Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals

Stephen C. FRY¹

The Edinburgh Cell Wall Group, Institute of Cell and Molecular Biology, The University of Edinburgh, The King's Buildings, Edinburgh EH9 3JH, U.K.

Scission of plant cell wall polysaccharides *in vivo* has generally been assumed to be enzymic. However, in the presence of L-ascorbate, such polysaccharides are shown to undergo non-enzymic scission under physiologically relevant conditions. Scission of xyloglucan by 1 mM ascorbate had a pH optimum of 4.5, and the maximum scission rate was reached after a 10–25-min delay. Catalase prevented the scission, whereas added H₂O₂ (0.1–10 mM) increased the scission rate and shortened the delay. Ascorbate caused detectable xyloglucan scission above approx. 5 μ M. Dehydroascorbate was much less effective. Added Cu²⁺ (> 0.3 μ M) also increased the rate of ascorbate-induced scission; EDTA was inhibitory. The rate of scission in the absence of added metals appeared to be attributable to the traces of Cu (2.8 mg·kg⁻¹) present in the xyloglucan. Ascorbate-induced

scission of xyloglucan was inhibited by radical scavengers; their effectiveness was proportional to their rate constants for reaction with hydroxyl radicals ([•]OH). It is proposed that ascorbate non-enzymically reduces O₂ to H₂O₂, and Cu²⁺ to Cu⁺, and that H₂O₂ and Cu⁺ react to form [•]OH, which causes oxidative scission of polysaccharide chains. Evidence is reviewed to suggest that, in the wall of a living plant cell, Cu⁺ and H₂O₂ are formed by reactions involving ascorbate and its products, dehydroascorbate and oxalate. Systems may thus be in place to produce apoplastic [•]OH radicals *in vivo*. Although [•]OH radicals are often regarded as detrimental, they are so short-lived that they could act as site-specific oxidants targeted to play a useful role in loosening the cell wall, e.g. during cell expansion, fruit ripening and organ abscission.

INTRODUCTION

Plant cell walls undergo controlled loosening *in vivo*, enabling cell expansion, fruit softening and organ abscission. Wall loosening is generally assumed to involve the scission (backbone cleavage) of structural polysaccharides by hydrolases [1] and transglycosylases [2], and/or the rearrangement of hydrogen bonds by 'expansin' proteins [3]. Little attention has been paid to the possibility that polysaccharides in the walls of living plant cells also undergo non-enzymic scission. Miller [4] found that several plant cell wall polysaccharides (xylan, galacturonan, arabinogalactan and cellulose) suffer slight non-enzymic scission when incubated in 0.1–10 mM H₂O₂. He suggested that the scission of polysaccharides by H₂O₂ is a biologically relevant mechanism of cell wall lysis. However, Miller's work has not been followed up.

Much more reactive than H₂O₂ is the hydroxyl radical ([•]OH), which can cause the scission of polysaccharides such as hyaluronate [5,6], chitosan [7] and pullulan [8]. [•]OH radicals can be generated by γ -radiolysis of water [9], by the action of light on periodate [10] or by reaction of H₂O₂ with a reduced transition metal ion, e.g. Ti³⁺ [11], Fe²⁺ [5] or Cu⁺ [8].

The apoplast (the aqueous solution that permeates the wall of a living plant cell) usually contains O₂, ascorbate [12–16] and Cu²⁺ [17], a combination that readily generates [•]OH [18]. Apoplastic H₂O₂ is also present in many plant tissues [19,20], and this would promote [•]OH formation in the presence of ascorbate and Cu²⁺ [18]. There is recent evidence for *in vivo* production of extracellular [•]OH in elicitor-treated rice cells [21]. The present

work was therefore undertaken with two main aims: (i) to test the ability of ascorbate, with and without exogenous H₂O₂, to generate [•]OH radicals under the mildly acidic conditions and low Cu²⁺ concentrations encountered in the plant cell wall, and (ii) to test the susceptibility of plant cell wall polysaccharides to [•]OH radicals thus generated. Emphasis was placed on one polysaccharide, xyloglucan, which is considered to be of particular significance in plant cell wall architecture because it hydrogen bonds to cellulose and may act to 'tether' adjacent cellulosic microfibrils [1].

MATERIALS AND METHODS

Materials

Pectin (apple), dextran (5–40 MDa), poly-L-histidine hydrochloride (DP 106), L-histidyl-L-lysine, salicylic acid, catalase (bovine liver), ascorbate oxidase (cucurbit), superoxide dismutase (bovine erythrocytes), H₂O₂, 2,6-dichlorophenol indophenol (DCPIP) and deferoxamine (or desferal; desferrioxamine) mesylate, were from Sigma Chemical Co., Poole, U.K. Carboxymethylcellulose, methylcellulose, alginate (Na⁺ salt; 'high viscosity') and L-ascorbic acid (AnalaR) were from BDH, Poole, U.K. Dehydroascorbic 'acid' was from Aldrich Chemical Co., Poole, U.K. Tamarind seed xyloglucan was a generous gift from Mr. K. Yamatoya, Dainippon Pharmaceutical Co., Osaka, Japan. Xyloglucan was freed from low-M_r contaminants by washing in 60 % ethanol followed by 100 % acetone, dried, and

Abbreviations used: C_{0.2}, concentration of an additive required to reduce by 80 % the ascorbate-induced increase in specific fluidity of polysaccharide solution; DCPIP, 2,6-dichlorophenol indophenol; k_([•]OH), rate constant for reaction with [•]OH; t₂, time required to double the specific fluidity of a polysaccharide solution.

