosynthesis of ASC [23,28,29,58], but when leaves start to age, their apoplastic ASC pool is depleted, and the symplastic ASC content is lowered significantly [13,89].

ASC concentration changes during different stages in seed development, too. A first stage, characterised by a high mitotic activity, comprises the actual formation of the embryo. At this point during plant development almost 90% of the total ascorbate (ASC + DHA) pool is in the reduced state. Later on, upon the onset of cell elongation, the DHA level rises above the ASC level [5]. When the embryo (and concomitantly, the seed) develop further, the ASC pool becomes even more oxidised. In the end, upon seed maturation, the only form present is DHA [5,22,90]. The stored DHA will be used during the development of the seedling, to ensure the presence of an ASC pool in the young plantlet, necessary for its development. Studies on wheat and pine embryos show that, during the first hours after germination, embryos provide themselves with ASC through the reduction of DHA, stored inside. The DHA reduction capacity of embryos is high in the first hours after germination, and decreases gradually afterwards [22,30,90]. ASC biosynthesis is initiated from the moment of germination onwards: wheat, pine and broad been seeds possess already ASC biosynthetic capacity, and while the seedling grows, the DHA reduction capacity decreases gradually, and ASC biosynthesis becomes more important than DHA reduction in determining the ASC pool size [22,90].

Apparently, changes in normal ASC metabolism have a profound effect on growth rates. For example, application of ASC or L-galactono-γ-lactone (GL, the last biosynthetic precursor in the ASC biosynthesis, [83]) to *Lupinus albus* seedlings increases the production of lateral roots and stimulate the activity of primary and secondary meristematic tissue [4]. An increase in the mitotic index of tobacco-cultured cells occurs as a consequence of ASC biosynthesis stimulation with GL [28]. On the other hand, although treating *Arabidopsis* suspension cells with ASC precursors (for example GL), does increase ASC synthesis rates and intracellular ASC concentrations, no changes in cell numbers were observed [20].

Decreases in the internal ASC concentration may de-ease growth rates. Two different strategies have been used try to lower cellular ASC levels: the application of a osynthesis inhibitor, and the isolation of biosynthetic utants, containing less ASC. The alkaloid lycorine was scribed as a potent and specific inhibitor of ASC biosyn-esis [2], in particular of the L-galactono-γ-lactone dehy-ogenase (GLDH) [25], the last enzyme of the Smirnoff heeler pathway [83]. Application of this alkaloid to lupine edlings inhibited growth [4]. It should, however, be entioned that the specificity of this compound is under bate. No inhibition by lycorine was observed of the rified GLDH from cauliflower [20]. On the other hand, corine did inhibit the purified GLDH from sweet potato 0]. Luckily, an ascorbate-deficient *Arabidopsis* mutant ontaining only 30% of the ASC concentration of wild type *abidopsis* plants) has been isolated, called the *vtc1* mutant itamin c) [16]. Significant reduction in growth could be served in these mutants [94], a fact that adds to the idence confirming a link between ASC and growth ntrol.

Inclusion of ASC in the growth medium in in vitro periments, promotes growth and development. Experi-ents on somatic embryogenesis in white spruce demon-rated that the higher levels correlate with an increased mbryogenic competence. In addition the ASC redox status efined as the ratio of the concentration of the reduced rm to the concentration of both the reduced and the idised forms, i.e. [ASC]/([ASC] + [DHA]), increases dur-g the development of these spruce embryos [85]. Addition exogenous ASC increases the germination frequency of ese embryos, as well as the size of the apical region, esumably because of the production of a larger number of af primordia [84]. Finally, in vitro systems frequently need SC application to enhance shoot formation [53].

The overall conclusion from these data is that ASC is eply linked to plant growth. However, plant growth nerally depends upon two distinct processes: cell elonga-on and cell division. The role of ASC, or rather, MDHA, in ll elongation has been amply documented (reviewed in 9]). It is assumed that MDHA is able to invoke an ergisation of the plasma membrane, an activation of the lasma membrane ATPase, which will then enhance cell all acidification. MDHA also causes an increased vacuoli-tion, a process that drives cell elongation even more. evertheless, the model presented in those reviews is not fficient to explain all growth-related actions of ASC. This d to the notion that ASC (and other redox compounds, ssibly even synergistically) may have an effect on the ant cell cycle.

2. ASC mediates transition into S-phase

The earliest reports, describing a possible link between SC metabolism and cell cycle regulation, date from 1984, owing that ASC is required for cell division, and even promotes the cell cycle progression of competent cells. In these reports, considerable increase in mitotic activity was obtained following the addition of 1 mM ascorbic acid to *Allium cepa* roots grown in water [58]. The clearest demonstration of the effect that ASC stimulates cells to enter the cell cycle came with observations on cells from a root tip quiescent centre (QC). QC cells are known to divide rarely, and to have their cell cycle extended mostly in G1. Treatment of *A. cepa* root tips with ascorbic acid, however, stimulates these to undergo DNA synthesis, leading to a shortening of G1 phase, and a stimulated entry into S phase. Additionally, ASC does not only stimulate the activity of the QC cells, but also cell proliferation in the entire root meristem, and in the pericycle [3,51,59]. This observation was repeated later with *Zea mays* root tips [57]. On the other hand, roots treated with lycorine displayed a decline in the amount of endogenous ASC in the root tip. Simultaneously the cell cycle was interrupted during G1 and G2 phases [58]. From all these data, it can be reasonably assured that ASC is in some way involved in the G1–S transition, and that the presence of ASC is necessary in order to complete this transition.

The necessity for ASC during cell cycle progression is not limited to plant cells only. ASC seems to play a similar role in animal cells, where it apparently stimulates the entry of quiescent 3T3 cells into S phase [69]. ASC is also considered essential for the in vitro growth of plasmacytoma cells, and it influences the proliferation of human leukemic and preleukemic cells [74]. Furthermore, both ASC and MDHA stimulate the proliferation of HL-60 cells in serum-limiting media, by shortening the overall cell cycle length [1,38].

It is rather significant that all these experiments apparently demonstrate that the ASC-enhanced cell cycle activity is not due to an ASC-mediated enhancement of the overall state of competence of the cell. Competence is a state which is necessary, but not sufficient, for cell cycling; the progression also has to be stimulated. For example, quiescent embryo cells speeded up the G0–G1 transition during germination in the presence of ascorbic acid. Moreover, ASC seems a decisive factor in this respect, but cannot induce non-competent cells to overcome proliferation arrest [19], suggesting that the primary function of ASC is to enable already competent cells to progress through the cell cycle phases. One might rephrase the definition of competence, then, taking into account the existence of so-called cell cycle checkpoints. It is generally assumed that the cell has to cross two checkpoints, one at the transition from G1 to S-phase, the other at the G2–M transition [40]. At each of these points, a decision has to be taken, namely whether a new cycle is to be initiated (G1–S checkpoint), or whether the cell is ready to divide (at the onset of mitosis). A competent cell may then as well be redefined as a cell, ready to cross the G1–S checkpoint (with the decision to go through another cell cycle round already taken), but it might still lack certain stimuli to actually do so. For example, an

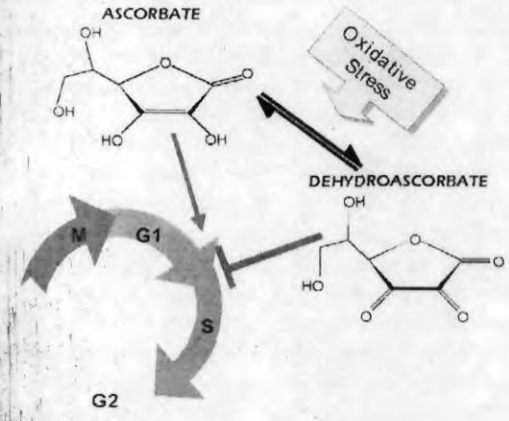


Fig. 2. Physiological meaning of dehydroascorbate-mediated cell cycle inhibition. While the presence of a sufficient amount of ascorbate is necessary for the occurrence of cell division, excess dehydroascorbate will effectively block it. The presence of dehydroascorbate is enhanced under oxidative stress conditions. The change in the ratio ascorbate vs. dehydroascorbate may therefore be crucial for the cell to sense stress, and take the appropriate action.

adequate ASC concentration (or a favourable redox status) may be necessary to complete one or more processes linked to the transition to DNA synthesis. The question remains as to how this is mediated.

Several possible mechanisms for an ASC-enhanced transition have been proposed over the years. To integrate the facts listed above on the effect of ASC on proper cell cycle progression, two distinctly different, not mutually exclusive hypotheses have been put forward, each focusing on a different process (Fig. 2). The first mechanism, explaining the effect of ASC on cell cycle progression, involves hydroxyproline synthesis. As stated before, ASC is being used as cofactor by the enzyme prolyl hydroxylase for the posttranslational hydroxylation of proline residues. Hydroxyproline-rich proteins are apparently needed during progression through the cell cycle [17,30]. It was hypothesised that a lack of ASC will prevent the functional hydroxyproline-rich proteins from forming, and, hence, will block the cell cycle. Other data show that underhydroxylation of proline residues induces a cell cycle block during metaphase [30].

Proliferating cells in the root tip of 3-day-old plantlets can be arrested at the G1–S boundary by hydroxyurea application. It was shown that addition of exogenous ASC made these cells resume the cell cycle earlier than the control cells [15]. This observation brought up a second hypothesis on the exact role of ASC in the enhancement of cell cycle activity. Apparently, the hydroxyurea treatment blocks the cells in their cell cycle by destabilising the iron centre present in the ribonucleotide reductase, the enzyme responsible for the production of deoxyribonucleotides [66]. The reconstitution of this centre requires the presence of Fe^{2+} ions. A plausible source may be provided by the Fe^{2+} binding protein ferritin. This protein is present in plant cells [19]. In vitro, it was demonstrated that ASC could increase the iron release from its ferritin stock [49]. If this reaction would work in vivo as well, an increased level of ASC in the cell would speed up the regeneration rate of the iron centre of the ribonucleotide reductase. In a more natural situation, ASC may be required to keep up a certain level of ribonucleotide reductase activity during S phase. ASC depletion would then slow down the regeneration, and, hence, keep the cells from entering S phase.

3.3. DHA blocks transition into S-phase

Nevertheless, several authors started to evaluate not only the effect of an increased or decreased concentration of ASC, but also of the role of the ASC redox status, and of the presence of the different oxidation forms. Indeed, also MDHA and DHA exert a certain influence on the cell cycle, not attributable to a rise in ASC concentration. For example, De Cabo et al. [21] demonstrated a shortening of the G1 phase in a dividing onion root meristem cells due to the action of monodehydroascorbate, not ASC. Moreover, while ASC (and MDHA) stimulate growth, DHA actually inhibits plant growth. For example, root elongation is inhibited when bulbs root in a hydroponic medium containing DHA [18]. Such a reduction may be caused by inhibition of cell expansion, but an inhibitory effect of DHA on cell proliferation seems equally likely. The latter hypothesis is supported by the low rates of incorporation of tritium-labelled thymidine in root meristems supplied with exogenous DHA [21]. Kerk and Feldman [57] were able to establish a direct correlation between ASC redox status and cell proliferation rates in the *Zea mays* QC, by pointing out the increased ASC oxidase activity in the QC. This enzyme causes the oxidised DHA to become the prevalent form in this particular region of the root. This is in contrast to the very low DHA concentrations in the meristem itself, situated next to the QC. As mentioned before, addition of (exogenously added) ASC to the root tip resulted in resumed cell division in the QC cells. In any case, while ASC and MDHA stimulate cell cycle activity, elevated DHA concentrations, in the culture medium or inside the cell, actually inhibit growth.

More recent research supporting this hypothesis focused mainly on the *Nicotiana tabacum* L. cv. Bright Yellow-2 cell suspension (BY-2). Due to its rapid growth and its highly synchronisable character, this suspension culture is most adequate for biochemical studies on the regulation of growth and cell division. During the growth of a BY-2 cell culture, a transient peak in the endogenous ASC level was shown, during the period when the cells were dividing exponentially. Upon entrance in the stationary phase, the ASC concentration and redox status decreased [28,54]. Kato and Esaka [54] provided further evidence pointing at a possible ASC-mediated redox control of the cell cycle by demonstrating a transient peak in the DHA concentration, with a concomitant decrease in the ASC/DHA ratio during M phase. This increase also correlated with a temporary

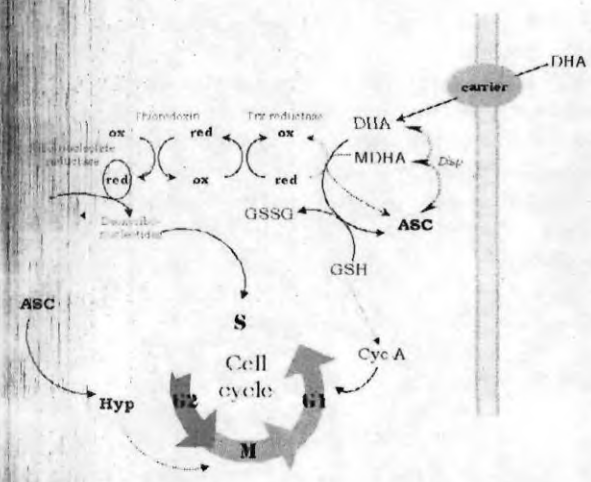


Fig. 3. Overview of different possible and hypothetical interactions between ascorbate metabolism and cell cycle regulation. For a detailed description, see text. Straight arrows, demonstrated links; double dashed arrow, monodehydroascorbate disproportionation reaction; dashed arrows, hypothetical connections.

increase in ASC oxidase expression during G2 and M phase [54]. These results brought up the hypothesis that intracellular levels of DHA may be controlled by the cell cycle, through the expression of ASC oxidase, which is, surprisingly, an apoplastic enzyme.

Regulating the DHA content throughout the cell cycle may at first seem to have no reason. Yet there may be a physiological explanation. Being the major antioxidant in the apoplast, ASC is a likely candidate to play a role in the signalling from the apoplast to the cytoplasm related to the oxidative properties of a possibly stressful environment. During stress conditions, the internal DHA concentration might remain high. A decrease in DHA during G1 could then function as a necessary and positive signal for the cell to proceed into S phase (Fig. 3) [54].

Also, the endogenous DHA signal originates in the apoplast, which implies that growth control, mediated by the ASC–DHA redox pair, is a process implicating different actions in various cell compartments. It may also point to the occurrence of specific processes, involving ASC, at the M–G1 transition. The formation of a new part of the cell wall (a process normally highly regulated by ASC) may as well be linked to the observed changes in ASC metabolism. An hypothesis for the effect of DHA on cell cycle progression has been raised in Chinese hamster ovary cells. When these AS52 cells, previously enriched with ASC, are subjected to an oxidative stress treatment (mediating the oxidation of ASC to DHA), the cyclin–cyclin-dependent kinase complex, responsible for G2–M transition was inhibited [11].

The explanation given above was easily checked by addition of excess DHA (1 mM) to a synchronised BY-2 culture, during G1 phase. This was assumed to raise internal DHA concentrations, leading to a delay in the onset of S phase. A high-affinity DHA transporter (Km = 139 μM) has been discovered at the plasma membrane of this cell line, ensuring efficient uptake [44]. Furthermore, this treatment resulted in the expected temporary delay in cell cycle progression, checked at the next occurrence of mitosis [75], confirming the role of DHA as a signal molecule, regulating cell division.

However, although internal ascorbate levels went up 20-fold after addition of DHA to BY-2 cells, an internal rise in DHA concentration did not occur [28,75]. This prompted the conclusion that *(a)* the observed delay in cell growth or cell cycle progression is not due to an increased DHA concentration, and *(b)* apparently the cell possesses a very efficient DHA reduction system (the nature of which will be discussed later). Now, an increase of internal ASC previously had been connected to cell cycle shortening and not in prolongation, as observed in this case. Cells treated with various biosynthetic precursors did not show any changes in cell cycle activity either [20]. It was therefore suspected that the reduction of DHA was probably the first step in a pathway, leading from DHA to the eventual cell cycle block. The flow of electrons, needed for the reduction of DHA, will alter the redox status of another molecule, and hence, its activity. Description of a pathway that would assist in the perceiving the DHA signal, and in communicating it with the cell cycle regulatory systems, would at least start with the identification of the proper reductants for DHA.

4. Evidence for the involvement of GSH in cell cycle control

While a fair amount of data has been collected on the involvement of ASC in the cell cycle, considerably less attention has been devoted to the role of GSH. On the whole, the effect of GSH on the cell cycle is still vague and needs more descriptive work, so that a more clear-cut picture emerges, like in the case of ASC. However, two reports point to an effective role for GSH in cell cycle regulation, each focusing on *Arabidopsis* root tips. Just like in the case of ASC [57], GSH tissue distribution levels are correlated quite nicely with the rate of division in parts of the root tip. High levels of GSH can be found in the epidermal and cortical initials, whereas the concentration in the slowly dividing cells of the QC was 27% lower. Treating roots with 100 μM GSH increased the mitotic activity, whereas a decrease in endogenous GSH, caused by application of the GSH biosynthesis inhibitor L-buthionine sulfoximine, lowered it [81]. ASC and dithiothreitol were apparently able to replace GSH in this respect, pointing to the fact that this phenomenon may rather be linked to a more general form of redox control, irrespective of the compound involved.