¹ e-mail: sfry@srv0.bio.ed.ac.uk

Figure 1 Scission of six polysaccharides by ascorbate and/or H_2O_2

The effect of 10 mM H_2O_2 ($\square$), 10 mM ascorbate ($\circ$), or 5 mM of each ($\triangle$) was tested on (final concentrations, w/v) 0.7% xyloglucan, 0.5% carboxymethylcellulose, 1.8% pectin, 1.8% dextran, 1.0% alginate and 1.5% methylcellulose (Me-cellulose). All solutions contained 100 mM succinate buffer (Na^+ , pH 5.5). ∇ , Control lacking ascorbate and H_2O_2 .

dissolved at 1% (w/v) in boiling water; denatured proteins were removed by centrifugation and the supernatant was used.

Viscometric assay of polysaccharide scission

Polysaccharide solutions (0.6 ml) were incubated at 20 °C with ascorbate and/or other additives and a buffer (specified in individual experiments), in 12-mm-diameter, open glass tubes (i.e., with O_2 present). Scission of polysaccharides was monitored as decrease in viscosity, a sensitive test for cleavage of polysaccharide backbones [22]: the efflux time of 0.05 ml of solution from a 0.1-ml vertical glass pipette, with its tip just submerged in the bulk solution, was measured at 20 °C. The efflux time of buffer solution was typically 0.6–0.7 s; that of the polysaccharide solutions was usually in the range 2.5–25 s. Specific fluidity, changes in which are related to the number of scission events, was calculated as (efflux time of buffer solution)/(efflux time of polysaccharide solution).

Assay of residual ascorbate

Aqueous samples (0.6 ml) plus 1 ml of 2.2 M formate buffer

(Na^+ , pH 3.7), were titrated with 0.1% (w/v) DCPIP until the A_{580} reached 0.2. Neither the dehydroascorbate used in the present work nor any of the monosaccharide solutions used were able to decolorize DCPIP (results not shown).

Determination of trace metals

Polysaccharides (1–5 g) were ashed and the residues assayed for Cu, Fe and Zn by atomic absorption spectroscopy. I thank Dr. John Farmer, Department of Chemistry, The University of Edinburgh, U.K., for performing these analyses.

RESULTS

Effect of ascorbate and H_2O_2 on plant cell wall polysaccharides

At pH 5.5, 10 mM H_2O_2 caused the slow scission of each polysaccharide tested (Figure 1). The time ($t_{1/2}$) required to double the specific fluidity was 15–72 h (Figure 1, and results not shown). This both confirms and extends a report published previously [4]. L-Ascorbate (10 mM) alone induced faster scission ($t_{1/2}$ 1–24 h). By far the most dramatic effect, however, was produced by 5 mM ascorbate + 5 mM H_2O_2 , which caused very rapid scission ($t_{1/2}$ 1–10 min).

Addition of ascorbate to a xyloglucan solution already containing 1 mM H_2O_2 caused a sudden burst of scission (Figure 2). The rate and extent of scission was determined by the dose of ascorbate added (0.25–2.0 mM). Ascorbate (2 mM), when added 4 h after the H_2O_2 , caused scission that was almost as rapid and as extensive as when added 1 h after the H_2O_2 (Figure 2), showing that a xyloglucan/ H_2O_2 mixture was relatively stable. Direct measurements after incubation of 1 mM H_2O_2 with 0.7% (w/v) xyloglucan, in the absence of ascorbate, confirmed that little loss of H_2O_2 occurred within 24 h (results not shown).

When ascorbate was added to xyloglucan, there was a delay of 10–25 min before the maximum rate of scission was achieved (Figure 3a), indicating that an initial reaction preceded the scission process. A pH of 4.5, thought to be typical of fast-growing plant cell walls, gave the shortest delay and the greatest rate of scission. When both ascorbate and H_2O_2 were added to xyloglucan, the maximum scission rate again occurred at pH 4.5, but there was negligible delay (Figure 3b). Thus it seemed likely that the effect of ascorbate depended on H_2O_2 and/or dehydroascorbate produced by the reactions featured in [23]:

or

where AH_2 is ascorbate, and A is dehydroascorbate (and in subsequent equations).

Catalase completely prevented ascorbate-induced scission (Figure 4), indicating that H_2O_2 production was necessary. Superoxide dismutase had little effect, showing that the superoxide radical ($\text{O}_2^{\cdot-}$) or its un-ionized form (the hydroperoxyl radical; $\text{HO}_2^{\cdot}$) were not required. Ascorbate oxidase, which oxidizes ascorbate to dehydroascorbate without forming H_2O_2 [23], strongly inhibited xyloglucan scission (Figure 4), showing that dehydroascorbate alone was not responsible. The low effectiveness of dehydroascorbate was confirmed (Figure 5): 0.01 mM ascorbate promoted xyloglucan scission, whereas dehydroascorbate was required at > 0.5 mM. D-Isoascorbate gave results similar to L-ascorbate (results not shown). In the presence (but not in the absence) of 1 mM H_2O_2 , 10–100 mM D-galacturonate or D-glucuronate caused slight scission of xylo-

Figure 2 Effect of H_2O_2 followed by ascorbate on the scission of xyloglucan

At zero time, H_2O_2 (final concentration 1 mM) was added to samples (a)–(e). Ascorbate was then added to each sample at the times indicated ($\uparrow$) to final concentrations of 2 mM [samples (a) and (e)], 1 mM (b), 0.5 mM (c) or 0.25 mM (d). All solutions contained 0.7% (w/v) xyloglucan and 85 mM succinate (Na^+ , pH 5.5).

glucan; D-galactonate, D-glucose, D-galactose, L-arabinose and D-fructose 6-phosphate had no effect on xyloglucan (results not shown). Equimolar ascorbate/dehydroascorbate mixtures, which spontaneously generate ascorbate free radical ($\text{AH}^\bullet$) [24], were neither more nor less effective than ascorbate alone (results not shown). The results indicate that H_2O_2 (rather than $\text{HO}_2^\bullet/\text{O}_2^{\bullet-}$, dehydroascorbate or $\text{AH}^\bullet$) was the important product formed during the delay period.

Evidence for a role of hydroxyl radicals

The dramatic effect of ascorbate and H_2O_2 in combination suggested that these two agents together generated a polysaccharide-cleaving principle. $\text{O}_2^{\bullet-}$, $\text{HO}_2^\bullet$ and singlet oxygen do not attack polysaccharides [9], but a plausible alternative active principle is $\cdot\text{OH}$. In support of this, $\cdot\text{OH}$ scavengers were found to inhibit xyloglucan scission (Figure 6). D-Glucose, D-xylose or D-mannitol (0.5 M) strongly inhibited the scission. Of several non-sugars tested at 0.1 M, there was a correlation between effectiveness as an inhibitor of scission and the published [25] rate constant, $k_{(\cdot\text{OH})}$, for reaction of the substance with $\cdot\text{OH}$. For example, 0.1 M DMSO [$k_{(\cdot\text{OH})} = 6.6 \text{ nM}^{-1}\cdot\text{s}^{-1}$] was a highly effective inhibitor, whereas 0.1 M acetate [$k_{(\cdot\text{OH})} = 0.09 \text{ nM}^{-1}\cdot\text{s}^{-1}$] was ineffective. All the effective compounds in Figure 6 inhibited both the rate and extent of scission, rather than acting to delay the process. This is as expected for an $\cdot\text{OH}$ -mediated process: the $\cdot\text{OH}$ radical is very short-lived and can only act on xyloglucan, or be scavenged, during a very short period after its generation. None of the compounds tested in Figure 6 prevented the oxidation of ascorbate (assayed after 21 h).

Many scavengers, such as L-histidyl-L-lysine, salicylate, glycerol, methanol and acetone, gave simple dose-response curves (Figure 7), as expected for competitive inhibition [26]. Among these compounds, there was a negative correlation between $C_{0.2}$ (the concentration of scavenger required to reduce

by 80% the ascorbate-induced increase in specific fluidity of xyloglucan) and $k_{(\cdot\text{OH})}$ (Figure 8, $\bullet$). Compounds with low $k_{(\cdot\text{OH})}$ values did not protect xyloglucan (Figure 8, Δ). Note that some published estimates of $k_{(\cdot\text{OH})}$ are imprecise: for example, the value ($1.5 \text{ nM}^{-1}\cdot\text{s}^{-1}$) quoted [25] for glucose is the mean of four independent reports (1.0, 1.7, 2.3, $1.1 \text{ nM}^{-1}\cdot\text{s}^{-1}$); thus some scatter of data points in Figure 8 is expected, owing to errors in published $k_{(\cdot\text{OH})}$ values.

Role of transition metal ions

The most likely source of $\cdot\text{OH}$ in the systems studied here is a Fenton reaction, which requires H_2O_2 and the reduced form of a transition metal ion, Cu^+ being 60 times more effective than Fe^{2+} [18]:

All the polysaccharide samples tested in Figure 1 contained measurable traces of Fe, Cu and Zn (Table 1). The Cu and Fe content of the xyloglucan was similar to that of several commercial polysaccharides. AnalaR-grade ascorbic acid also contained measurable traces of Cu and Fe. Therefore, even when no transition metal was deliberately added, Fenton reactions would be feasible.