Further evidence linking GSH to cell cycle regulation came with the discovery of a GSH-dependent developmental pathway, necessary for cell division initiation and maintenance in the root meristem. Apparently the *ROOT*

MERISTEMLESS1 (RML1) gene actually codes for the γ-glutamylcysteine synthase (γ-ECS), the rate-determining step in GSH biosynthesis [65,71]. Plants homozygous for a mutation in *RML1*, had only 3% of the wild type GSH level. GSH depletion in a BY-2 cell suspension culture blocked cell cycle activity at the G1–S transition. Two different A-type cyclins, involved in this transition, were also down regulated [95] (Fig. 2).

Also in animals, de novo GSH synthesis was proven to be necessary for entry into and passage through S-phase. For example, DNA synthesis itself seemed regulated by the GSH [86], and the typical cyclin–cyclin-dependent kinase activity necessary for G1–S transition appears to be modulated by the redox state of several regulatory proteins [79], leaving clues for the action mechanism whereby GSH is supposed to operate [65]. On the other hand, GSH depletion by buthionine sulfoximine treatment does not affect cell cycle progression in the ovary cell line AS52 [11].

On the other hand, while exogenous ASC and DHA have a distinct effect on the progression of the cell cycle, addition of GSSG or GSH (and internal rise of these compounds) does not influence the regular growth division of the tobacco BY-2 cell suspension, but rather the expansion of these cells [28]. This may as well indicate that the GSH requirement for cell cycling involves just the presence of a critical concentration, without which division does not occur, while any rise above this level is only superfluous in terms of the cell cycle. This is best illustrated by the phenotype of another mutant of the γ-ECS gene. The so-called *cad2-1* mutation (inferring higher sensitivity to cadmium to the plant) decreases GSH levels to 30% of the wild type plants, but does not influence cell division [48]. A larger drop in GSH concentration (like in *rml-1* mutants, which contain no more than 3% GSH, compared to control plants) blocks division in the root tip [95]. As stated above, this is not the case for ASC, where a higher ASC availability increases mitotic activity, whereas ASC depletion halts the cell cycle.

Even more general oxidative stress treatments, like the application of menadione [77], lead to cell cycle blocking. These experiments gave rise to the concept of an 'oxidative stress checkpoint pathway'. During oxidative stress, particular signals may converge on one (or more) specific regulatory elements from the cell cycle to block or prevent normal progression through the different phases. Indeed, plants need mechanisms to probe the levels of oxidative stress. Their sessile nature renders them incapable of flight, leaving them no other option than to collect adequate information on their surroundings, to be able to adapt and endure environmental conditions in the best possible manner. The ASC and DHA redox pair is suitable for this kind of scouting work, and several reports indicate that different types of environmental stress result in an increased oxidation of the apoplastic ASC pool [60,87]. DHA has also been proposed elsewhere [28] as a specific redox link between the apoplast and the cytoplasm.

The actual pathways and mechanisms, which are responsible for oxidative stress sensing, or, to sense a change in apoplastic ASC redox status, are yet to be discovered. However, both ASC and GSH are major team players in the antioxidative defence pathways, and have been linked in a biochemical mechanism, the Halliwell–Asada cycle [35]. In the following paragraphs, we will give a brief survey on the possible pathways involved in the connection between DHA and GSH, and of the possible links towards cell cycle control.

5. ASC and GSH, an intimate couple...

The first product of many redox reactions, which use ASC as an electron donor, is MDHA. However, as stated above, the different redox forms of ASC are not only interconversible by enzymatic steps, spontaneous disproportionation (a reaction striving to equilibrium) itself is already sufficient to form a certain amount of DHA from MDHA. Detoxification of the oxidation products, and therefore regeneration of the ASC pool, will start with the reduction of these two compounds.

In the chloroplast, two enzymes are responsible for ASC regeneration from its two oxidation products, MDHA and DHA. The MDHA reductase (MDHAR) will use electrons from NADPH, to ensure ASC regeneration; ferredoxin can reduce MDHA directly [8,67]. DHA reductase (DHAR) links ascorbate to glutathione, which is the other major soluble antioxidant in plant cells. GSH functions as an electron donor for the DHA, converting it into ASC, while GSH itself is being oxidised to the dimer GSSG (oxidised glutathione). A GSSG reductase will then re-reduce the GSSG to GSH, thereby consuming two electrons from NADPH. These reactions, together with APX, constitute the Halliwell–Asada cycle [35] (for a recent review, see [72]).

The ascorbate–glutathione cycle does not only occur in the chloroplast. Components of the ASC–GSH cycle have been reported in the cytoplasm, in mitochondria and peroxisomes, and represent an important antioxidant protection system against H_2O_2 generated in these organelles [52]. For example the enzymes involved in the Halliwell–Asada cycle are present as soluble proteins in the cytoplasm of *Solanum* tuber cells [12] and of *Zea* seedling and leaf cells. APX, MDHAR and DHAR activities are also demonstrated in a mitochondrial fraction of *Solanum* tuber cells possibly indicating a similar mitochondrial ASC regeneration system [27]. Bérczi and Møller [10] purified a MDHAR, situated on the cytoplasmic side of the plasma membrane, that converts MDHA to ASC with the aid of NADH. The ASC regeneration pathway, as described in the chloroplast, is apparently present in the cytoplasm as well.

However, does this connection hold up as well when it comes to cell cycle regulation? Evidence is scarce, to date, but an answer starts to become clearer. Addition of 1 mM DHA to *Lupinus* or *Allium* roots did not only invoke a cell

Table 1
Overview of different DHA reductases, their Michaelis–Menten constant and their source

Protein	Kinetic param.	Source	Ref.
DHAR only	Km (DHA)= 0.34 mM Km (GSH) = 4.43 mM pH optimum 7.5	Spinach leaves	[36]
DHAR only	Km (DHA)= 0.07 mM Km (GSH) = 2.5 mM pH optimum 7.8	Spinach leaves	[45]
DHAR only	Km (DHA)= 0.39 mM Km (GSH) = 4.35 mM pH optimum 8	Potato tubers	[34]
DHAR only	Km (DHA)= 0.35 mM Km (GSH) = 0.84 mM at pH 7.8 pH optimum 8.2	Rice bran	[55,92]
Kunitz-type trypsin inhibitor	Km (DHA) = 1 mM Km (GSH) = 7 mM at pH 7 pH optimum 8.3	Spinach chloroplasts	[91]
Thioredoxin f	Not determined	Spinach chloroplasts	[68,91]
Thioredoxin m	Not determined	Spinach chloroplasts	[91]
Storage proteins	Not determined	*Ipomoea* tubers *Dioscorea* tubers	[46,47]
Protein disulfide isomerase	Km (DHA) = 1 mM Km (GSH) = 3.9 mM	Bovine liver	[96]
Glutaredoxin	Km (DHA) = 0.2–2.2 mM Km (GSH) = 1.6–8.7 mM	Porcine liver Bovine thymus Human placenta	[96]
Thioredoxin reductase	Km (DHA) = 2.5 mM Km (DHA) = 0.7 mM[a]	Rat liver	[63]
3α-Hydroxysteroid dehydrogenase	Km (DHA) = 4.6 mM Km (NADPH) = 4.3 μM pH optimum 6.2	Rat liver	[31]

[a] After addition of 2.8 μM thioredoxin.

division block [73], reminiscent of the data obtained with the BY-2 cell suspension [28,75], but had also a profound, but transient, effect on the GSH pool in these roots [73]. However, even in this last report, the authors state that the cell cycle inhibition resulting from DHA treatment cannot be explained only by this decrease in GSH content. Moreover, where DHA is capable of retardation of the normal growth or division cycles, this is apparently not the case with GSSG [28]. As a last element, ASC could not rescue the *rml-1* phenotype [95]. These observations led to the suggestion that there must be other pathways for ASC regeneration, besides the Halliwell–Asada cycle.

6. ... or perfect strangers?

The enzyme connecting DHA and GSH, is named a GSH-dependent DHAR. It can be argued that the cytosol needs DHAR activity, to detoxify any DHA being brought in from the apoplast, by means of the plasma membrane carrier [43]. However, exactly this cytosolic DHA reductase remains rather enigmatic.

Several authors have reported the purification of proteins with DHA reduction capacity, from spinach leaves [45], potato tubers [34] or rice bran [55] or even cloned a gene from rice [92]. The latter protein exhibits an amino acid sequence, which is significantly dissimilar of any other protein. On the other hand, there are different proteins, both from animal and plant sources, known to have complete different functions, but also exhibiting a DHA reductase activity (see Table 1).

However, none of these proteins (except for the one isolated from spinach leaves [45]) exhibits a very high affinity for DHA. This point becomes more clear when the Km of these proteins is compared with the Km of the plasma membrane DHA transporter (139 μM for BY-2 cells [44]). Why should a plant have a high affinity transporter for DHA, only to stock DHA in the cytoplasm? Moreover, 50 μM DHA fully inhibit the activity of different chloroplastic enzymes [68], which shows that an inefficient reduction (with low-affinity proteins as described in Table 1) will lead to metabolic problems. Another question arises from the fact that even after addition of 1 mM exogenous DHA, no rise in internal DHA concentration can be observed [28,73,75]—if the cell indeed stocks DHA brought in from the apoplast, then why is it impossible to localise it? It is quite difficult to fathom that the DHARs listed in Table 1 are able to handle adequately the substantial influx of DHA in to the cytoplasm, under these circumstances. This implies that other mechanisms may as well contribute to the reduction of DHA.

One possible mechanism for DHA reduction concerns the chemical balance between ASC, DHA and the intermediary MDHA. The spontaneous reaction of ASC and DHA forms two MDHA molecules, with a constant equilibrium constant of 10^{-8}, and a second order rate constant of 10^5 M^{-1} s^{-1}. A continuous influx of DHA is bound to change the subtle balance between the three ASC redox forms, promoting formation of the MDHA radical. Reduction mechanisms for this compound (e.g. by a plasma membrane-bound MDHA reductase [10]) have been described, and this very reaction may provide the necessary drive to keep the disproportionation, which in essence is converging to an equilibrium point, going on. Further evidence (for example careful measurements of MDHA concentration) should provide the means to confirm or reject this hypothesis. Now, MDHA may not only be reducible by a ferredoxin- or NADH-dependent MDHA reductase. In rat liver, an alternative enzyme has been proposed. The thioredoxin reductase has been implicated in MDHA reduction with a Km of 2.8 μM for MDHA, a value decreased to 0.95 μM upon addition of selenocystine to the reaction medium [62]. The thioredoxin system is directly linked to the synthesis of deoxyribonucleotides, by the ribonucleotide reductase, necessary for DNA synthesis. When these enzymes would be occupied, regenerating ASC, less reducing power could be shuttled to this latter enzyme, effectively delaying cell cycle progression. This mechanism (as illustrated in Fig. 3) has not yet been demonstrated in plant cells, however.

In conclusion, we can state that, while GSH is still acknowledged as the most probable electron donor, many other proteins may as well fulfil this role. As far as the link to the cell cycle is concerned, different possibilities are still open for exploration. Anyway, instead of being a protein without a function (like the ascorbate oxidase or the plasma membrane cytochrome b561 [9]), we can safely say that the DHAR is still a function without a specific protein assigned to. A survey to unravel the different possible DHA reductases and their kinetic constants may prove beneficial to our understanding of the proper position of ASC, MDHA and DHA in cell metabolism.

7. Conclusions and perspectives

The body of evidence uncovering the existence of a direct control of the cell cycle, mediated by ASC and GSH, two major redox molecules in a plant cell, cannot be denied any longer. Different theories have been put forward to explain the observed effects. However, many questions are waiting to be answered, for the picture to be completed. First, the endogenous link of the ASC metabolism with the cell cycle needs a more exhaustive description. How about a cell cycle-dependent regulation of ASC biosynthesis or DHA regeneration capacity? What is the place of glutathione in cellular metabolism, providing it is not involved in the DHA mediated cell cycle delay? What is the exact mode of action of DHA and ASC by which each of them influences plant cell growth? Is the ASC metabolism tied into the general thioredoxin–thioredoxin reductase system, and will this pathway lead to changes in protein activity, by redox signalling, or in transcription? For some time, the existence of an ASC-dependent transcription factor has been discussed [72]. Nevertheless, no definitive proof has been put forward yet.

Throughout the story, one compartment seems to play a more interesting part than might have been expected. ASC oxidation in the apoplast seems to be an unmistakably well integrated process in the whole of cell cycle regulation, not only in times of stress, but also during normal cell cycle progression. A reason for this may lie in the fact that mitosis cannot be completed without cell wall synthesis, to separate the two daughter cells. Different authors have investigated the equilibrium between ASC oxidation and cell wall formation. Is this the cause for the observed changes in ASC metabolism, around the M–G1 transition [54]? And as a last remark, adding to an already complex picture, is there a pathway integrating the different dioxygenase reactions where ASC is required [6]?

Come to it—enough questions have been formulated, but few answers have been provided, still. Ironically, when first isolating the 'antiscorbutic factor', Dr. Szent-Györgyi first gave his new compound the prophetic name 'godnose' ("God knows"), not grasping its function in general metabolism. Seven decades later, we have still not figured out the answer.

Acknowledgements

Geert Potters and Nele Horemans are respectively Aspirant and Postdoctoral Researcher at the Fund for Scientific Research—Flanders (FWO–Vlaanderen). Professor Dr. Roland Caubergs and Dr. Alajos Bérczi are gratefully acknowledged for helpful discussion on this matter.

References

[1] F.J. Alcaín, M.I. Burón, J.C. Rodriguez-Aguilera, J.M. Villalba, P. Navas, Ascorbate free radical stimulates the growth of a human promyelocytic leukemia cell line, Cancer Res. 50 (1990) 5887–5891.

[2] O. Arrigoni, R. Arrigoni-Liso, G. Calabrese, Lycorine as an inhibitor of ascorbic acid biosynthesis, Nature 256 (1975) 513–514.

[3] O. Arrigoni, M.B. Bitonti, R. Cozza, A.M. Innocenti, R. Liso, R. Veltri, Ascorbic acid effect on pericycle cell line in *Allium cepa* root, Caryologia 42 (1989) 213–216.

[4] O. Arrigoni, G. Calabrese, L. De Gara, M.B. Bitonti, R. Liso, Correlation between changes in cell ascorbate and growth of *Lupinus albus* seedlings, J. Plant Physiol. 150 (1997) 302–308.

[5] O. Arrigoni, L. De Gara, F. Tommasi, R. Liso, Changes in the ascorbate system during seed development of *Vicia faba* L., Plant Physiol. 99 (1992) 235–238.

[6] O. Arrigoni, M.C. De Tullio, The role of ascorbic acid in cell metabolism: between gene-directed functions and unpredictable chemical reactions, J. Plant Physiol. 157 (2000) 481–488.

[7] K. Asada, Production and action of active oxygen species in photosynthetic tissues, in: C.H. Foyer, P.M. Mullineaux (Eds.), Causes of Photooxidative Stress and Amelioration of Defence Systems in Plants, CRC Press, Boca Raton, 1994, pp. 77–104.

[8] K. Asada, The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 601–639.

[9] H. Asard, J. Kapila, W. Verelst, A. Bérczi, Higher-plant plasma membrane cytochrome b561: a protein in search of a function, Protoplasma 217 (2001) 77–93.

[10] A. Bérczi, I.M. Møller, NADH-monodehydroascorbate oxidoreductase is one of the redox enzymes in spinach leaf plasma membrane, Plant Physiol. 116 (1998) 1029–1036.

[11] G.N. Bijur, B. Briggs, C.L. Hitchcock, M.V. Williams, Ascorbic acid–dehydroascorbate induces cell cycle arrest at G2–M DNA damage' checkpoint during oxidative stress, Environ. Mol. Mutagen. 33 (1999) 144–152.

[12] G. Borracino, S. Dipierro, O. Arrigoni, Purification and properties of ascorbate free radical reductase from potato tubers, Planta 167 (1986) 521–526.

[13] G. Borraccino, L. Mastropasqua, S. De Leonardis, S. Dipierro, The role of ascorbic acid system in delaying the senescence of oat (*Avena sativa* L.) leaf segments, J. Plant. Physiol. 144 (1994) 161–166.

[14] W.L. Chameides, The chemistry of ozone deposition to plant leaves: Role of ascorbic acid, Environ. Sci. Technol. 23 (1989) 595–600.

[15] S. Citterio, S. Sgorbati, S. Scippa, E. Sparvoli, Ascorbic acid effect on the onset of cell proliferation in pea root, Physiol. Plant. 92 (1994) 601–607.

[16] P.L. Conklin, E.H. Williams, R.L. Last, Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant, Proc. Natl. Acad. Sci. USA 93 (1996) 9970–9974.

[17] J.B. Cooper, J.B. Heuser, J.E. Varner, 3,4-dehydroproline inhibits cell wall assembly and cell division in tobacco protoplasts, Plant Physiol. 104 (1994) 747–752.

[18] M.C. Cordoba-Pedregosa, J.A. González-Reyes, M. del Sagrario Canadillas, P. Navas, F. Cordoba, Role of apoplastic and cell wall peroxidases on the stimulation of root elongation by ascorbate, Plant Physiol. 112 (1996) 1119–1126.

[19] R.R. Crichton, Y. Ponce-Otiz, M.H.J. Koch, R. Parfait, H.B. Stuhrmann, Isolation and characterization of phytoferritin from pea (*Pisum sativum*) and lentil (*Lens esculenta*), Biochem. J. 171 (1978) 349–356.

[20] M.W. Davey, C. Gilot, G. Persiau, J. Østergaard, Y. Han, G.C. Bauw, et al., Ascorbate biosynthesis in *Arabidopsis* cell suspension culture, Plant Physiol. 121 (1999) 535–544.

[21] R.C. De Cabo, J.A. González-Reyes, P. Navas, The onset of cell proliferation is stimulated by ascorbate free radical in onion root primordia, Biol. Cell. 77 (1993) 231–233.

[22] L. De Gara, M.C. de Pinto, O. Arrigoni, Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination, Physiol. Plant. 100 (1997) 894–900.

[23] L. De Gara, C. Paciolla, M.C. De Tullio, M. Motto, O. Arrigoni, Ascorbate-dependent hydrogen peroxide detoxification and ascorbate regeneration during germination of a highly productive maize hybrid: evidence of an improved detoxification mechanism against reactive oxygen species, Physiol. Plant. 109 (2000) 7–13.

[24] L. De Gara, C. Paciolla, R. Liso, A. Stefani, A. Blanco, O. Arrigoni, Ascorbate metabolism in mature pollen grains of *Dasypyrum villosum* (L.) Borb. during imbibition, J. Plant Physiol. 141 (1993) 405–409.

[25] L. De Gara, C. Paciolla, F. Tommasi, R. Liso, O. Arrigoni, In vivo inhibition of galactono-γ-lactone conversion to ascorbate by lycorine, J. Plant Physiol. 144 (1994) 649–653.

[26] L. De Gara, F. Tommasi, R. Liso, O. Arrigoni, Ascorbic acid utilization by prolyl hydroxylase in vivo, Phytochemistry 30 (1991) 1397–1399.

[27] S. De Leonardis, G. De Lorezo, G. Borracino, S. Dipierro, A specific ascorbate free radical reductase insozyme participates in the regeneration of ascorbate for scavenging toxic oxygen species in potato tuber mitochondria, Plant Physiol. 109 (1995) 847–851.

[28] M.C. De Pinto, D. Francis, L. De Gara, The redox state of the ascorbate–dehydroascorbate pair as a specific sensor of cell division in tobacco BY-2 cells, Protoplasma 209 (1999) 90–97.

[29] M.C. De Pinto, F. Tommasi, L. De Gara, Enzymes of the ascorbate biosynthesis and ascorbate–glutathione cycle in cultured cells of tobacco Bright Yellow-2, Plant Physiol. Biochem. 38 (2000) 541–550.

[30] M.C. De Tullio, C. Paciolla, F. Della Vecchia, N. Rascio, S. D'Emerico, L. De Gara, et al., Changes in onion root development induced by the inhibition of peptidyl prolyl hydroxylase and influence of the ascorbate system on cell division and elongation, Planta 209 (1999) 424–434.

[31] B. Del Bello, E. Maellaro, L. Sugherini, A. Santucci, M. Comporti, A.F. Casini, Purification of NADPH-dependent dehydroascorbate reductase from rat liver and its identification with 3α-hydroxysteroid dehydrogenase, Biochem. J. 304 (1994) 385–390.

[32] J.C. Deutsch, Dehydroascorbic acid, J. Chromat. 881 (2000) 301–309.

[33] R. Di Cagno, L. Guidi, L. De Gara, G.F. Soldatini, Combined cadmium and ozone treatments affects photosynthesis and ascorbate-dependent defences in sunflower, New Phytolog. 151 (2001) 627–636.

[34] S. Dipierro, G. Borracino, Dehydroascorbate reductase from potato tubers, Phytochemistry 30 (1991) 427–429.

[35] C.H. Foyer, B. Halliwell, Presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism, Planta 133 (1976) 21–25.

[36] C.H. Foyer, B. Halliwell, Purification and properties of dehydroascorbate reductase from spinach leaves, Phytochemistry 16 (1977) 1347–1350.

[37] C.H. Foyer, F.L. Theodoulou, S. Delrot, The functions of intracellular glutathione transport systems in plants, Trends Plant Sci. 6 (2001) 486–492.

[38] M. González, F.J. de Quevedo, J.A. Alcaín, P. González-Reyes, Navas Growth patters of the HL-60 human promyelocyte cell line stimulated by ascorbate free radical, Cancer J. 4 (1991) 262–266.

[39] J.A. González-Reyes, F. Córdoba, P. Navas, Involvement of plasma membrane redox systems in growth control of animal and plant cells, in: H. Asard, A. Bérczi, R.J. Caubergs (Eds.), Plasma membrane redox systems and their role in biological stress and disease, Kluwer Academic Publishers, Dordrecht, 1998, pp. 193–213.

[40] L.H. Hartwell, T.A. Weinert, Checkpoints: controls that ensure the order of cell cycle events, Science 246 (1989) 629–634.

[41] U. Heber, C. Miyake, J. Mano, C. Ohno, K. Asada, Monodehydroascorbate radical detected by electron paramagnetic resonance spectrometry is a sensitive probe of oxidative stress in intact leaves, Plant Cell Physiol. 37 (1996) 1066–1072.

[42] C. Herschbach, H. Rennenberg, Influence of glutathione on net uptake of sulphate and sulphate transport in tobacco plants, J. Exp. Bot. 45 (1994) 1069–1076.

[43] N. Horemans, C.H. Foyer, G. Potters, H. Asard, Ascorbate function and associated transport systems in plants, Plant Physiol. Biochem. 38 (2000) 531–540.

[44] N. Horemans, G. Potters, R.J. Caubergs, H. Asard, Transport of ascorbate into protoplasts of *Nicotiana tabacum* Bright Yellow-2 cell line, Protoplasma 205 (1998) 114–121.
[45] M.A. Hossain, K. Asada, Purification of dehydroascorbate reductase from spinach and its characterisation as a thiol enzyme, Plant Cell Physiol. 25 (1984) 85–92.
[46] W.C. Hou, H.J. Chen, Y.H. Lin, Dioscorins, the major tuber storage proteins of yam (*Dioscorea batatas* Decne), with dehydroascorbate reductase and monodehydroascorbate reductase activities, Plant Sci. 149 (1999) 151–156.
[47] W.C. Hou, Y.H. Lin, Dehydroascorbate reductase and monodehydroascorbate reductase activities of trypsin inhibitors, the major sweet potato (*Ipomoea batatas* (L.) Lam) root storage protein, Plant Sci. 128 (1997) 151–158.
[48] R. Howden, C.R. Andersen, P.B. Goldsbrough, C.S. Cobbett, Cadmium sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*, Plant Physiol. 107 (1995) 1067–1073.
[49] T. Hunter, T. Hunt, R.J. Jackson, H.D. Robertson, The characteristics of inhibition of protein synthesis by double stranded ribonucleic acid in reticulocyte lysates, J. Biol. Chem. 250 (1975) 409–417.
[50] T. Imai, S. Karita, G. Shiratori, M. Hattori, T. Nunome, K. Oba, et al., L-galactono-γ-lactone dehydrogenase from sweet potato: purification and cDNA sequence analysis, Plant Cell Physiol. 39 (1998) 1350–1358.
[51] A.M. Innocenti, M.B. Bitonti, O. Arrigoni, R. Liso, The size of quiescent centre in roots of *Allium cepa* L. grown with ascorbic acid, New Phytol. 110 (1990) 507–509.
[52] A. Jiménez, J.A. Hernández, L.A. Del Río, F. Sevilla, Evidence for the presence of the ascorbate–glutathione cycle in mitochondria and peroxisomes of pea leaves, Plant Physiol. 114 (1997) 275–284.
[53] R.W. Joy, K.R. Patel, T.A. Thorpe, Ascorbic acid enhancement of organogenesis in tobacco, Plant Cell Tissue Organ. Cult. 13 (1988) 219–228.
[54] N. Kato, M. Esaka, Changes in ascorbate oxidase gene expression and ascorbate levels in cell division and cell elongation in tobacco cells, Physiol. Plant. 105 (1999) 321–329.
[55] Y. Kato, J. Urano, Y. Maki, T. Ushimaru, Purification and characterization of dehydroascorbate reductase from rice, Plant Cell Physiol. 38 (1997) 173–178.
[56] S.E. Keates, N.M. Tarlyn, F.A. Loewus, V.R. Franceschi, L-ascorbic acid and L-galactose are sources for oxalic acid and calicium oxalate in *Pistia stratiotes*, Phytochemistry 53 (2000) 433–440.
[57] N.M. Kerk, L.J. Feldman, A biochemical model for the initiation and maintenance of the quiescent center: implications for organization of root meristems, Development 121 (1995) 2825–2833.
[58] R. Liso, G. Calabrese, M.B. Bitonti, O. Arrigoni, Relationship between ascorbic acid and cell division, Exp. Cell Res. 150 (1984) 314–320.
[59] R. Liso, A.M. Innocenti, M.B. Bitonti, O. Arrigoni, Ascorbic acid-induced progression of quiescent centre cells from G1 to S-phase, New Phytol. 110 (1988) 469–471.
[60] M.W.F. Luwe, U. Takahama, U. Heber, Role of ascorbate in detoxifying ozone in the apoplast of spinach (*Spinacia oleracea* L.) leaves, Plant Physiol. 101 (1993) 969–976.
[61] K. Marrs, The functions and regulation of glutathione S-transferases in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 127–158.
[62] J.M. May, C.E. Cobb, S. Mendiretta, K.E. Hill, R.F. Burk, Reduction of the ascorbyl free radical to ascorbate by thioredoxin reductase, J. Biol. Chem. 273 (1998) 23039–23045.
[63] J.M. May, S. Mendiretta, K.E. Hill, R.F. Burk, Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase, J. Biol. Chem. 272 (1997) 22607–22610.
[64] M.J. May, C.J. Leaver, Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures, Plant Physiol. 103 (1993) 621–627.
[65] M.J. May, T. Vernoux, C. Leaver, M. Van Montagu, D. Inzé, Glutathione homeostasis in plants: implications for environmental sensing and plant development, J. Exp. Bot. 49 (1998) 649–667.
[66] G.A. McClarty, A.K. Chan, B.K. Choy, J.A. Wright, Increased ferritin gene expression is associated with ribonucleotide reductase gene expression and the establishment of hydroxyurea resistance in mammalian cells, J. Biol. Chem. 265 (1990) 7539–7547.
[67] C. Miyake, K. Asada, Ferredoxin-dependent photoreduction of the monodehydroascorbate radical in spinach thylakoids, Plant Cell Physiol. 35 (1995) 539–549.
[68] S. Morell, H. Follmann, M. De Tullio, I. Häberlein, Dehydroascorbate and dehydroascorbate reductase are phantom indicators of oxidative stress in plants, FEBS Lett. 414 (1997) 567–570.
[69] F. Navarro, J.C. Rodriguez-Anguilera, F.J. Alcain, M.I. Buron, P. Navas, Ascorbate potentiates serum-induced S-phase entry of quiescent 3T3 cells, Protoplasma 169 (1992) 85–87.
[70] P. Navas, J.M. Villalba, F. Córdoba, Ascorbate function at the plasma membrane, Biochim. Biophys. Acta 1197 (1994) 1–13.
[71] G. Noctor, A.C.M. Arisi, L. Jouanin, K.J. Kunert, H. Rennenberg, C.H. Foyer, Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants, J. Exp. Bot. 49 (1998) 623–647.
[72] G. Noctor, C.H. Foyer, Ascorbate and glutathione: keeping active oxygen under control, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 249–279.
[73] C. Paciolla, M.C. De Tullio, A. Chiappetta, A.M. Innocenti, M.B. Bitonti, R. Liso, et al., Short- and long-term effects of dehydroascorbate in *Lupinus albus* and *Allium cepa* roots, Plant Cell Physiol. 42 (2001) 857–863.
[74] C.H. Park, B.F. Kimler, Growth modulation of human leukemic, preleukemic and myeloma progenitor cells by L-ascorbic acid, Am. J. Clin. Nutr. 54 (1991) 12415–12465.
[75] G. Potters, N. Horemans, R.J. Caubergs, H. Asard, Ascorbate and dehydroascorbate influence cell cycle progression in a *Nicotiana tabacum* cell suspension, Plant Physiol. 124 (2000) 17–20.
[76] W.E. Rauser, Phytochelatins, Annu. Rev. Biochem. 59 (1990) 61–86.
[77] J.P. Reichheld, T. Vernoux, F. Lardon, M. Van Montagu, D. Inzé, Specific checkpoint regulate plant cell cycle progression in response to oxidative stress, Plant J. 17 (1999) 647–656.
[78] M.E. Reid, Relation of vitamin C to cell size in the growing region of the primary root of cowpea seedlings, Am. J. Bot. 28 (1941) 410–415.
[79] T. Russo, N. Zambrano, F. Esposito, R. Ammendola, F. Cimino, M. Fiscella, et al., A p53-independent pathway for activation of *WAF1/CIP1* expression following oxidative stress, J. Biol. Chem. 270 (1995) 29386–29391.
[80] M. Sánchez, E. Queijeiro, G. Revilla, I. Zarra, Changes in ascorbic acid levels in apoplastic fluid during growth of pine hypocotyls. Effect on peroxidase activities associated with cell walls, Physiol. Plant. 101 (1997) 815–820.
[81] R. Sánchez-Fernández, M. Fricker, L.B. Corben, N.S. White, N. Sheard, C.J. Leaver, et al., Cell proliferation and hair tip growth in the *Arabidopsis* root under mechanistically different forms of redox control, Proc. Natl. Acad. Sci. USA 94 (1997) 2745–2750.
[82] A. Sen Gupta, R.G. Alscher, D. McCume, Response of photosynthesis and cellular antioxidants to ozone in *Populus* leaves, Plant Physiol. 96 (1991) 650–655.
[83] N. Smirnoff, P.L. Conklin, F.A. Loewus, Biosynthesis of ascorbic acid in plants: a renaissance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 437–468.
[84] C. Stasolla, E.C. Yeung, Ascorbic acid improves conversion of white spruce somatic embryos, In Vitro Cell Dev. Biol. 35 (1999) 316–319.
[85] C. Stasolla, E.C. Yeung, Ascorbic acid metabolism during white spruce somatic embryo maturation and germination, Physiol. Plant. 111 (2001) 196–206.

M. Suthantiran, M.E. Anderson, V.K. Sharma, A. Meister, Glutathione regulates activation-dependent DNA synthesis in highly purified normal human T lyphocytes via the CD2 and CD3 antigens, Proc. Natl. Acad. Sci. USA 87 (1990) 3343–3347.

U. Takahama, Redox state of ascorbic acid in the apoplast of stems of *Kalanchoe daigremontiana*, Physiol. Plant. 89 (1993) 791–798.

U. Takahama, Changes induced by abscisic acid and light in the redox state of ascorbate in the apoplast of epicotyls of *Vigna angularis*, Plant Cell Physiol. 35 (1994) 975–978.

U. Takahama, M. Hirotsu, T. Oniki, Age-dependent changes in levels of ascorbic acid and chlorogenic acid, and activities of peroxidase and superoxide dismutase in the apoplast of tobacco leaves: mechanism of the oxidation of chlorogenic acid in the apoplast, Plant Cell Physiol. 40 (1999) 716–724.

F. Tommasi, C. Paciolla, M.C. de Pinto, L. De Gara, A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seeds, J. Exp. Bot. 52 (2001) 1647–1654.

S. Trümper, H. Follmann, I. Häberlein, A novel dehydroascorbate reductase from spinach chloroplasts homologous to plant trypsin inhibitor, FEBS Lett. 352 (1994) 159–162.

J. Urano, T. Nakagawa, Y. Maki, T. Masumura, K. Tanaka, N. Murata, et al., Molecular cloning and characterization of a rice dehydroascorbate reductase, FEBS Lett. 466 (2000) 107–111.

[93] H. Vanacker, T.L.W. Carter, C.H. Foyer, Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves, Plant Physiol. 117 (1998) 1103–1114.

[94] S.D. Veljovic-Jovanovic, C. Pignocchi, G. Noctor, C.H. Foyer, Low ascorbic acid in the *vtc-1* mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system, Plant Physiol. 127 (2001) 426–435.

[95] T. Vernoux, R.C. Wilson, K.A. Seeley, J.P. Reichheld, S. Muroy, S. Brown, et al., The *ROOT MERISTEMLESS1/CADMIUM SENSITIVE2* gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development, Plant Cell 12 (2000) 97–109.

[96] W.W. Wells, D.P. Xu, Y. Yanf, P.A. Rocque, Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity, J. Biol. Chem. 265 (1990) 15361–15364.

[97] C. Xiang, B.L. Werner, E.M. Christensen, D.J. Oliver, The biological functions of glutathione revisited in *Arabidopsis* transgenic plants with altered glutathione levels, Plant Physiol. 126 (2001) 564–574.