To explore the role of metal ions in ascorbate-induced xyloglucan scission, salts were added at zero time, followed at 30 min by ascorbate (to 1 mM). All solutions contained 0.8% (w/v) xyloglucan in 20 mM succinate (Na^+ , pH 5.5). K^+ , Mg^{2+} and Ca^{2+} (2 mM) and Mn^{2+} and Zn^{2+} (0.2 mM) had little effect on the ascorbate-induced scission; Fe^{3+} (0.2 mM) slightly promoted scission for approx. 20 min, but later became inhibitory (results not shown). Cu^{2+} (0.6–20 μM) strongly promoted scission and the effect was proportional to Cu^{2+} concentration (Figure 9a). Extrapolation to zero scission rate showed that the xyloglucan solution behaved as if it contained 0.6 μM Cu^{2+} (Figure 9b). If its

Figure 3 Effect of pH on ascorbate-induced scission of xyloglucan

At zero time, H_2O_2 was added to 0.25 mM (b), or an equal volume of H_2O was added (a). This was followed immediately by ascorbate (to 1 mM). All samples contained 0.8% (w/v) xyloglucan, 100 mM phosphate and 100 mM succinate (pH adjusted with NaOH).

behaviour were indeed due to 'endogenous' Cu^{2+} , this would imply that the Cu content of the xyloglucan was 4.8 p.p.m., which is close to the measured Cu content (Table 1) of 2.8 p.p.m. (approx. 1 Cu atom per 150000 sugar residues, or approx. 1 Cu atom per 20–30 xyloglucan molecules of $M_r \approx 10^6$).

The effects of ascorbate, Cu^{2+} , EDTA and H_2O_2 were tested in all combinations to investigate their individual effects (Table 2). Ascorbate always promoted xyloglucan scission. Cu^{2+} had no effect in the absence of ascorbate but greatly promoted scission in one specific case (+ascorbate, –EDTA, – H_2O_2). H_2O_2 promoted ascorbate-induced scission, especially in the absence of EDTA and Cu^{2+} . EDTA tended to diminish the effect of ascorbate. In general, conditions favouring xyloglucan scission promoted ascorbate oxidation (Table 2).

Added Fe^{3+} did not increase the extent of ascorbate-induced xyloglucan scission. Although EDTA inhibited scission in the

Figure 4 Effect of oxidoreductases on ascorbate-induced xyloglucan scission

Catalase (3000 units), ascorbate oxidase (0.4 units), or superoxide dismutase (SOD; 300 'Sigma Units') were added at zero time, followed by ascorbate (to 1 mM; denoted by $\downarrow$). All solutions contained 0.8% (w/v) xyloglucan, 80 mM phosphate and 80 mM succinate (Na^+ , pH 5.5).

Figure 5 Effects of various doses of ascorbate and dehydroascorbate (with and without exogenous H_2O_2) on the extent of xyloglucan scission

H_2O_2 was added (to 1 mM), where appropriate, followed by ascorbate (to 0.001–1 mM) or dehydroascorbate (to 0.03–30 mM). All samples contained 0.65% (w/v) xyloglucan, 40 mM acetate (Na^+ , pH 4.7) and 2 μM CuSO_4 . Incubation time was 18 h.

presence of added Cu^{2+} and in the absence of deliberately added metals, it promoted scission in the presence of added Fe^{3+} (Table 3). This indicates that traces of endogenous Cu, rather than Fe,

Figure 6 Kinetics of the effect of $\cdot\text{OH}$ scavengers on the ascorbate-induced scission of xyloglucan

Scavengers tested were as indicated on the graph; the control had no scavenger (but note that the succinate buffer used had weak scavenging activity; $k_{(\cdot\text{OH})} \approx 0.3 \text{ nM}^{-1} \cdot \text{s}^{-1}$ [25]). Specific fluidity data are plotted on a logarithmic scale. The $k_{(\cdot\text{OH})}$ values (in $\text{nM}^{-1} \cdot \text{s}^{-1}$; after [25]) of the scavengers tested (in the predominant ionic form at pH 5.5) are: xylose, 2.2; glucose, 1.5; mannitol, 1.7; DMSO, 6.6; benzoate, 5.9; formate, 3.2; Tris, 1.5; *t*-BuOH (2-methylpropan-2-ol), 0.6; acetate, 0.09. Each sample contained (final concentrations) 0.96% (w/v) xyloglucan, 100 mM succinate (Na^+ , pH 5.5), $5 \mu\text{M}$ CuSO_4 , the scavenger indicated and 1 mM ascorbate (added last). (.....), specific fluidity in absence of ascorbate.

Figure 7 Dose-dependence of the effect of scavengers and chelators on ascorbate-induced scission of xyloglucan

Final concentrations were 0.65% (w/v) xyloglucan, 40 mM acetate (Na^+ , pH 4.7), $2 \mu\text{M}$ CuSO_4 and 1 mM ascorbate (added last); viscosity was measured after 16 h.

were primarily responsible for ascorbate-induced xyloglucan scission in the absence of deliberately added metals.

Deferoxamine, which effectively chelates Fe^{3+} , strongly protected xyloglucan in the absence of added transition metal ions (Table 3). However, deferoxamine also provided substantial protection against the (much more extensive) scission caused in the presence of added Cu^{2+} . Thus deferoxamine is not a specific tool with which to demonstrate a role for Fe^{3+} .