Ascorbate biosynthesis and function in photoprotection

Nicholas Smirnoff

School of Biological Sciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter EX4 4PS, UK (n.smirnoff@exeter.ac.uk)

Ascorbate (vitamin C) can reach very high concentrations in chloroplasts (20–300 mM). The pool size in leaves and chloroplasts increases during acclimation to high light intensity and the highest concentrations recorded are in high alpine plants. Multiple functions for ascorbate in photosynthesis have been proposed, including scavenging of active oxygen species generated by oxygen photoreduction and photorespiration, regeneration of α-tocopherol from α-tocopheryl radicals, cofactor for violaxanthin de-epoxidase and donation of electrons to photosystem II. Hydrogen peroxide scavenging is catalysed by ascorbate peroxidase (Mehler peroxidase reaction) and the subsequent regeneration of ascorbate by reductant derived from photosystem I allows electron flow in addition to that used for CO_2 assimilation. Ascorbate is synthesized from guanosine diphosphate-mannose *via* L-galactose and L-galactono-1,4-lactone. The last step, catalysed by L-galactono-1,4-lactone dehydrogenase, is located on the inner mitochondrial membrane and uses cytochrome *c* as electron acceptor. L-galactono-1,4-lactone oxidation to ascorbate by intact leaves is faster in high-light acclimated leaves and is also enhanced by high light, suggesting that this step contributes to the control of pool size by light. Ascorbate-deficient *Arabidopsis thaliana vtc* mutants are hypersensitive to a number of oxidative stresses including ozone and ultraviolet B radiation. Further investigation of these mutants shows that they have reduced zeaxanthin-dependent non-photochemical quenching, confirming that ascorbate is the cofactor for violaxanthin de-epoxidase and that availability of thylakoid lumen ascorbate could limit this reaction. The *vtc* mutants are also more sensitive to photooxidation imposed by combined high light and salt treatments.

Keywords: *Arabidopsis thaliana vtc* mutants; chlorophyll fluorescence; L-galactono-1,4-lactone dehydrogenase; non-photochemical quenching; vitamin C

1. INTRODUCTION

n is potentially toxic and even more so when ned with light, pigments and electron transport y: such conditions are provided in chloroplasts. ynthesis releases oxygen, absorbs light and carries ctron transport so the chloroplast therefore needs tion from reactive oxygen species. These include xide and hydrogen peroxide formed by oxygen eduction, and singlet oxygen formed by transfer of ion energy to oxygen (Asada 1999). Additionally espiration generates hydrogen peroxide from the ate oxidase reaction in the peroxisomes. Antits and free radical scavengers are needed to deal hese toxic products of photosynthesis (Noctor & 1998; Asada 1999; Niyogi 1999). Ascorbate is the bundant soluble antioxidant in chloroplasts. Inter-y, in mammals, the eye is amongst the tissues ning the highest ascorbate concentration (Halliwell tteridge 1999), which suggests that ascorbate is larly important in situations where cells contain nts designed to absorb light effectively.

n its abundance and the importance of plants as a source of ascorbate (vitamin C) for humans (who able to synthesize it), it is surprising that very little wn about ascorbate metabolism in plants. Its thetic pathway is different from mammals and has only recently been established. New information on the role of ascorbate in photosynthesis and photoprotection is beginning to emerge, aided by knowledge of the biosynthetic pathway and the isolation of ascorbate-deficient (*vtc*) mutants of *Arabidopsis thaliana*. This paper reviews the role of ascorbate in photoprotection. New data on the relationship between light and biosynthesis, and the use of ascorbate-deficient *A. thaliana* mutants to investigate photoprotective roles of ascorbate, are presented.

2. THE ROLE OF ASCORBATE IN PHOTOSYNTHESIS

The involvement of ascorbate in photosynthesis has been recognized for some time and has been reviewed relatively recently with emphasis on photoprotection (Smirnoff 2000; Noctor & Foyer 1998; Asada 1999; Niyogi 1999). Possible roles for ascorbate in photosynthesis were suggested by Arnon and co-workers in the early 1950s (Marrè *et al.* 1959; Forti & Jagendorf 1961; Mapson 1962). Initially a role as electron carrier was considered but later as a protectant. Our current knowledge of the roles of ascorbate in photosynthesis was foreshadowed by Marrè *et al.* (1959) who proposed that chloroplasts could both oxidize ascorbate to monodehydroascorbate (MDA) and reduce MDA to ascorbate, these processes being dependent on water splitting and electron flow. They also suggested the involvement of a monodehydroascorbate

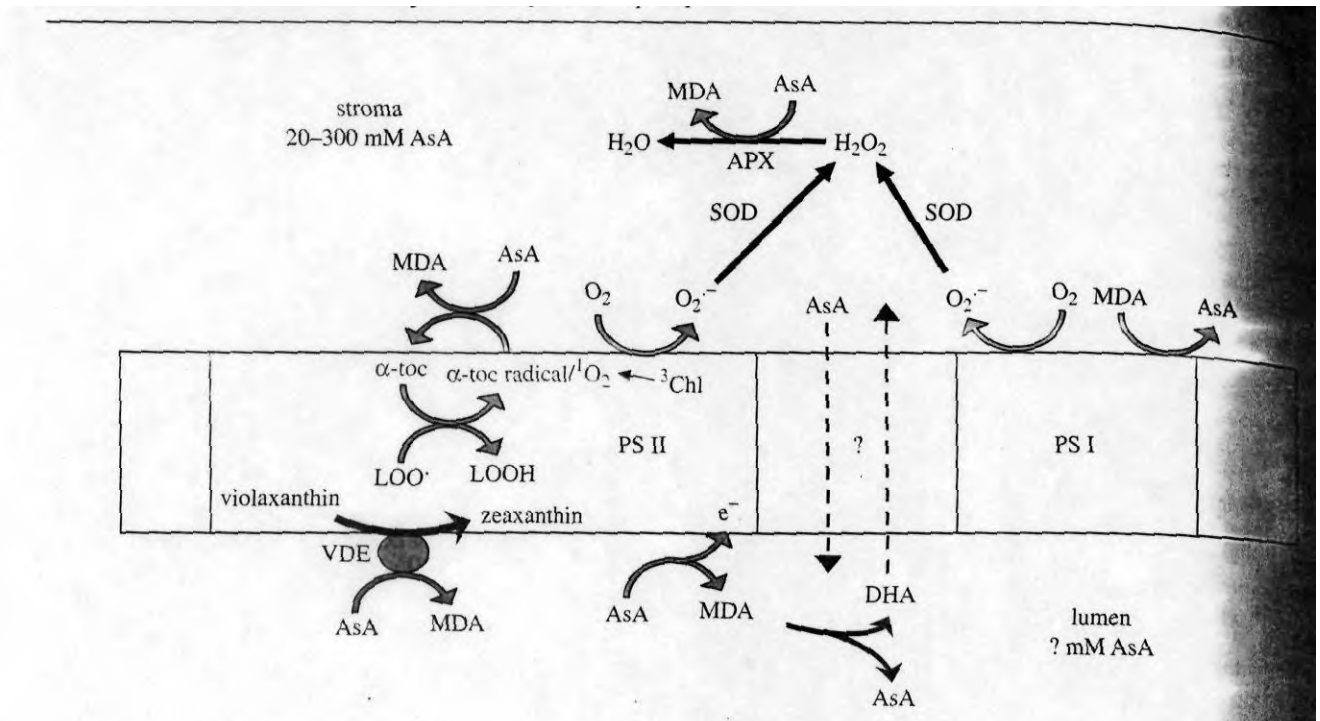


Figure 1. The roles of ascorbate in photosynthesis. The points at which ascorbate acts as an electron donor and monodehy ascorbate acts as an electron acceptor are shown diagrammatically, along with its roles as a cofactor for violaxanthin de-epoxidase, removal of singlet oxygen and regeneration of α-tocopherol from α-tocopheryl radicals. Movement of ascor and dehydroascorbate across the thylakoid lumen is shown by dashed lines—a carrier system has not been identified. Rea that regenerate ascorbate from monodehydroascorbate (NADPH-dependent monodehydroascorbate reductase) and dehyd ascorbate (glutathione-dependent dehydroascorbate reductase) are not shown. APX, ascorbate peroxidase; AsA, ascorbic ascorbate; ^{3}Chl, triplet chlorophyll; DHA, dehydroascorbate; LOO·, lipid peroxyl radical; LOOH, lipid hydroperoxide; M monodehydroascorbate radical; $^1O_2$, singlet oxygen; PS I, photosystem I; PS II, photosystem II; SOD, superoxide dismut α-toc, α-tocopherol; VDE, violaxanthin de-epoxidase.

reductase enzyme. Forti & Jagendorf (1961) showed that ascorbate stimulates photophosphorylation and allows electron transport by acting catalytically in the Mehler reaction. Mapson (1962) showed that ascorbate is subject to photo-oxidation and that both oxidation and reduction of dehydroascorbate were inhibited by blocking photosynthetic electron transport. Later, when the details of photosynthetic electron transport were more clearly understood, it become clear that ascorbate and glutathione in chloroplasts are oxidized by hydrogen peroxide produced by the Mehler reaction and that reduction of their oxidized forms uses reductant from photosystem I (PS I) (Foyer & Halliwell 1976; Anderson *et al.* 1983; Asada 1999). Current understanding of the roles of ascorbate in photosynthesis can be summarized as follows (figure 1).

(i) Hydrogen peroxide scavenging catalysed by ascorbate peroxidase (APX).
(ii) Direct scavenging of superoxide, hydroxyl radicals and singlet oxygen.
(iii) Regeneration of α-tocopheryl radicals produced when α-tocopherol reduces lipid peroxyl radicals.
(iv) Electron donation to photosystem II (PS II) by lumenal ascorbate.
(v) Cofactor of violaxanthin de-epoxidase (VDE) involved in zeaxanthin-dependent dissipation of excess excitation energy (a component of non-photochemical quenching).

Oxidants formed during photosynthesis (Asada 1999) can be scavenged non-enzymatically while hydrogen peroxide reduction is also catalysed by APX of there are stromal- and thylakoid-bound forms 1999). Hydrogen peroxide is formed from dismutat superoxide that itself is produced by oxygen redu either *via* reduced ferredoxin (Mehler reaction) or PS II (Cleland & Grace 1999). The primary oxid product of ascorbate is the relatively stable mo dehydroascorbate radical (MDA). This can be detect *vivo* by electron paramagentic resonance spectrosco leaves. In some cases it is detected after illumination bright light but is particularly prominent after impos of oxidative stress by paraquat, ultraviolet B (UVB drought (Heber *et al.* 1996; Hideg *et al.* 1997). It is p able that the importance of ascorbate as a free-ra scavenger depends on the relative stability of the M radical: thilyl radicals, formed when thiols such glutathione scavenge free radicals, are more reactive are themselves relatively dangerous (Sturgeon *et al.* 1 The MDA radical disporportionates to ascorbate dehydroascorbate. Normally the ascorbate pool in le and chloroplasts is 90% reduced and this is achieve systems that reduce MDA and dehydroascorbate (D back to ascorbate. This is important because DH unstable, particularly at the pH of illuminated stro The ascorbate regeneration systems include direct re tion of MDA by PS I, NADPH-dependent MDA ductase and glutathione-dependent dehydroascor reductase. NADPH-dependent glutathione reduc regenerates reduced glutathione (GSH) from oxid glutathione (GSSG). The net result of the Mehler rea is that water is the final product of oxygen photoredu

hat photosynthetically generated reductant is used to erate the ascorbate used in this reaction. This allows ron flow in addition to that used for CO_2, NO_3^- and assimilation. The consequences and significance of o-called Mehler peroxidase or water–water reaction discussed by Asada (1999, this issue).

4. THE RELATIONSHIP BETWEEN LIGHT AND THE ASCORBATE CONCENTRATION IN LEAVES AND CHLOROPLASTS

The total concentration of ascorbate in leaves is light ndent. Growth at high light intensity produces leaves higher ascorbate content than at low light intensity rnoff & Pallanca 1996; Grace & Logan 1996; Logan 1996). The readjustment of ascorbate concentration latively slow, occurring over a period of several days transfer (Eskling & Åkerlund 1998). Conversely, transfer to low light, ascorbate content decreases to w steady state within a few days (Eskling & Åkerlund The concentration range found in leaves varies around 2–20 $\mu mol\, g^{-1}$ fresh weight (Wildi & Lutz Streb *et al.* 1997). The higher concentrations seem to ur more frequently in temperate evergreen and alpine ies. Alpine species are also characterized by relatively concentrations of other antioxidants (glutathione α-tocopherol) and carotenoids (Wildi & Lutz 1996; reb *et al.* 1997). GSH, the other major soluble anti-dant, is generally tenfold less concentrated than rbate (Noctor & Foyer 1998).

A number of estimates of ascorbate concentration in loroplasts have been made, generally using chloroplasts ated in aqueous media. The values quoted range from 230 mM (Foyer *et al.* 1983; Rautenkranz *et al.* 1994; reb *et al.* 1997). Ascorbate is therefore one of the most ndant chloroplast metabolites, even at the most oted concentration of 20–50 mM, and in alpine plants concentration is enormous, reaching 300 mM (Streb *al.* 1997). The pool is generally highly reduced (90% +), less the chloroplasts are subjected to oxidative stress by raquat or H_2O_2 treatment (Law *et al.* 1983). The crease in leaf content is also mirrored by equivalent creases in chloroplast ascorbate (Schöner & Krause 90). Ascorbate is also much more abundant in chloro-asts than GSH, which has a concentration *ca.* 3–4 mM oyer & Halliwell 1976; Streb *et al.* 1997). The concentra-on of ascorbate in the thylakoid lumen is not known for rtain and it has been suggested that thylakoids have no corbate transport system (Foyer & Lelandais 1996). suming there is 50 mM ascorbate in the stroma at H 8 and that undissociated ascorbic acid (the pK_a of corbic acid is 4.2) diffuses across the thylakoid mbrane, Eskling *et al.* (1997) calculated that the ncentration of ascorbic acid in the thylakoid lumen ould be 8 μM in the absence of a carrier. The concentra-on of ascorbate in the lumen is critical for operation of xanthophyll cycle, since it is a cofactor of VDE kling *et al.* 1997). VDE prefers undissociated ascorbic id that predominates over the ascorbate anion at low H (Bratt *et al.* 1995; Eskling *et al.* 1997). VDE catalyses nversion of antheroxanthin and violaxanthin to axanthin in leaves exposed to high light. Zeaxanthin is volved in non-photochemical quenching by dissipating excitation energy as heat (Niyogi 1999). The predicted concentration of 8 μM is much lower than the measured K_m of 100 μM for VDE. They therefore proposed that there must be thylakoid carriers to transport ascorbate in and DHA out. On the other hand, Mano *et al.* (1997) suggested that 10–20% of chloroplast ascorbate occurs in the thylakoid lumen. Since the thylakoid lumen volume of a typical chloroplast is about 20% of the total chloroplast volume (Lawlor 1993) this measurement implies that lumen and stroma have a similar ascorbate concentration. Further measurements are needed but it is possible that the light responsiveness of the ascorbate pool and the high concentration in the stroma is related to the need to maintain sufficient ascorbate for VDE activity in the thylakoid lumen.

The last step of ascorbate biosynthesis occurs in the mitochondria (see §4) and there is little evidence that chloroplasts can synthesize ascorbate. It must therefore be transported from the cytosol. Ascorbate uptake has been measured by isolated chloroplasts and is a facilitated diffusion mechanism with a K_m of 20 mM (Anderson *et al.* 1983; Beck *et al.* 1983; Foyer & Lelandais 1996). The rate of uptake is not sufficient to keep pace with the rate of ascorbate oxidation in the chloroplast (Anderson *et al.* 1983), so ascorbate concentration inside is maintained by the well-known regeneration systems (Asada 1999). The uptake rate must be sufficient to accommodate the relatively slow adjustment in ascorbate pool size that occurs when light intensity changes.

With the exception of inorganic ions such as magnesium, potassium and phosphate (Lawlor 1993), ascorbate is probably the most abundant metabolite in chloroplasts. The question of why so much ascorbate is required in chloroplasts arises. The alpine plants with exceptionally high chloroplast ascorbate may provide a clue—these plants are exposed to a combination of conditions that are conducive to photo-oxidative stress: high light, low temperature and relatively high UVB radiation. Also, given that the rate of ascorbate biosynthesis is relatively sluggish, that transport into the chloroplast is slow and that DHA is unstable, it is possible that a high concentration is needed as an insurance against episodes of increased photo-oxidative stress. It is notable that the ascorbate-deficient *vtc1 A. thaliana* mutant is hypersensitive to UVB radiation, as well as ozone and sulphur dioxide (Conklin *et al.* 1996). Further evidence for the importance of ascorbate in resistance to oxidative stress comes from overexpression and antisense suppression of various APX genes. Antisense suppression of cytosolic APX increases ozone sensitivity (Orvar & Ellis 1997) while overexpression of peroxisomal APX increases hydrogen peroxide resistance (Wang *et al.* 1999). Transcript levels of cytosolic APX increase rapidly when *A. thaliana* leaves are exposed to high light (Karpinski *et al.* 1997, 1999). It seems likely that the cytosolic APX has a role in scavenging photorespiratory hydrogen peroxide that leaks from the peroxisomes as well as any leaking from chloroplasts (Foyer & Noctor 1999). There is evidence that hydrogen peroxide and/or redox signals from Q_B or PQ are the signal inducing APX (Karpinski *et al.* 1999; Morita *et al.* 1999) and that a systemic response is induced in brightly illuminated leaves so that APX transcripts increase in shaded leaves of the same

plant (Karpinski *et al.* 1999). Assuming that the increased cytosolic APX transcript levels translate into increased APX activity, it seems that rapid induction of APX by high light could compensate for the slow response of the ascorbate pool itself.

4. BIOSYNTHESIS PATHWAY

The plant ascorbate biosynthesis pathway differs from that in mammals and a complete scheme has recently been proposed (Wheeler *et al.* 1998; Smirnoff & Wheeler 1999; Conklin *et al.* 1999; Loewus 1999; Smirnoff 2000). The immediate precursor is L-galactose, which is oxidized to L-galactono-1,4-lactone by an NAD-dependent L-galactose dehydrogenase. L-galactono-1,4-lactone is oxidized to ascorbate by L-galactono-1,4-lactone dehydrogenase. This enzyme is located on the inner mitochondrial membrane and donates electrons to cytochrome *c* (Siendones *et al.* 1999; Bartoli *et al.* 2000). L-galactose and L-galactono-1,4-lactone are readily converted to ascorbate when supplied to intact tissue and can elevate the ascorbate pool up to tenfold within a few hours. L-galactose is derived from mannose-1-phosphate *via* guanosine diphosphate (GDP)-mannose and GDP-L-galactose. Evidence for this pathway is derived from ^{14}C-labelling studies (Wheeler *et al.* 1998) and from the ascorbate-deficient *vtc1* mutant of *A. thaliana*. *Vtc1* has *ca.* 30% of wild-type ascorbate and has a lower rate of biosynthesis (Conklin *et al.* 1997). *VTC1* encodes GDP-mannose pyrophosphorylase (Conklin *et al.* 1999). Leaf extracts from mutant plants have about 30% of the activity of GDP-mannose pyrophosphorylase and ascorbate content can be restored by transforming the mutant with the wild-type gene (Conklin *et al.* 1999). Further confirmation of the role of GDP-mannose pyrophosphorylase comes from reduction of its expression by antisense technology in the potato. The resulting plants have lower ascorbate content (Keller *et al.* 1999). A number of details remain to be resolved. The GDP-mannose-3,5-epimerase, proposed to convert GDP-mannose to GDP-L-galactose, has been detected but not characterized in any detail. Conversion of GDP-L-galactose to L-galactose occurs in cell-free extracts but the nature of the reactions is not fully resolved (G. L. Wheeler and N. Smirnoff, unpublished data). It is likely that further ascorbate-deficient *vtc* mutants currently being characterized will be useful for identifying the genes involved in ascorbate biosynthesis (Conklin *et al.* 2000).

L-galactose appears to be a dedicated precursor for ascorbate, while earlier intermediates have other roles. The GDP-sugars are used as cell wall polysaccharide precursors and for protein glycosylation. Antisense potato plants with reduced GDP-mannose pyrophosphorylase activity also have reduced mannose content in their wall polysaccharides (Keller *et al.* 1999). It is currently assumed that the pathway occurs in the cytosol with the exception of the final step in the mitochondria. The integration of L-galactono-1,4-lactone oxidation into the mitochondrial electron transport chain *via* cytochrome *c* could have implications for coordinating ascorbate metabolism with the energy metabolism and redox state of the cell. Currently very little is known about the control of the pathway. Evidence for feedback inhibition or

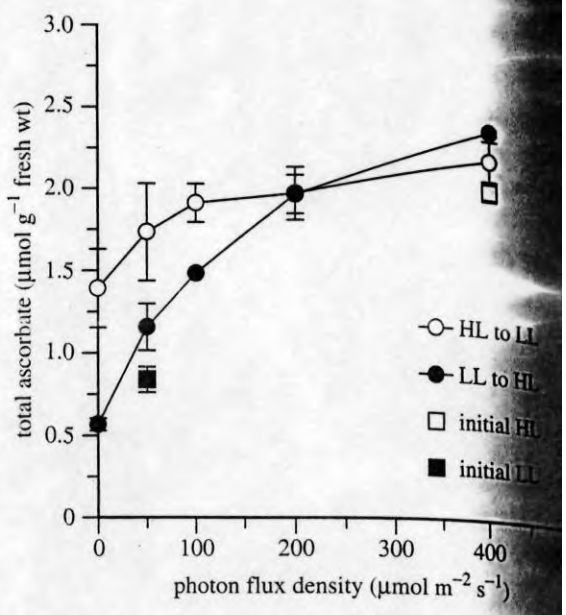


Figure 2. Light dependence of the ascorbate pool in barley leaves (*Hordeum vulgare*). Barley seedlings were grown at photon flux densities of 50 µmol m^{-2} s^{-1} (initial LL) and 200 µmol m^{-2} s^{-1} (initial HL) for seven days. Leaf slices from HL-grown plants (HL to LL) and LL-grown plants (LL to HL) were then floated on water and exposed to a range of light intensities for 24 h before determining total ascorbate concentration. The values are means of three replicates ± standard deviation.

repression of synthesis by ascorbate pool size has been provided (Pallanca & Smirnoff 2000) but nothing is known about the mechanism. Gene probes, antibodies, transgenic plants and quantitative enzyme assays for enzymes involved in the proposed pathway are now becoming available and the next few years should see advances in understanding how ascorbate biosynthesis is controlled.

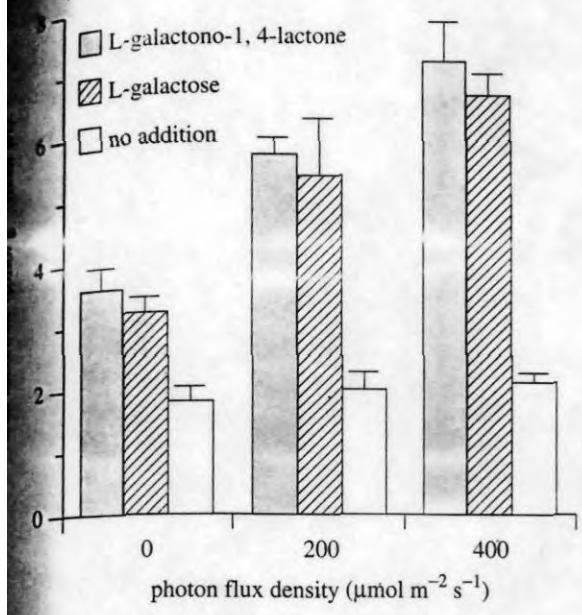
5. MATERIAL AND METHODS

(a) *The effect of light on ascorbate pool size and synthesis*

The primary leaf of seven-day-old barley seedlings was used for investigations of the light dependence of ascorbate biosynthesis. Plants were grown at 20 °C with a 16 h light period. Portions of leaf 3 cm long were cut from the middle of the primary leaf and sliced transversely into 2 mm segments. Leaf segments (0.2 g per replicate) were floated on water in petri dishes. L-galactose and L-galactono-1,4-lactone were added at concentrations designated for each experiment. The leaf slices were exposed to continuous light provided by fluorescent tubes at 20 °C. Light intensities are indicated for each experiment. After incubation for the designated time, the leaf segments were extracted and their total ascorbate content (ascorbate + dehydroascorbate) was measured by an ascorbate oxidase assay (Conklin *et al.* 1997).

(b) *Determination of non-photochemical quenching and other chlorophyll fluorescence parameters*

Wild-type *A. thaliana* Col-0) and *vtc1*, *2*, *3* and *4* mutants (Conklin *et al.* 2000) were grown at 20 °C with a 12 h



re 3. The effect of light on *in vivo* conversion of lactose and L-galactono-1,4-lactone to ascorbate. slices from barley (*Hordeum vulgare*) plants grown at mol m^{-2} s^{-1} were floated on water with 15 mM lactono-1,4-lactone or 15 mM L-galactose. The rbate concentration in the leaf slices was determined incubation for 5.5 h. The values are means of three cates ± s.d.

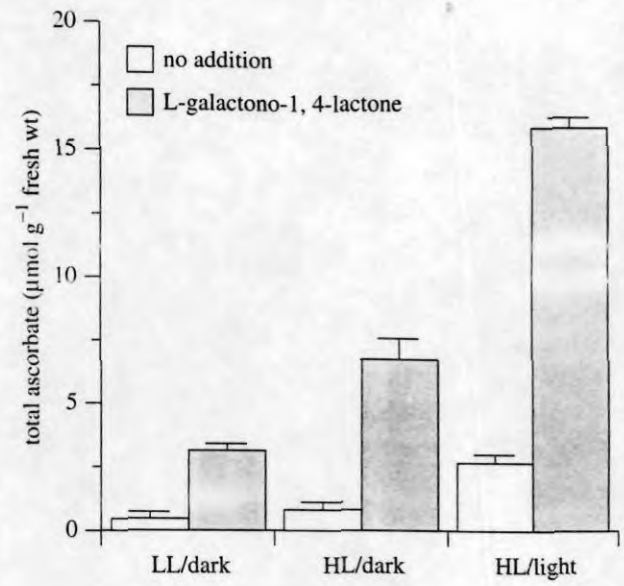


Figure 4. The effect of light on *in vivo* conversion of L-galactono-1,4-lactone to ascorbate. Leaf slices from barley (*Hordeum vulgare*) plants grown at photon flux densities of 80 μmol m^{-2} s^{-1} (LL) and 360 μmol m^{-2} s^{-1} (HL) for three days were floated on water with and without 10 mM L-galactono-1,4-lactone in the dark and light (photon flux density 250 μmol m^{-2} s^{-1}). The ascorbate concentration in the leaf slices was determined after incubation for 24 h. The values are means of three replicates ± s.d.

d at a photon flux density of 200 μmol m^{-2} s^{-1}. Fully nded leaves from preflowering rosettes were used for ophyll fluorescence measurements using a Hansatech FMS1 ulated fluorometer (Hansatech Instruments, King's Lynn, . The modulated beam was set at a level that had no ochemical effect and the high intensity light pulses (*ca.*) μmol m^{-2} s^{-1}) were saturating. Leaves were dark adapted h. After an initial dark-adapted measurement of F_v/F_m, a -response curve for chlorophyll fluorescence parameters was tructed by exposure to a sequence of increasing actinic light s. After 5 min at each level, F'_o (in far-red light), F_s and F'_m measured. Chlorophyll fluorescence parameters were ulated as follows: photochemical quenching coefficient $= (F'_m - F_s)/(F'_m - F'_o)$]; quantum efficiency of PS II [ΦPS II = $F_s)/F'_m$]; non-photochemical quenching coefficient $= (F_m - F'_m)/(F_m - F'_o)$] and non-photochemical quenching $Q = (F_m - F'_m)/F'_m$] (Schreiber *et al.* 1995). A time-course of lopment of chlorophyll fluorescence parameters was deter- d after illumination of dark-adapted leaves with a photon density of 1000 μmol m^{-2} s^{-1}.

(c) *Salt-induced photo-oxidation of* Arabidopsis thaliana *seedlings*

edlings of wild-type and *vtc1, 2, 3* and *4* mutants (Conklin 2000) were grown at 20 °C with a 12 h light period at a on flux density of 200 μmol m^{-2} s^{-1} in Petri dishes aining MS medium (minus sucrose) with or without mM NaCl (water potential, −0.5 MPa). The dishes were sferred to high light (400 μmol m^{-2} s^{-1}) when indicated. proportion of seedlings showing complete bleaching of their es was recorded at intervals.

6. RESULTS AND DISCUSSION

(a) *The effect of light on ascorbate pool size and synthesis*

The ascorbate pool size in barley leaves is higher when the plants are grown at higher light intensity (Smirnoff 1995; Smirnoff & Pallanca 1996). The dependence of the pool size on photon flux density (PFD) was further investigated by growing barley seedlings at relatively low PFD and then exposing leaf slices to a range of PFDs for 20 h. The ascorbate pool increased to new levels after this time (figure 2). At the highest PFD of 400 μmol m^{-2} s^{-1} it reached the same concentration as intact plants acclimated to this PFD over seven days. Leaf slices from intact plants acclimated to 400 μmol m^{-2} s^{-1} did not show a reduction in the ascorbate pool when incubated at lower light intensities unless kept in the dark. This shows that the readjustment is relatively slow, as has been found in other studies (see § 1) and that the ascorbate pool in the leaf slices behaves in a similar manner to intact leaves on exposure to high light. Therefore barley leaf slices provide a useful system to investigate ascorbate metabolism in relation to light. Exogenous L-galactose and L-galactono-1,4-lactone are readily absorbed and converted to ascorbate by leaves, the reactions being catalysed by L-galactose dehydrogenase and mitochondrial L-galactono-1,4-lactone dehydrogenase (Wheeler *et al.* 1998; Loewus 1999; Bartoli *et al.* 2000). Barley leaf slices supplied with these precursors convert both substrates in a light-stimulated manner over 5.5 h (figure 3). Over 24 h conversion of L-galactono-1,4-lactone to ascorbate is also greater in the