In addition to EDTA, some, but not all, of the compounds that complex Cu^{2+} were effective inhibitors of Cu^{2+} /ascorbate-induced scission (Figure 8, $\square$). Each was effective above a characteristic threshold concentration (e.g. $5 \mu\text{M}$ EDTA, 10 mM oxalate, 200 mM citrate; Figure 7, and results not shown). Thiosulphate and thiourea completely inhibited scission, whereas with oxalate, citrate, EDTA, 2,2'-bipyridyl and poly-L-histidine, the effect reached a plateau at partial inhibition (Figure 7; results not shown). The thresholds probably represented the concentrations required to chelate most of the Cu^{2+} into a form that no longer catalysed ascorbate oxidation so efficiently. In line with this, EDTA (Table 2), oxalate, thiourea and thiosulphate (results not shown) strongly inhibited ascorbate oxidation. Cysteine's effect was biphasic: the plateau of partial inhibition at 0.05–10 mM was probably due to Cu^{2+} chelation, whereas the almost complete inhibition at $> 50 \text{ mM}$ was probably due to $\cdot\text{OH}$ scavenging. Histidine and proline can complex Cu^{2+} , but neither was able to prevent ascorbate oxidation or protect xyloglucan. L-Histidyl-L-lysine, which has a high affinity for Cu^{2+} but for which no $k_{(\cdot\text{OH})}$ has been published, had a $C_{0.2}$ value of 10 mM (Figure

Figure 8 Correlation between a compound's effectiveness at inhibiting ascorbate-induced xyloglucan scission and its rate constant for reaction with $\cdot\text{OH}$

Data are from Figure 7 and several similar experiments. (Δ), compounds whose $C_{0.2}$ was not reached (highest concentration tested is plotted on y-axis); ($\square$), compounds for which $C_{0.2}$ could be measured but which are known to complex strongly with Cu^{2+} ; ($\bullet$), all other compounds. The line of best fit is for the data described by the plot with closed circles. Compounds tested were: 1, acetone; 2, L-serine; 3, methanol; 4, D-xylose; 5, D-glucose; 6, maltose; 7, ethanol; 8, sucrose; 9, cellobiose; 10, formate; 11, glycerol; 12, propan-1-ol; 13, DMSO; 14, butan-1-ol; 15, imidazolium; 16, acetate/acetic acid; 17, L-proline; 18, glycine; 19, L-alanine; 20, L-malate; 21, D-tartrate; 22, L-threonine; 23, L-asparagine; 24, citrate; 25, L-histidine; 26, oxalate; 27, thiourea; 28, 2,2'-bipyridyl; 29, thiosulphate; 30, L-cysteine; 31, EDTA; 32, salicylate.

Table 1 Trace metal content of polysaccharides used in the present work

Abbreviation: N.D., not determined.

	Trace metal ($\text{mg} \cdot \text{kg}^{-1}$)		
	Cu	Fe	Zn
Xyloglucan*			
(i)	2.8	19	6.2
(ii)	2.8	20	6.2
(iii)	2.8	20	6.1
(iv)	2.8	19	6.2
Pectin	7.8	133	12.0
Carboxymethylcellulose	1.0	3.0	0.3
Methylcellulose	0.4	26	0.3
Dextran	1.2	27	2.9
Ascorbic acid	0.6	0.7	N.D.

* Analyses of four portions of the tamarind xyloglucan preparation that had been ethanol-washed on separate occasions.

7); its effect showed no tendency to reach a plateau, suggesting that it acted principally as an $\cdot\text{OH}$ scavenger rather than as a Cu^{2+} chelator.

Figure 9 Effect of Cu^{2+} on ascorbate-induced scission of xyloglucan

CuSO_4 solution or an equal volume of water was added at zero time, followed at 25 min by ascorbate (to 1 mM). (a) Time course; (b) copper concentration dependence of the rate of scission, estimated as $1/(\text{time taken to double the specific fluidity})$. All solutions contained 0.8% (w/v) xyloglucan in 20 mM succinate (Na^+ , pH 5.5).

Poly-L-histidine afforded partial protection, reducing the increase in specific fluidity by about 79% at all concentrations tested (0.03–6.7 g/l).

DISCUSSION

Source of $\cdot\text{OH}$ *in vitro*

The simple reaction mixtures discussed in this work led to extensive scission of xyloglucan and other polysaccharide chains. For a Fenton reaction [eqn. (3)] to account for ascorbate-induced scission, the simplest reaction mixture used (xyloglucan, ascorbate, O_2 and traces of Cu^{2+}) would need to generate $\text{Cu}^+ + \text{H}_2\text{O}_2$, and hence $\cdot\text{OH}$. Ascorbate is a reducing agent, widely viewed as providing protection against the damaging effects of free radicals [14,18,23,27,28]. It is thus interesting that ascorbate can also act to generate $\cdot\text{OH}$. This apparent contradiction may be explained by a mechanism in which ascorbate effects two separate reductions [23]: in the presence of traces of Cu^{2+} , ascorbate reduces O_2 to H_2O_2 [eqn. (1)]; ascorbate also reduces Cu^{2+} to Cu^+ :

The two products ($\text{H}_2\text{O}_2 + \text{Cu}^+$) can participate in a Fenton reaction [eqn. (3)], regenerating Cu^{2+} . Overall, ascorbate reduces O_2 to $\cdot\text{OH}$ with the stoichiometry:

Table 2 Effect of ascorbate, Cu^{2+} , EDTA and H_2O_2 on the extent of scission of xyloglucan

Xyloglucan [0.65% (w/v) in 40 mM acetate (Na^+), pH 4.7] was incubated at 20 °C with 0 or 2 μM CuSO_4 , 0 or 3 mM EDTA, 0 or 1 mM H_2O_2 , and 0 or 1 mM ascorbate. After 18 h, viscosity was recorded (means of three experiments $\pm$ S.E.M.) and the remaining ascorbate was assayed. +, Present; —, absent.

Additives				$10^2 \times$ Final specific fluidity (means $\pm$ S.E.M.)	Final [ascorbate] (mM)
Cu^{2+} (2 μM)	EDTA (3 mM)	H_2O_2 (1 mM)	Ascorbate (1 mM)		
—	—	—	—	3.37 ± 0.10	—
—	—	—	+	9.29 ± 1.06	0.43
—	—	+	—	3.53 ± 0.12	—
—	—	+	+	27.1 ± 1.1	0.00
—	+	—	—	3.39 ± 0.12	—
—	+	—	+	7.30 ± 1.31	0.51
—	+	+	—	3.33 ± 0.10	—
—	+	+	+	9.56 ± 2.53	0.45
+	—	—	—	3.51 ± 0.06	—
+	—	—	+	21.0 ± 2.0	0.00
+	—	+	—	3.44 ± 0.18	—
+	—	+	+	34.2 ± 3.6	0.00
+	+	—	—	3.49 ± 0.11	—
+	+	—	+	7.56 ± 1.67	0.55
+	+	+	—	3.46 ± 0.09	—
+	+	+	+	10.5 ± 2.9	0.42

Table 3 The effect of Cu^{2+} , Fe^{3+} , EDTA, deferoxamine and H_2O_2 on ascorbate-induced scission of xyloglucan

Xyloglucan [final concentration 0.65% (w/v)] in 110 mM acetate buffer (Na^+ , pH 4.7) containing 1 mM ascorbate was incubated at 20 °C in the presence of the additives listed. FeCl_3 and CuSO_4 were added as stock solutions in 100 mM HCl; samples without added metals received an equivalent amount of HCl (final Cl^- concentration 1.6 mM). After 18 h, the viscometer efflux time was recorded and the final ascorbate was assessed qualitatively. The final specific fluidity of controls lacking ascorbate was 0.030.

Additives (final concentration)					Final specific fluidity	Final [ascorbate]
Cu^{2+} (2 μM)	Fe^{3+} (20 μM)	EDTA (3 mM)	DF* (3 mM)	H_2O_2 (1.2 mM)		
—	—	—	—	—	0.104	+
—	—	+	—	—	0.097	+
—	—	—	+	—	0.034	+
+	—	—	—	—	0.35	—
+	—	+	—	—	0.12	+
+	—	—	+	—	0.12	$\pm$
—	+	—	—	—	0.092	+
—	+	+	—	—	0.26	—
—	+	—	+	—	0.031	+
—	—	—	—	+	0.55	—
—	—	+	—	+	0.17	+
—	—	—	+	+	0.082	+
+	—	—	—	+	0.60	—
+	—	+	—	+	0.17	+
+	—	—	+	+	0.23	—
—	+	—	—	+	0.55	$\pm$
—	+	+	—	+	0.35	—
—	+	—	+	+	0.078	+

* DF, deferoxamine.

This dual role of ascorbate would explain why dehydroascorbate-induced scission of xyloglucan was more dependent on exogenous H_2O_2 than was ascorbate-induced scission (Figure 5): dehydroascorbate can evidently reduce Cu^{2+} , but not O_2 .

Effect of $\cdot\text{OH}$ on carbohydrates

$\cdot\text{OH}$, although a reduction product of O_2 , is a much more powerful oxidant (standard reduction potential +1.8 to +2.7 V,

depending on pH) [25]. Studies of the γ -irradiation of sugar solutions show that $\cdot\text{OH}$ reacts with carbohydrates exceedingly rapidly [$k_{(\cdot\text{OH})}$ 1–4 $\text{nM}^{-1}\cdot\text{s}^{-1}$] [25], abstracting a C-bonded H atom according to the general equation [29,30]:

In the presence of O_2 , the $\text{R}\cdot$ radical quickly forms a carbonyl derivative, e.g. glucose yields a mixture of products including dialdehydoglucose, four glucosuloses and gluconate [9,29,30].