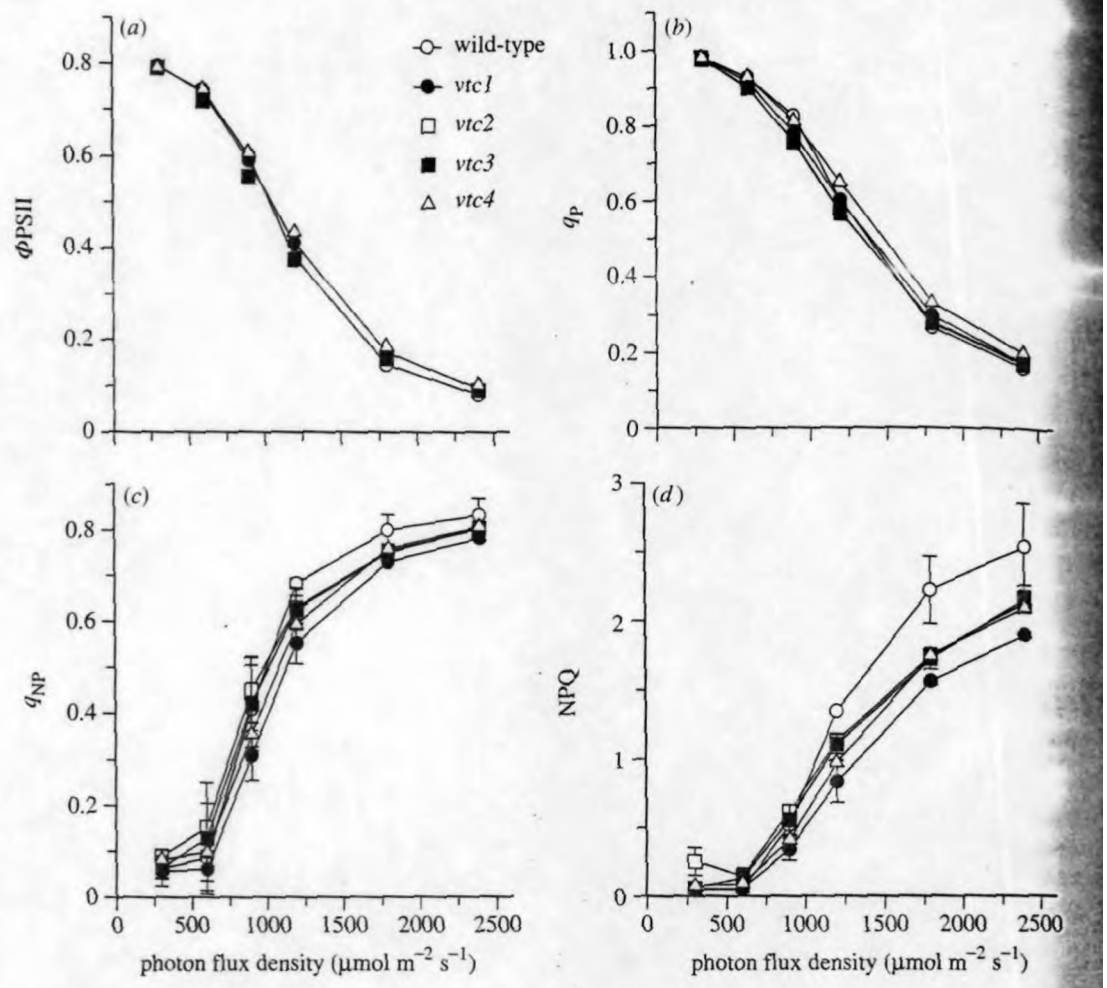


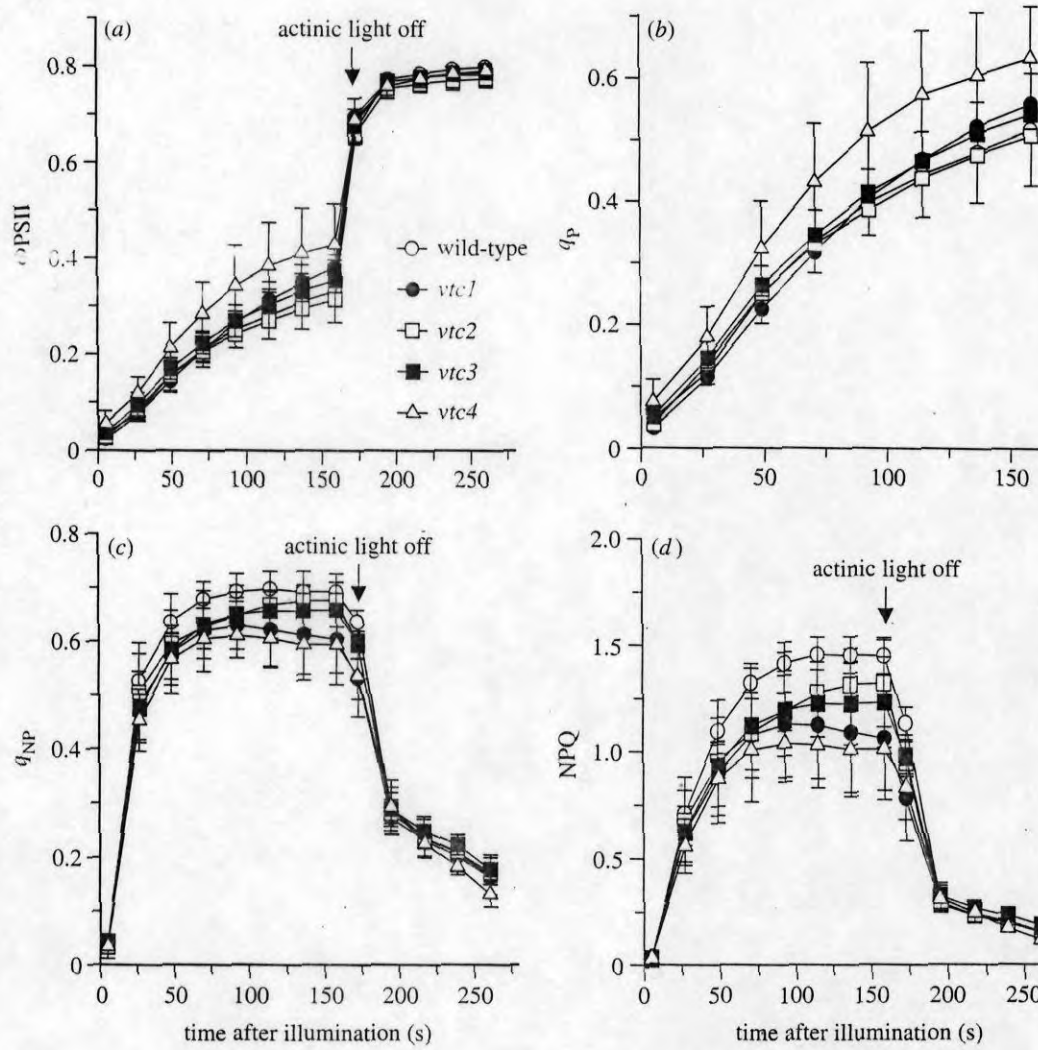
Figure 5. A comparison of the light response of steady-state chlorophyll fluorescence parameters measured in leaves from type and ascorbate-deficient *vtc* mutants of *Arabidopsis thaliana*. (*a*) Quantum efficiency of PS II (ΦPS II). (*b*) Photochemic quenching coefficient (q_P). (*c*) Non-photochemical quenching coefficient (q_{NP}). (*d*) Non-photochemical quenching (NPQ) values are means of four replicates ± s.e.

light than in the dark (figure 4). Furthermore, when leaves were taken from plants previously acclimated to high or low light, conversion of L-galactono-1,4-lactone to ascorbate in the dark was greater in the previously high-light acclimated leaves (figure 4). These results suggest that L-galactono-1,4-lactone oxidation is enhanced by light. The persistent increase in ascorbate synthesis from L-galactono-1,4-lactone in high light acclimated leaves in the dark suggests that their mitochondria have a larger L-galactono-1,4-lactone dehydrogenase capacity. The further increase in ascorbate synthesis when high-light acclimated leaves were kept in the light suggests that there is also a direct enhancement of L-galactono-1,4-lactone oxidation by light. This is under further investigation to determine if light affects expression of L-galactono-1,4-lactone dehydrogenase and if there is an interaction between light and mitochondrial metabolism that affects L-galactono-1,4-lactone activity. It is surprising that the last step of the pathway, which apparently has a large capacity compared to earlier steps, is affected by light. To understand fully the close coordination between ascorbate pool size and light it will also be necessary to determine the effect of light on earlier steps in the pathway.

(b) *Ascorbate-deficient* Arabidopsis thalia *mutants have reduced non-photochemical que*

Four ascorbate-deficient *A. thaliana* mutant reduced ascorbate concentration have been (Conklin *et al.* 1996, 2000). They have been name *3* and *4*. The young leaves of each mutant have 3 of the wild-type ascorbate content. The ascorbate tration in the rosette leaves of some *vtc2* alleles d even further after flowering to 10% of wild-ty cloning of *VTC1* and its contribution to confirm proposed ascorbate biosynthesis pathway was d in §1.

Some, but not all, of the *vtc* mutants are ozone sensitive while *vtc1* is also more sensitive to dioxide and UVB (Conklin *et al.* 1996, 2000 exogenous ascorbate reverses the ozone hypersensi *vtc1*, the phenotype is unlikely to be caused by ple effects and this provides strong genetic evidence antioxidant role for ascorbate (Conklin *et al.* 1996 bate is also proposed to play a role in photoprote acting as a cofactor for VDE, thus facilitating zea synthesis and dissipation of excess excitation en heat. The background was reviewed in §1. The non-photochemical quenching (measured as N



ure 6. A comparison of the time-course of induction and relaxation (actinic light off) of chlorophyll fluorescence parameters asured in leaves from wild-type and ascorbate-deficient *vtc* mutants of *Arabidopsis thaliana*. Dark-adapted leaves were exposed actinic light (photon flux density 1000 μmol $m^{-2} s^{-1}$) at zero time. (*a*) Quantum efficiency of PS II (ΦPS II). (*b*) Photo-emical quenching coefficient (q_P). (*c*) Non-photochemical quenching coefficient (q_{NP}). (*d*) Non-photochemical quenching PQ). The values are means of three replicates ± s.d.

thaliana can be attributed to zeaxanthin synthesis iyogi *et al.* 1998). Therefore the development of NPQ in ld-type and *vtc* mutants has been assessed by analysis of lorophyll fluorescence quenching. The plants were own at low light intensity (200 μmol $m^{-2} s^{-1}$) to avoid oto-oxidative stress and the maximum quantum iciency (F_v/F_m) was the same in all the strains. Chloro-yll fluorescence measurements were used to compare otochemical quenching (q_P), non-photochemical enching (calculated as q_{NP} and NPQ) and the antum efficiency of PS II (ΦPS II) at steady state over range of light intensities in wild-type and *vtc* mutants gure 5). There were no significant differences in ΦPS II q_P, although the latter was very slightly higher in *vtc4*. n-photochemical quenching measured as q_{NP} was her in the wild-type leaves, while expression as NPQ wed that all the *vtc* mutants developed lower non-otochemical quenching than the wild-type at irra-nces above 1000 μmol $m^{-2} s^{-1}$. A time-course of elopment of the chlorophyll fluorescence parameters r illuminating dark-adapted leaves was determined and revealed the same differences between wild-type and *vtc* mutants (figure 6). Non-photochemical quenching measured by q_{NP} or NPQ was higher in wild-type leaves by 40 s after illumination. This initial 40 s phase corresponds to zeaxanthin-independent NPQ seen in *npq1*, a VDE-deficient mutant (Niyogi *et al.* 1998). Divergence of the mutants and wild-type after this point suggests that the mutants have reduced zeaxanthin-dependent NPQ that could be attributed to a limitation of VDE activity by ascorbate supply. Because the effect occurs in four non-allelic mutants, it is very likely that ascorbate deficiency is the direct cause of reduced NPQ. The results support previous assumptions that ascorbate supply in the thylakoid lumen is potentially limiting to VDE activity (Neubauer & Yamamoto 1994). It is not known if the reduction in ascorbate concentration in the *vtc* mutants is equal in all subcellular compartments. However, because a relatively small decrease in ascorbate reduces VDE activity, it can be tentatively suggested that the concentration of ascorbic acid in the lumen must be near the K_m of VDE (100 μM; see § 1) and not near the stromal

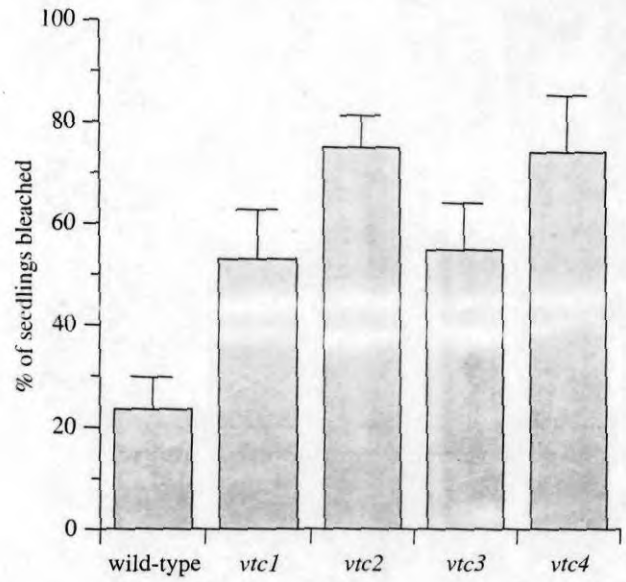


Figure 7. A comparison of the sensitivity of wild-type and ascorbate-deficient *vtc* mutants of *Arabidopsis thaliana* to NaCl-induced photo-oxidation. Ten-day-old seedlings grown with 122 mM NaCl at low light intensity (photon flux density 75 $\mu mol\, m^{-2} s^{-1}$) were transferred to higher light intensity (photon flux density 240 $\mu mol\, m^{-2} s^{-1}$). After 12 days the proportion of seedlings with all their leaves bleached was determined. The values are means of four replicates ± s.e.

concentration. A very high stromal ascorbate concentration may be required to maintain lumen ascorbate in the apparent absence (Foyer & Lelandais 1996) of a carrier (figure 1). In contrast to the effect of loss of VDE activity in the *npq1* mutant (Niyogi *et al.* 1998) there were no measurable corresponding changes in q_P, ΦPS II and F'_v/F'_m (data not shown) in the *vtc* mutants. This is probably because the reduction in NPQ is smaller than in the VDE-deficient plants.

(c) *The* vtc *mutants are susceptible to salt-induced photo-oxidation*

When young *A. thaliana* seedlings are exposed to a combination of high light intensity and NaCl in the rooting medium, the leaves bleach within a few days (figure 7). NaCl at low light intensity or high light alone does not cause this response, which provides an easily monitored bioassay for photo-oxidation. Under conditions that cause slow bleaching of wild-type seedlings, all the ascorbate-deficient *vtc* mutants bleach more rapidly (figure 7). This provides further evidence for the role of ascorbate in photoprotection. At present the mechanism of protection against salt and light-induced photo-oxidation is not known. It could be related to protection by NPQ, scavenging of superoxide and hydrogen peroxide in chloroplasts and peroxisomes, or regeneration of α-tocopherol. APX and superoxide dismutase activity are induced by this treatment (Tsugane *et al.* 1999), the increase in APX further underlining a role for ascorbate. Tsugane *et al.* (1999) isolated a recessive *A. thaliana* mutant (*pstI*) that is more tolerant to NaCl-induced photo-oxidation and which has higher APX activity. This system and the *vtc* mutants should be useful in assessing the role of a... in photoprotection.

7. CONCLUSIONS

The results presented here identify multiple ... ascorbate in photosynthesis and photoprotect... show that its synthesis in leaves is controlled ... However, the question why chloroplasts fre... contain enormous ascorbate concentrations is ... fully answered. The ascorbate-deficient *vtc* ... appear to function normally until exposed to ox... stress, implying that very high ascorbate, as seen ... alpine species, must be maintained to deal with ... extreme episodes of photo-oxidative stress. Manip... of the biosynthetic pathway of ascorbate might ... possible as a number of genes have been cloned a... should provide further insights into its function.

The work described here was funded by the Biotechnol... Biological Sciences Research Council (UK) and Bio-I... Resources (Manitowoc, WI, USA). P. L. Conklin and R. ... provided the *vtc* mutants. I thank Marjorie Raymond and ... Kingdon for technical assistance. Bethan Wynne-Jones ... out some of the experiments on the light control of a... synthesis.

REFERENCES

Anderson, J. W., Foyer, C. H. & Walker, D. A. 198... dependent reduction of dehydroascorbate and up... exogenous ascorbate by spinach chloroplasts. *Pla...* 442–450.

Asada, K. 1999 The water–water cycle in chloroplasts ... ging of active oxygens and dissipation of excess ... *A. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 601–639.

Bartoli, C., Pastori, G. & Foyer, C. H. 2000 Ascorbate bi... esis in mitochondria is linked to electron transport ... between complexes III and IV. *Plant Physiol.* **123**, 335–3...

Beck, E., Burkert, A. & Hofman, M. 1983 Uptake of L-as... by intact spinach chloroplasts. *Plant Physiol.* **73**, 41–45.

Bratt, C. E., Arvidsson, P. O., Carlsson, M. & Akerlund, ... 1995 Regulation of violaxanthin de-epoxidase activity ... and ascorbate concentration. *Photosynth. Res.* **45**, 169–175.

Cleland, R. E. & Grace, S. C. 1999 Voltammetric detec... superoxide production by photosystem II. *FEBS Lett.* ... 348–352.

Conklin, P. L., Williams, E. H. & Last, R. L. ... Environmental stress tolerance of an ascorbic acid-def... *Arabidopsis* mutant. *Proc. Natl Acad. Sci. USA* **93**, 9970–997...

Conklin, P. L., Pallanca, J. E., Last, R. L. & Smirnoff, N. ... L-ascorbic acid metabolism in the ascorbate-def... *Arabidopsis* mutant *vtc1*. *Plant Physiol.* **115**, 1277–1285.

Conklin, P. L., Norris, S. R., Wheeler, G. L., Williams, E. ... Smirnoff, N. & Last, R. L. 1999 Genetic evidence for the ... of GDP-mannose in plant ascorbic acid (vitamin C) biosy... esis. *Proc. Natl Acad. Sci. USA.* **96**, 4198–4203.

Conklin, P. L., Saracco, S. A., Norris, S. R. & Last, R. L. ... Identification of ascorbic acid-deficient *Arabidopsis* ... mutants. *Genetics* **154**, 847–856.

Eskling, M. & Åkerlund, H. E. 1998 Changes in the qua... of violaxanthin de-epoxidase, xanthophylls and ascorb... spinach upon shift from low to high light. *Photosynth. R...* 41–50.

Eskling, M., Arvidsson, P.-O. & Åkerlund, H.-E. 199... xanthophyll cycle, its regulation and components. *P...* *Plant* **100**, 806–816.

G. & Jagendorf, A. T. 1961 Photosynthetic phosphorylation in the absence of redox dyes: oxygen and ascorbate effects. *Biochim. Biophys. Acta* **54**, 322–330.

C. H. & Halliwell, B. 1976 The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta* **133**, 21–25.

C. & Lelandais, M. 1996 A comparison of the relative rates of transport of ascorbate and glucose across the thylakoid, chloroplast and plasmalemma membranes of pea leaf mesophyll cells. *J. Plant Physiol.* **148**, 391–398.

C. H. & Noctor, G. 1999 Leaves in the dark see in the light. *Science* **284**, 599–601.

C. H., Rowell, J. & Walker, D. A. 1983 Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. *Planta* **157**, 239–244.

S. C. & Logan, B. A. 1996 Acclimation of foliar antioxidant systems to growth irradiance in three broadleaved evergreen species. *Plant Physiol.* **112**, 1631–1640.

Halliwell, B. & Gutteridge, J. M. C. 1999 *Free radicals in biology and medicine*, 3rd edn. Oxford, UK: Clarendon Press.

U., Miyake, C., Mano, J., Ohno, C. & Asada, K. 1996 Monodehydroascorbate radical detected by electron paramagnetic resonance spectrosocopy is a sensitive probe of oxidative stress in intact leaves. *Plant Cell Physiol.* **37**, 1066–1072.

E., Mano, J., Ohno, C. & Asada, K. 1997 Increased levels of monodehydroascorbate radical in UV-B-irradiated broad bean leaves. *Plant Cell Physiol.* **38**, 684–690.

Karpinski, S., Escobar, C., Karpinska, B., Creissen, G. & Mullineaux, P. 1997 Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light. *Plant Cell* **9**, 627–640.

Karpinski, S., Reynolds, H., Karpinska, B., Wingsle, G., Criessen, G. & Mullineaux, P. 1999 Systemic signalling and acclimation in response to excess excitation energy in *Arabidopsis*. *Science* **284**, 654–657.

Keller, R., Springer, F., Renz, A. & Kossmann, J. 1999 Antisense inhibition of the GDP-mannose pyrophosphorylase reduces the ascorbate content in transgenic plants leading to developmental changes during senescence. *Plant J.* **19**, 131–141.

Law, M. Y., Charles, S. A. & Halliwell, B. 1983 Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. *Biochem. J.* **210**, 899–903.

Lawlor, D. W. 1993 *Photosynthesis: molecular, physiological and environmental approaches*, 2nd edn. London: Longman.

Loewus, F. A. 1999 Biosynthesis and metabolism of ascorbic acid in plants and of analogs of ascorbic acid in fungi. *Phytochem.* **52**, 193–210.

Logan, B. A., Barker, D. H., Demmig-Adams, B. & Adams II, W. W. 1996 Acclimation of leaf carotenoid composition and ascorbate levels to gradients in the light environment within an Australian rainforest. *Plant Cell Environ.* **19**, 1083–1090.

Mano, J., Ushimaru, T. & Asada, K. 1997 Ascorbate in thylakoid lumen as an endogenous electron donor to photosystem II: protection of thylakoids from photoinhibition and regeneration of ascorbate in stroma by dehydroascorbate reductase. *Photosynth. Res.* **53**, 197–204.

Mapson, L. W. 1962 Photo-oxidation of ascorbic acid in leaves. *Biochem. J.* **85**, 360–369.

Marrè, E., Arrigoni, O. & Rossi, G. 1959 Ascorbic acid and photosynthesis. II. Anaerobic photo-oxidation of ascorbic acid by chloroplast fragments and by whole chloroplasts. *Biochim. Biophys. Acta* **36**, 56–64.

Mittova, S., Kaminaka, H., Masumura, T. & Tanaka, K. 1999 Induction of rice cytosolic ascorbate peroxidase mRNA by oxidative stress; the involvement of hydrogen peroxide in oxidative stress signalling. *Plant Cell Physiol.* **40**, 417–422.

Neubauer, C. & Yamamoto, H. Y. 1994 Membrane barriers and Mehler-peroxidase limit the ascorbate available for violaxanthin de-epoxidase activity in intact chloroplasts. *Photosynth. Res.* **39**, 137–147.

Niyogi, K. K. 1999 Photoprotection revisited: genetic and molecular approaches. *A. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 333–359.

Niyogi, K. K., Grossman, A. R. and Björkman, O. 1998 *Arabidopsis* mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *Plant Cell* **10**, 1121–1134.

Noctor, G. & Foyer, C. H. 1998 Ascorbate and glutathione: keeping active oxygen under control. *A. Rev. Plant Physiol. Mol. Biol.* **49**, 249–279.

Orvar, B. L. & Ellis, B. E. 1997 Transgenic tobacco plants expressing antisense RNA for cytosolic ascorbate peroxidase show increased susceptibility to ozone injury. *Plant J.* **11**, 1297–1305.

Pallanca, J. E. & Smirnoff, N. 2000 The control of ascorbic acid synthesis and turnover in pea seedlings. *J. Exp. Bot.* **51**, 669–674.

Rautenkranz, A. A. F., Li, L., Machler, F., Martinoia, E. & Oertli, J. J. 1994 Transport of ascorbic and dehydroascorbic acids across protoplast and vacuole membranes isolated from barley (*Hordeum vulgare* cv. Gerbil) leaves. *Plant Physiol.* **106**, 187–193.

Schöner, S. & Krause, G. H. 1990 Protective systems against active oxygen species in spinach: response to cold acclimation in excess light. *Planta* **180**, 383–389.

Schreiber, U., Bilger, W. & Neubauer, C. 1995 Chlorophyll fluorescence as a non-intrusive indicator for rapid assessment of *in vivo* photosynthesis. In *Ecophysiology of photosynthesis* (ed. E.-D. Schulze & M. M. Caldwell), pp. 49–70. Berlin: Springer.

Siendones, E., Gonzlez-Reyes, J. A., Santos-Ocaña, C., Navas, P. & Córdoba, F. 1999 Biosynthesis of ascorbic acid in kidney bean. L-galactono-γ-lactone dehydrogenase is an intrinsic protein located at the mitochondrial inner membrane. *Plant Physiol.* **120**, 907–912.

Smirnoff, N. 1995 Antioxidant systems and plant response to the environment. In *Environment and plant metabolism: flexibility and acclimation* (ed. N. Smirnoff), pp. 217–243. Oxford, UK: BIOS Scientific Publishers.

Smirnoff, N. 2000 Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Curr. Opin. Plant Biol.* **3**, 229–235.

Smirnoff, N. & Pallanca, J. E. 1996 Ascorbate metabolism in relation to oxidative stress. *Biochem. Soc. Trans.* **24**, 472–478.

Smirnoff, N. & Wheeler, G. L. 1999 Ascorbic acid metabolism in plants. In *Plant carbohydrate biochemistry* (ed. J. A. Bryant, M. M. Burrell & N. J. Kruger), pp. 215–229. Oxford, UK: BIOS Scientific Publishers.

Streb, P., Feierabend, J. & Bligny, R. 1997 Resistance to photoinhibition of photosystem II and catalase and antioxidative protection in high mountain plants. *Plant Cell Environ.* **20**, 1030–1040.

Sturgeon, B. E., Sipe, H. J., Barr, D. P., Corbett, J. T., Martinez, J. G. & Mason, R. P. 1998 The fate of the oxidizing tyrosyl radical in the presence of glutathione and ascorbate—implications for the radical sink hypothesis. *J. Biol. Chem.* **273**, 30 116–30 121.

Tsugane, K., Kobayashi, K., Niwa, Y., Ohba, Y., Wada, K. & Kobayshi, H. 1999 A recessive *Arabidopsis* mutant that grows photoautotrophically under salt stress shows enhanced active oxygen detoxification. *Plant Cell* **11**, 1195–1206.

Wang, J., Zhang, H. & Allen, R. D. 1999 Overexpression of an *Arabidopsis* peroxisomal ascorbate peroxidase gene in tobacco increases protection against oxidative stress. *Plant Cell Physiol.* **40**, 725–732.

Wheeler, G. L., Jones, M. A. & Smirnoff, N. 1998 The biosynthetic pathway of vitamin C in higher plants. *Nature* **393**, 365–369.

Wildi, B. & Lutz, C. 1996 Antioxidant composition of selected high alpine plant species from different altitudes. *Plant Cell Environ.* **19**, 138–146.

Discussion

C. Critchley (*Department of Botany, University of Queensland, Australia*). Is there a correlation between PS II activity and ascorbate contents? Chloroplasts and mitochondria are much more closely and intimately associated in the cell than we generally believe.

N. Smirnoff. There appears to be a general correlation between the photosynthetic capacity and leaf ascorbate concentration within a species. For example, in leaves from barley (*Hordeum vulgare*) seedlings, both photosynthetic capacity and ascorbate concentration from the leaf base to the tip (N. Smirnoff, ur data). Also, as discussed in the paper, acclimati light increases ascorbate content perhaps by st biosynthesis. However, there is a tenfold ran ascorbate concentration between different spec apparently not correlated with PS II activ generalization, it seems that alpine plants and evergreens have the highest concentrations b necessarily have a high photosynthetic capacity.

C. Critchley. The close association observed chloroplast and mitochondria could facil exchange of metabolites and messages involv light stimulation of galactonolactone dehy activity in mitochondria. This enzyme oxidize nolactone to ascorbate.

Hindawi Publishing Corporation
Advances in Materials Science and Engineering
Volume 2015, Article ID 701940, 6 pages
http://dx.doi.org/10.1155/2015/701940

Hindawi

Research Article

Asphalt Mixture for the First Asphalt Concrete Directly Fastened Track in Korea

Seong-Hyeok Lee,[1] Dae-Wook Park,[2] Hai Viet Vo,[2] and Samer Dessouky[3]

[1]*Korea Railway Research Institute, 176 Cheldo Bangmulgwan-ro, Uiwang, Gyeonggi-do 437-757, Republic of Korea*
[2]*Department of Civil Engineering, Kunsan National University, 558 Daehak-ro, Kunsan, Chellabuk-do 573-701, Republic of Korea*
[3]*Department of Civil and Environmental Engineering, University of Texas, San Antonio, TX 78249, USA*

Correspondence should be addressed to Dae-Wook Park; parkdw@hotmail.com

Received 5 March 2015; Accepted 18 March 2015

Academic Editor: João M. P. Q. Delgado

The research has been initiated to develop the asphalt mixtures which are suitable for the surface of asphalt concrete directly fastened track (ADFT) system and evaluate the performance of the asphalt mixture. Three aggregate gradations which are upper (finer), medium, and below (coarser). The nominal maximum aggregate size of asphalt mixture was 10 mm. Asphalt mixture design was conducted at 3 percent air voids using Marshall mix design method. To make impermeable asphalt mixture surface, the laboratory permeability test was conducted for asphalt mixtures of three different aggregate gradations using asphalt mixture permeability tester. Moisture susceptibility test was conducted based on AASHTO T 283. The stripping percentage of asphalt mixtures was measured using a digital camera and analyzed based on image analysis techniques. Based on the limited research results, the finer aggregate gradation is the most suitable for asphalt mixture for ADFT system with the high TSR value and the low stripping percentage and permeable coefficient. Flow number and beam fatigue tests for finer aggregate asphalt mixture were conducted to characterize the performance of asphalt mixtures containing two modified asphalt binders: STE-10 which is styrene-butadiene-styrene (SBS) polymer and ARMA which is Crum rubber modified asphalt. The performance tests indicate that the STE-10 shows the higher rutting life and fatigue life.

1. Introduction

Hot mix asphalt (HMA) was first developed for applying to the track structure to partly replace the conventional granular material and now is selectively considered as an option for new mainline tracks, yards, and terminal construction due to lower delivering and placing cost compared to conventional granular subballast [1]. Hensley and Rose [2] found that HMA mat is capable of performing as an elastic layer under the railway instead of open-graded unbound ballast layer. HMA is suitable for railway substructure to enhance performance, support dynamic, and vibrated loading especially at the effective depth of 0–2 meters [3]. Viscoelastic strength and modulus of HMA can make it more sustainable for using as high-speed railway substructures [4] and also no damage or crack of the asphalt is detected after many years of heavy traffic under various conditions [5]. Fang et al. [6] concluded that air voids content and permeability of asphalt mixture is strongly affected by the aggregate gradation but not correlated with normal maximum aggregate size. Rose et al. [7] suggested that the loading conditions in trackbeds are different from those in highway pavements, so the asphalt content of HMA trackbeds should be 0.5% higher than that considered optimum for highway applications with air voids of 1 to 3% to facilitate adequate strength and an impermeable mat. Sangsefidi et al. [8] postulated to adopt the optimum mixture; the aggregate gradation is considered as an important characteristic, since it affects the rutting resistance and the moisture susceptibility of the asphalt mixtures. Merusi et al. [9] applied a digital image analysis method to identify the stripping surface to quantitatively evaluate the stripping resistant of asphalt mixture. It offers a good observation and qualification of stripping. The impermeable asphalt layer can prevent stripping and possible contamination of the lower layer by vertical hydraulic transport of mud and fines [10].

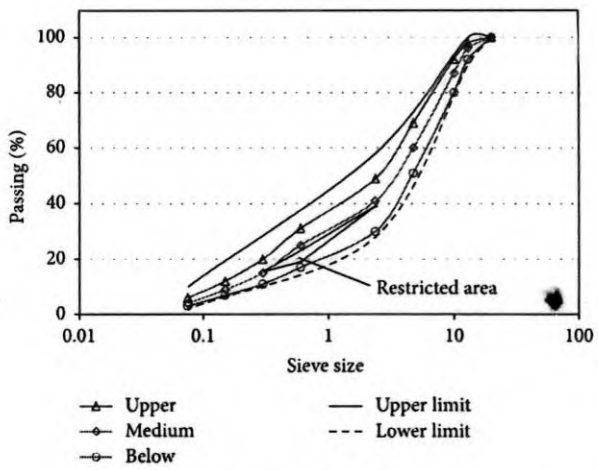


FIGURE 1: Aggregate gradations in 0.45 power.

The purposes of this study are to evaluate the asphalt mixtures with three aggregate gradations, upper, medium, and below, to evaluate which are suitable for the surface of Asogakt Concrete Directly Fastened (ADFT) system. The Marshall mix design method was conducted to determine the optimum asphalt binder content for asphalt mixes with different aggregate gradation. Digital images captured from wet indirect tensile (IDT) test were analyzed and compared with its TSR obtained from dry and wet conditioned specimens in AASHTO T 283 test. The falling head permeameter was used to determine the permeability of the asphalt mixtures in the laboratory in order to find the impermeable mixtures correlated with the aggregate gradations. The performance of asphalt concrete mixtures which has upper gradation was investigated using flow number and beam fatigue test using two types of modified asphalt binders.

2. Materials and Mix Design

2.1. Materials. Asphalt binder STE-10 which is styrene-butadiene-styrene (SBS) polymer was used for evaluating aggregate gradations. The aggregates with three gradations, upper (finer), medium, and below (coarser), have bulk specific gravities, G_{sb}, of 2.563, 2.578, and 2.594 g/cm^3, respectively, determined according to AASHTO T 85 [11] and AASHTO T 84 [12] tests. The aggregate gradations were chosen between the upper and lower control limits without passing through the restricted area based on Superpave mix design method with aggregate maximum size of 12.5 mm [4]. The details of aggregate gradations are shown in Figure 1.

2.2. Mix Design. Asphalt mix design is conducted based on Marshall mix design method [4], and samples of asphalt mixtures were prepared with three replicates at three different asphalt contents. The optimum asphalt contents selected at an air void of 3% were 5.5%, 5.2%, and 5.3% for upper, medium, and below mixes, respectively. As shown in Table 1, asphalt mixture with below gradation did not meet the flow value criteria as 43.1.

TABLE 1: Marshall test results at the optimum asphalt content.

Marshall properties	Criteria limit	Upper	Medium	Below
Optimum AC (%)	**At 3**	**5.5**	**5.2**	**5.3**
Marshall stability (kgf)	>500	2536.7	2162.9	1776.1
VMA (%)	>13	13.6	13.0	12.9
VFA (%)	70–80	79.3	78.8	77.9
Flow (0.01 cm)	20–40	35.8	31.8	43.1
G_{mb}(g/cm^3)	—	2.344	2.353	2.358

3. Evaluation of Aggregate Gradations

3.1. Indirect Tensile (IDT) Test. HMA moisture susceptibility is mostly determined based on the AASHTO T 283 [13]. The damage due to moisture is controlled by the specific limits of the tensile strength ratios (TSR). All specimens were compacted by the gyratory compactor and had air voids of $7 \pm 0.5\%$. Total six specimens were separated into two subsets: one subset for dry IDT test and the other for wet IDT test. The maximum load was recorded from the indirect tensile tester and the corresponding IDT strength was calculated by

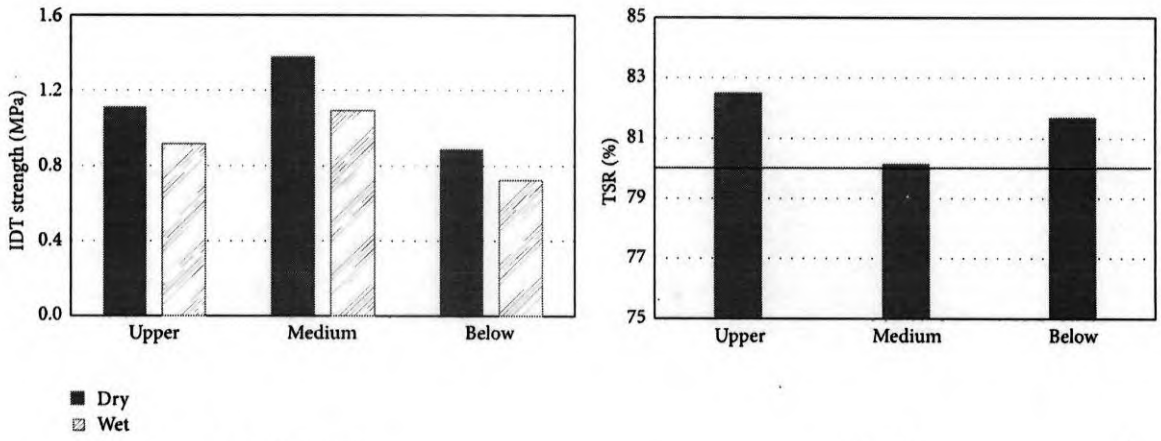
$$S_t = \frac{2000P}{\pi Dt}, \tag{1}$$

where S_t is the IDT strength, kPa, P is the maximum load, t is the thickness of specimen, and D is the diameter of specimen. The tensile strength ratio (TSR) is determined by the following equation from the dry and wet IDT test results:

$$\text{TSR} = \frac{S_W}{S_D}, \tag{2}$$

where S_W is the average wet IDT strength and S_D is the average dry IDT strength. Figure 2 indicates that the aggregate gradation affects to some extent the moisture susceptibility of the asphalt mixtures. The mixtures with upper, medium, and below gradations have TSR values of 82.5%, 80.2%, and 81.7%, respectively. Accordingly, all the mixtures met the criteria of a minimum TSR value of 80% [13], and the mixes with upper (finer) gradation showed better TSR than mixes with medium or below (coarser) gradation. Basically, the water penetration depends on the gradation of aggregate: dense or open graded. Since finer materials tend to fill voids and increase the density, therefore its reduction also affected the TSR in coarser graded mixes. Furthermore, due to breaking of coarser aggregate during compaction, uncoated surface absorbs the water more than the other particles and then leads to stripping. There is low amount of fine material in medium and below aggregate gradation. As a result, although the asphalt mixture with medium aggregate gradation has high dry and wet IDT strength values (Figure 2(a)), but, as seen in Figure 2(b), its moisture resistance or TSR value is the lowest as compared to the others.