Less work has been conducted on the effects of $\cdot\text{OH}$ on polysaccharides. Model studies on cellobiose suggest that hydrogen abstraction from C-1, C-4 or C-5 of a sugar residue in a (1 $\rightarrow$ 4)-linked polysaccharide chain leads to scission of a glycosidic bond [31]. In the case of hydrogen abstraction from C-1, an intermediary hemi-*o*-ester is likely to be formed which, on subsequent hydrolysis, could cause backbone scission. Some splitting of C-C bonds within sugar residues also occurs, but this is more prevalent in the absence of O_2 [30]. Analysis of the termini of fragments released from hyaluronate by the action of $\cdot\text{OH}$ supports this general mechanism of polysaccharide scission [5].

Oxidation by ozone at C-1 of a sugar residue is strongly influenced by the anomeric configuration, β -D-glycosides being more readily oxidized than α -D-glycosides [32,33]. If this rule also applies to attack by $\cdot\text{OH}$, then it could explain the observation (Figure 1) that xyloglucan and carboxymethylcellulose, which have β -D-linked backbones, exhibit a greater susceptibility to ascorbate-initiated scission than do dextran and pectin, which have α -D-linked backbones. However, $\cdot\text{OH}$ oxidizes sugars much more indiscriminately than ozone, readily abstracting hydrogens from any carbon atom of a sugar residue, not predominantly C-1. Another factor that will influence the ability of $\cdot\text{OH}$ to reduce the viscosity of a polysaccharide solution is the number of chains that associate: the scission of a single strand of a double helix would have relatively little effect on viscosity [8].

Production of $\cdot\text{OH}$ in the cell wall *in vivo*?

Since $\cdot\text{OH}$ can cleave polysaccharides, it is pertinent to consider how it could be generated in a plant cell wall *in vivo*. The basic requirements are O_2 , a transition metal, such as Cu^{2+} , and appropriate electron donor(s). O_2 , the first of these requirements, is ubiquitous in healthy plants [34].

Concerning the second requirement for a transition metal, plant tissues typically have Cu contents within a fairly narrow range (3–30 mg/kg dry weight), regardless of the Cu concentration of the soil in which the plant was grown [35]. Stems, leaves, fruits and roots all have similar mean Cu contents [36]. Much of the Cu is located in the cell walls [17]; thus walls typically have a Cu content at least equal to that of the polysaccharides used in the present work (Table 1). Cu-wall bonds are strong; thus wall-bound Cu can only be displaced by other Cu ions (e.g. ^{63}Cu versus ^{64}Cu), by the chemically similar Pb, or by 0.1 M HCl [37]. The Cu in the cell wall may be bound to histidine-rich glycoproteins [38]; however, Cu bound in this form may still participate in eqns. (1), (3) and (4), as indicated by the observation that polyhistidine did not prevent ascorbate-induced xyloglucan scission. Other biologically relevant chelating agents tested, e.g. citrate, oxalate, His-Lys, tartrate, etc. did not prevent ascorbate-induced xyloglucan scission except at very high concentrations (Figure 7). Some ligands, e.g. histidine, may even have promoted the participation of Cu in $\cdot\text{OH}$ production. The affinity of Cu^{2+} for the cell wall means that $\cdot\text{OH}$ production may be 'targeted' near the polymer molecules whose scission it can cause. A similar targeting has been proposed for DNA-bound Cu [39].

The third requirement for $\cdot\text{OH}$ formation in the wall is the electron donor(s) needed to reduce apoplastic Cu^{2+} to Cu^+ and O_2 to H_2O_2 . Ascorbate can perform both reductions non-enzymically, dehydroascorbate only the former. Ascorbate and dehydroascorbate have been reported to be present in the apoplast, e.g. of spinach leaves [14], *Vigna* stems [13,15], *Kalanchoë* stems [12] and spruce needles [16]. The total ascorbate plus dehydroascorbate concentration in the spinach leaf apoplast was

estimated to be 0.15–0.6 mM [14]. Another potentially relevant electron donor is $\text{O}_2^{\cdot-}$, which can reduce Cu^{2+} to Cu^+ . $\text{O}_2^{\cdot-}$ may be released into the apoplast by NAD(P)H oxidases located in the plasma membrane. The action of NADH oxidase *in vitro* is promoted by treatment with an auxin-type growth promoter [40]. This suggests that $\text{O}_2^{\cdot-}$ may also be produced *in vivo* in healthy, fast-growing plant cells. However, the production of $\text{O}_2^{\cdot-}$ *in vivo* has so far been reported only in cells infected by pathogens or treated with elicitors of defence responses [41,42]. Production of $\cdot\text{OH}$, speculated to be dependent on $\text{O}_2^{\cdot-}$, was elicited in cultured rice cells treated with oligosaccharins [21]. Reduction of O_2 to H_2O_2 can also be achieved enzymically by the action of apoplastic oxidases, e.g. oxalate oxidase [43], amine oxidases [44,45], and some peroxidases [42,46]. Although dehydroascorbate cannot directly reduce O_2 to H_2O_2 , it is a precursor of oxalate [47], which can enzymically reduce O_2 to H_2O_2 . In addition, $\text{O}_2^{\cdot-}$ undergoes rapid dismutation to form H_2O_2 . Whatever its precise origin(s), H_2O_2 is indeed detectable in the apoplast of most plant tissues [19,20,46].