3.2. Image Analysis. Two-halves of the samples from the wet IDT test were kept for image stripping analysis. The images were captured to the fractured surface by digital camera



(a) IDT strength (b) TSR

FIGURE 2: Indirect tensile (IDT) test result.

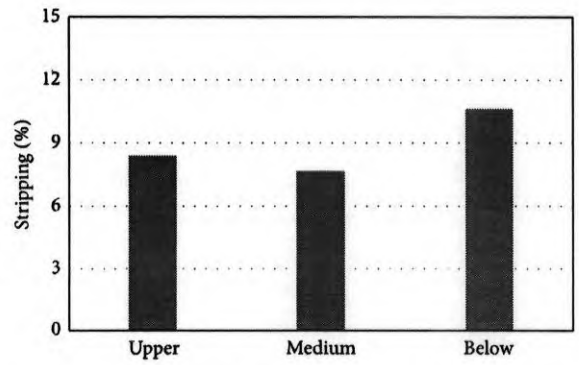


FIGURE 3: Image analysis result.

(SONY DSC-T100 8.1 mega pixels) in an adequate and indirect light condition to prevent creation of light reflection. It is recommended to use a light green color as background during taking picture. With this color, background separation can be done accurately and evenly. Moreover, the angle of images capturing should be perpendicular to the surface of sample. The image analysis program developed using Matlab [14] was employed for determining stripping percentage. The numbers of black and white pixels were counted to determine the stripping area. By determining a threshold value within the grayscale, white and black pixels can be distinguished in binary image. Applying different threshold levels indicated that the global threshold values between 0.16 and 0.17 did not significantly affect the stripping percentage results; however, for an accurate comparison, the analyses of all the images should be done with the same threshold value. The threshold value of 0.165 was chosen to distinguish the white and black pixels. This threshold level seems to be a realistic value to recognize the stripping percentage.

In image analysis test, the lower the stripping percentage is, the less the moisture damage occurs. As seen in Figure 3, the results indicate that the image analysis is basically reliable when its results, stripping percentage, have almost the same trend with the wet IDT values. The IDT test result of the asphalt mixture with medium aggregate gradation is high, and also the image analysis indicates a high stripping resistance (lower stripping percentage), relatively matching the wet IDT strength value. In this study, the stripping percentage values have yet denoted the correlation with TSR values.

3.3. Permeability Test. The permeability of asphalt concrete samples was measured by the permeameter developed by Florida Department of Transportation (FDOT) for testing in the laboratory [15]. The permeable coefficient was computed according to (3). Three replicated tests on the same specimen were performed and averaged:

$$k = \frac{aL}{At} \ln\left(\frac{h_1}{h_2}\right) t_c, \tag{3}$$

where k is the permeable coefficient, a is the inside cross-sectional area of the graduated cylinder, L is the average thickness of the test specimen, A is the cross-sectional area of the test specimen, t is the elapsed time between h_1 and h_2, h_1 and h_2 are initial and final head across the test specimen, and t_c is the temperature correction for viscosity of water. According to FDOT's criteria for impermeable asphalt mixture, the permeable coefficient measured by the falling head permeameter must not exceed 0.1 cm/s. The permeability of asphalt concrete is mostly affected by the porosity and air voids distribution. As seen in Figure 4, all mixtures met criteria for impermeable mixture; however, the permeability of the mix with below (coarse) gradation is higher by far than the others. All the air void contents are almost at the same level. Therefore, it is noted that the upper and medium mixes can produce smaller air void pockets than the below.

4. Performance Evaluation

According to the previous work [16], the modified asphalt mixtures are suitable for railway substructures compared to

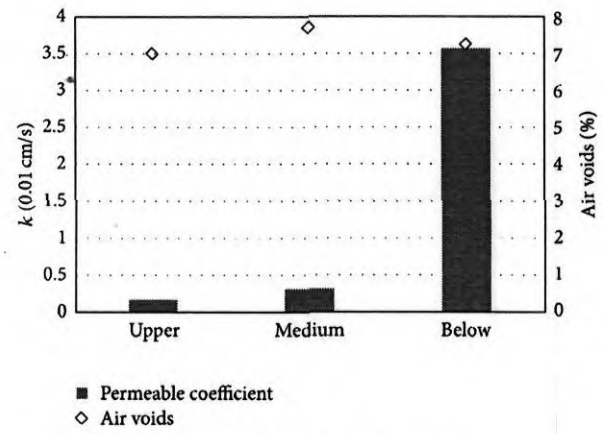


FIGURE 4: Permeability test result.

the original one. For performance evaluation, asphalt mixtures with two types of modified asphalt binder, STE-10 and ARMA, were used for flow number and four point beam fatigue tests.

4.1. Flow Number. The flow number (FN) is a measure of the rutting potential of asphalt mixes [17]. Mixes with high flow number are more resistant to permanent deformation and vice versa. The cylindrical specimen geometry is similar to the dynamic modulus test. Asphalt specimens were subjected to repeated haversine axial cycle with 0.1 and 0.9 sec loading time and rest period, respectively. The test was performed under unconfined conditions at 54°C and deviatoric stress of 600 kPa. The failure criterion is determined at 15,000 cycles or 50,000 microstrain, whichever occurs first. During FN test, the mix follows three different stages of deformation: primary (Consolidation), steady state, and tertiary [17]. Flow number is the number of load cycles at the minimum rate of permanent axial strain (e.g., constant volume) at which tertiary flow begins. The minimum strain rate was determined at the junction point in between the steady state and tertiary of deformation. The following equation is applied to calculate the strain rate;

$$\frac{\delta_{s_i}}{\delta_N} = \frac{\varepsilon_{N_{i+1}} - \varepsilon_{N_{i-1}}}{2\Delta N}, \tag{4}$$

where δ_{s_i}/δ_N is the strain rate at Nth cycle, $\varepsilon_{N_{i+1}}$ is the strain at $(i+1)$th cycle, $\varepsilon_{N_{i+1}}$ is the strain at $(i-1)$th cycle, and ΔN is the number of cycles in between $(i+1)$th and $(i-1)$th cycle. In this study, the test was conducted on two replicates with an average FN of 733 and 226 cycles for STE-10 and ARMA mixtures, respectively. Figure 5 represents the strain rate evolution for the two replicates of STE-10 mixtures. Results suggest that the asphalt mixture containing STE-10 exhibits the higher rutting life compared to that of ARMA.

4.2. Four Point Beam Fatigue Test. This test was conducted to determine the fatigue life of asphalt pavement using a 380 × 63 × 50 mm beam. Repeated loads were applied until reaching fatigue. Fatigue life is the number of cycles recorded at the failure of asphalt mixtures according to AASHTO T 321 [18].

An in-house steel mold was used to fabricate 405 × 240 × 75 mm asphalt concrete slab. The slab is compacted by a steel rod to reach target air voids of 7 to 9%. The slab was sawn approximately 6 mm from all sides to avoid the air voids clustering at the edge of the slab due to the mold temperature. After that, two standard beams of 380 × 63 × 50 mm were cut from the slab.

The beams were tested by four point loading clamps. The loads on the two inner clamps were cycled with a repeated haversine (sinusoidal) load at frequency of 10 Hz while the outer ones remained fixed to provide a reaction support, providing a constant bending moment over the center part of the beam. The deflection caused by the loading is measured at the center of the beam. The deformation of the beam centroid was calculated to produce the tensile strain values in the bottom fiber of the beam and collects load and deformation data every 100 cycles.

The test was performed on a strain-controlled condition using a strain level of 650 microstrain at room temperature of 19 ± 1°C. The sample was conditioned in a chamber for about two hours at the testing temperature prior to testing. As loading is applied, the beam resilience reduces gradually. The test was terminated when the beam stiffness reduced to 50% of the initial stiffness, and the number of cycles at that time is referred to as the fatigue life. The flexural stiffness (S) is determined using

$$S = \frac{\sigma_t}{\varepsilon_t}, \tag{5}$$

where σ_t and ε_t are the maximum tensile stress and strain determined as follows:

$$\sigma_t = \frac{0.357P}{bh^2},$$
$$\varepsilon_t = \frac{12\delta h}{3L^2 + 4a^2}, \tag{6}$$

where P is the applied load by actuator, b is the average beam width, h is the average beam height, δ is the maximum deflection at center of beam, L is the average beam length (m), and a is the space between inner clamps (0.119 m). Examples of the normalized fatigue stiffness reduction with number of cycles for all mixtures are shown in Figure 6. Results suggest that the STE-10 exhibits the higher fatigue life with 50 times greater than the other.

5. Conclusions

The ADFT system requires a durable and impermeable asphalt layer under the sleepers. Three types of mixes made with combination of three different aggregate gradations were designed and evaluated for their moisture susceptibility and permeability. The mixtures were designed by Marshall mix design method and tested with IDT test, image analysis

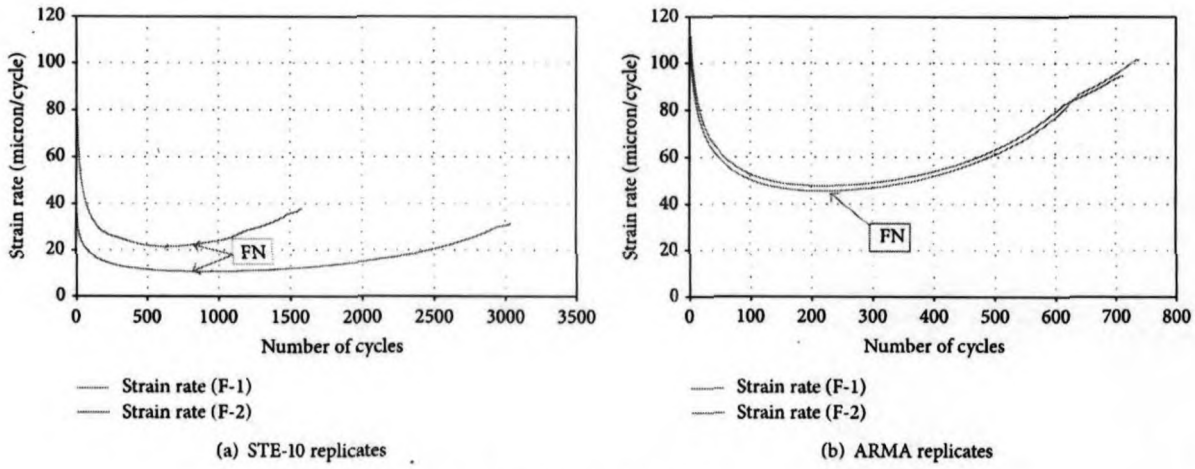


FIGURE 5: Permanent strain rate curves.

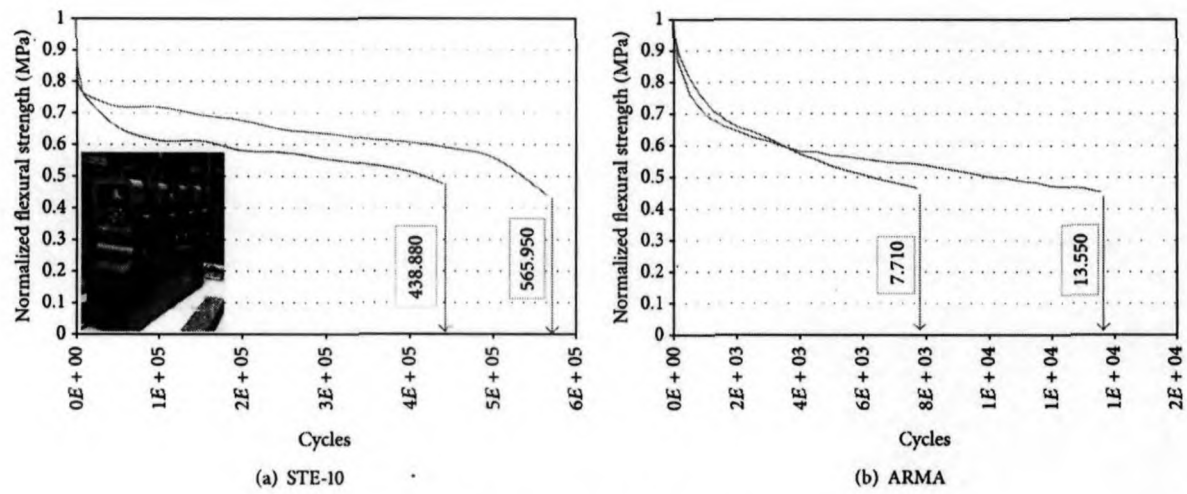


FIGURE 6: Beam fatigue test.

method, and permeability test. Only below aggregate gradation mix does not meet the design criteria of Marshall flow and VMA values at 3 percent air voids. The results indicate that the mix prepared by using upper aggregate gradation has the best performance on moisture susceptibility with the optimum asphalt content of 5.5 percent; however, the IDT strength of medium mixture is the highest among all. Further, the mixes of upper and medium gradation have lower value of permeable coefficient and stripping percentage. The authors suggest developing threshold value in image analysis method for different types of mixtures and conditions and learning more about the relationship between stripping percentage and IDT test results. Based on the test results and authors' experience, it is recommended to use aggregate gradations between medium and upper (fine graded aggregate) for ADFT system due to their appropriate moisture resistance and permeability. Also, the modified asphalt binder STE-10 which provides higher fatigue life and rutting life for the asphalt mixture is more suitable for ADFT system compared to the ARMA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This research was supported by a grant from R&D Program of the Korea Railroad Research Institute, Republic of Korea.

References

[1] J. G. Rose, P. F. Teixeira, and P. Veit, "International design practices, applications, and performances of asphalt /bituminous railway trackbeds," in *GEORAIL 2011: Symposium international*

Géotechnique ferroviaire, Paris, France, 19-20 mai 2011, Volume 1, 2011.

[2] M. J. Hensley and J. G. Rose, "Design, construction and performance of hot mix asphalt for railway trackbeds," in *Proceedings of the 1st World Conference of Asphalt Pavements*, Sydney, Australia, 2000.

[3] M. Fang, Y. Qiu, J. G. Rose, R. C. West, and C. Ai, "Comparative analysis on dynamic behavior of two HMA railway substructures," *Journal of Modern Transportation*, vol. 19, no. 1, pp. 26–34, 2011.

[4] E. R. Brown, P. S. Khal, F. L. Roberts, Y. R. Kim, D. Y. Lee, and T. W. Kennedy, *Hot Mix Asphalt Materials, Mixture Design, and Construction*, NAPA Research and Education Foundation, Lanham, Md, USA, 3rd edition, 2009.

[5] J. G. Rose and R. S. Bryson, "Hot mix asphalt railway trackbeds: trackbed materials, performance evaluations, and significant implications," in *Proceedings of the International Conference on Perpetual Pavements*, Columbus, Ohio, USA, 2009.

[6] M. Fang, Y. Qiu, C. Ai, and Y. Wei, "Gradation determination of impermeable asphalt mix on subgrade surface layer for ballastless track in high-speed railway lines," in *Proceedings of the 3rd International Conference on Transportation Engineering (ICTE '11)*, pp. 1926–1931, July 2011.

[7] J. G. Rose, P. F. Teixeira, and N. E. Ridgway, "Utilization of asphalt/bituminous layers and coatings in railway trackbeds: a compendium of international applications," in *Proceedings of the ASME Joint Rail Conference (JRC '10)*, pp. 239–255, Urbana, Ill, USA, April 2010.

[8] E. Sangsefidi, H. Ziari, and A. Mansourkhaki, "The effect of aggregate gradation on creep and moisture susceptibility performance of warm mix asphalt," *International Journal of Pavement Engineering*, vol. 15, no. 2, pp. 133–141, 2014.

[9] F. Merusi, A. Caruso, R. Roncella, and F. Giuliani, "Moisture susceptibility and stripping resistance of asphalt mixtures modified with different synthetic waxes," *Journal of the Transportation Research Board*, no. 2180, pp. 110–120, 2010.

[10] European Asphalt Pavement Association (EAPA), *Asphalt in Railway Tracks*, European Asphalt Pavement Association, Breukelen, The Netherlands, 2010.

[11] AASHTO, "Specific gravity and absorption of coarse aggregate," AASHTO T 85-10, 2010.

[12] AASHTO, "Specific gravity and absorption of fine aggregate," Tech. Rep. T 84-10, American Association of State Highway and Transportation Officials, 2010.

[13] AASHTO, "Standard method of test for resistance of compacted asphalt mixtures to moisture-induced damage," AASHTO T 283-03, 2003.

[14] Matlab, "The MathWorks," 2013, http://www.mathworks.com/.

[15] Florida Department of Transportation (FDOT), "Measurement of water permeability of compacted asphalt paving mixtures," Tech. Rep. FM 5-565, Florida Department of Transportation, Tallahassee, Fla, USA, 2000.

[16] S. H. Lee, J. W. Lee, D. W. Park, and H. V. Vo, "Evaluation of asphalt concrete mixtures for railway track," *Construction and Building Materials*, vol. 73, pp. 13–18, 2014.

[17] R. Dongré, J. D'Angelo, and A. Copeland, "Refinement of flow number as determined by asphalt mixture performance tester: use in routine quality control-quality assurance practice," *Transportation Research Record*, no. 2127, pp. 127–136, 2009.

[18] AASHTO, "Standard method of test for determining the fatigue life of compacted hot-mix asphalt (HMA) subjected to repeated flexural bending," Tech. Rep. AASHTO T 321-03, 2003.