The pH of the plant cell wall is thought to vary between about 4 and 6; low pH values are often associated with rapid cell expansion [3]. Many wall enzymes have low pH optima (about pH 4 in the case of the H_2O_2 -generating oxalate oxidase) [43]. Thus the enzymic reactions discussed above are likely to operate effectively at the slightly acidic pH values typical of a growing plant cell wall. Some of the non-enzymic reactions discussed are also pH-dependent within the pH range 4–6. For example, the dismutation of superoxide to form H_2O_2 is maximal at pH 4.75, where an equimolar mixture of $\text{O}_2^{\cdot-}$ and $\text{HO}_2^{\cdot}$ exists [48]. The results in Figure 3 show that when exogenous H_2O_2 is supplied (and thus the only role of the ascorbate is to reduce Cu^{2+}), the rate of xyloglucan scission is maximal at approx. pH 4.5, although still high at pH 3.5. Such a pH optimum could arise from the need for both Cu^{2+} reduction [eqn. (4)] and the Fenton reaction [eqn. (3)], which generate H^+ and OH^- ions respectively, and may therefore differ in their individual pH optima. When exogenous H_2O_2 is not supplied, the rate of xyloglucan scission still peaks at about pH 4.5, but is considerably lower at pH 3.5. This greater susceptibility to acid conditions could be because the ascorbate now also has to reduce O_2 to H_2O_2 , which it is best able to do as the monoanion (AH^-), the species prevalent above pH 4.25 [23].

Physiological consequences of $\cdot\text{OH}$ production *in vivo*

Thus there are several means by which Cu^+ and H_2O_2 can be generated in the apoplast. This combination will produce $\cdot\text{OH}$ by a Fenton reaction [eqn. (3)], with a rate constant of $4700 \text{ M}^{-1} \cdot \text{s}^{-1}$ [18]. A proportion of the $\cdot\text{OH}$ will be scavenged by solutes present in the apoplast. However, high concentrations of scavengers, e.g. 50 mM cysteine or > 30 mM salicylate, are required to block xyloglucan scission completely (Figure 7). Thus any apoplastic $\cdot\text{OH}$ formed would be likely to cause polysaccharide scission in the cell wall.

The concept that apoplastic ascorbate could cause polysaccharide scission, and hence wall loosening, may explain some interesting observations on plant growth. For example, lycorine, which blocks ascorbate biosynthesis, inhibits cell elongation in oat coleoptiles [49] and exogenous ascorbate can promote cell expansion [50–52], especially in the presence of H_2O_2 [28]. It may also be relevant that added dithiothreitol inhibits both the action of NADH oxidase (whose product, $\text{O}_2^{\cdot-}$, can lead to $\cdot\text{OH}$ formation) and auxin-stimulated elongation [40], which requires wall loosening. Similarly, the dramatic induction of oxalate oxidase [43], an apoplastic H_2O_2 -generating enzyme, during

imbibition of some graminaceous seeds could be related to the explosive cell expansion that occurs at the onset of germination.

If H_2O_2 (+ Cu^+) can lead to wall loosening, it is intriguing that H_2O_2 can also participate in peroxidase-catalysed phenolic coupling reactions that 'tighten' the wall [53–55]. 'OH-mediated loosening and peroxidase-mediated tightening reactions would compete for available H_2O_2 , and the net effect could thus critically depend on the activity of peroxidase in the wall. This suggests a new significance for wall peroxidases.

Solubilization and depolymerization of wall polysaccharides also contribute to the softening of ripe fruit [56]. Intriguingly, pectin solubilization and degradation can occur in some fruits that contain little or no pectinase (endopolygalacturonase), e.g. persimmon [57] and kiwi fruit [58]. The present work shows that 'OH production would be a plausible non-enzymic mechanism for polysaccharide scission in fruits. Ascorbate is certainly plentiful in most fruits, although there appears to be no information on whether it is present in the fruit apoplast. Evidence that H_2O_2 -dependent oxidative processes contribute to fruit softening was presented [59], but has been largely ignored. In the light of the findings reported here that H_2O_2 readily leads to scission of wall polysaccharides under physiological conditions, the possible role of H_2O_2 in fruit softening deserves more thorough investigation.

Conclusion

In conclusion, 'OH is readily produced in simple mixtures of reagents known to be present in the plant cell wall, and causes extensive scission of plant cell wall polysaccharides *in vitro*. The 'devastating' 'OH radical is generally regarded as detrimental to life, causing membrane damage, enzyme inactivation and mutation. However, 'OH is very short-lived and is thus a site-specific oxidant. Therefore if produced at the right time and in the right place, 'OH could be targeted to play a useful role in loosening the cell wall during physiologically important processes, such as germination, growth, fruit ripening and abscission. These considerations invite a reconsideration of the prevailing view that proteins (enzymes and expansins) are the sole means of loosening plant cell walls *in vivo*.

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