Oxalate oxidases and differentiating surface structure in wheat: germins

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Oxalate oxidases (OXOs) have been found to be concentrated in the surface tissues of wheat embryos and grains: germin is concentrated in root and leaf sheaths that surround germinated embryos; pseudogermin (OXO-ψ) is concentrated in the epidermis and bracts that 'encircle' mature grains. Most strikingly, the epidermal accumulation of OXO-ψ was found to presage the transition of a delicate 'skin', similar to the fragile epidermis of human skin, into the tough shell (the miller's 'beeswing') that is typical of mature wheat grains. A narrow range of oxalate concentration (1–2 mM) in the hydrated tissues of major crop cereals (barley, maize, oat, rice, rye and wheat) contrasted with wide variations in their OXO expression, e.g. cold-tolerant and cold-sensitive varieties of maize have similar oxalate contents but the former was found to contain approx. 20-fold more germin than did the latter. Well-known OXOs in sorghum, a minor cereal, and beet, a dicotyledon, were found to have little antigenic relatedness to the germins, but the beet enzyme did share some of the unique stability properties that are peculiar to the germin-like OXOs that are found only in the major crop cereals. Their concentration in surface structures of domesticated wheat suggests a biochemical role for germin-like OXOs: programmed cell death in surface tissues might be a constitutive as well as an adaptive form of differentiation that helps to produce refractory barriers against tissue invasion by predators. Incidental to the principal investigation, and using an OXO assay (oxalate-dependent release of CO_2) that did not rely on detecting H_2O_2, which is often fully degraded in cell extracts, it was found that OXO activity in soluble extracts of wheat was manifested only in standard solution assays if the extract was pretreated in a variety of ways, which included preincubation with pepsin or highly substituted glucuronogalactoarabinoxylans (cell-wall polysaccharides).

Key words: apoptosis, cereals, maturation, pathogenesis, seeds.

INTRODUCTION

Oxalates have been studied in the basic and applied sciences for centuries [1]. In biology, oxalates have usually been regarded as inert end products of carbon assimilation that, in the forms of their insoluble calcium salts, are well-tolerated 'storage forms' of calcium in plants, and serious contributors to chronic, sometimes acute, 'stone diseases' in humans [1]. Entrenched and traditional views of oxalate as a 'static substance' were challenged by the discovery that germin, long known as a marker of growth onset in germinating cereals [2,3] and long suspected of being an agent in host-plant resistance to disease [4], is an oxalate oxidase (OXO) [5,6]. A focused vision of plant oxalate as a dynamic resource, as a source of H_2O_2, which can, at different concentrations, mediate developmental signalling, cross-linking and defence reactions in higher plants [3], has met with increasing favour [7].

Germins are remarkable water-soluble oligomeric proteins: they are refractory to dissociation in SDS/PAGE gels [8] and to hydrolysis by known proteases under non-denaturing conditions [9]. Germins [10] and their coding elements [11] have so far been found only in what Hill dubbed the 'true cereals', the major crop cereals barley (*Hordeum*), maize (*Zea*), oat (*Avena*), rice (*Oryza*), rye (*Secale*) and wheat (*Triticum*) [12]. Wheat [5], barley [5,6], maize (the present paper), oat, rice and rye (B. G. Lane, unpublished work) germins are OXOs (EC 1.2.3.4) that generate H_2O_2 from oxalate, confirming an early suspicion [4], partly based on an association with wheat cell walls [4,13], that germins might have a role in plant defence [3] as well as development [2]. A 1.6 Å X-ray diffraction structure of barley germin is complete (J. Dunwell, personal communication) and is compatible with a proposed octahedral co-ordination of 6 mol of Mn per mol of catalytic hexamer [14].

As shown here, an OXO in a minor cereal, sorghum (*Sorghum*) [15], lacks the stability of germins, and an OXO in the garden beet (*Beta vulgaris*) [16], a dicotyledon, shares some of their stability but does not react with germin antibodies. Beginning with the most idiosyncratic sequence (PHIHPRATEI; single-letter amino acid codes) in wheat germin [11], called the 'germin box' in PROSITE analyses (PHXHPRATEI), it was shown that there is broad dispersal of coding elements for germin-like proteins (GLPs) [17–19] beyond the monocotyledons. GLPs are found in protists [11], bryophytes [20], gymnosperms [21] and dicotyledons [22–27]. Study of GLP proteins, as opposed to coding elements, has been rare, but a moss GLP has been shown to be a superoxide dismutase [20] and two *Arabidopsis thalania* GLPs have been shown to share the stability of germins [26].

Classical botanical studies had forecast roles for oxalate and 'wound oxalate' in the germinative development of lupin ([28], cited in [1]) and in the defence responses of *Tradescantia fulminensis* [29] respectively but the possible significance of these isolated observations faded with the passage of time. More recently, projected roles of germins and germin-like OXOs (gl-OXOs) in phytopathology were proposed [3,4] and soon validated when three groups showed independently that infection with powdery mildew induces the synthesis of gl-OXOs in barley [30,31] and wheat [32]. The germinative emergence of germin in structures that envelop the embryo (3% of grain mass) [33] is

Abbreviations used: d.p.a., days post-anthesis; gl-OXO, germin-like OXO, i.e. an OXO oligomer that has the unique stability properties of germin (refractory to hydrolysis by all known proteases and resistant to dissociation in standard SDS/PAGE gels); GLP, germin-like protein, i.e. a protein encoded by a germin-like coding element; hsGGAX, highly substituted glucuronogalactoarabinoxylan(s); OXO, oxalate oxidase; OXO-ψ, pseudogermin; P16, insoluble (pellet) fraction after centrifugation of a homogenate at 16000 ***g***; S16, soluble (supernatant) fraction after centrifugation of a homogenate at 16000 ***g***; XG, xyloglucan.

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broadly extended in the present study to structures comprising most of the mass of the wheat flower (embryo, grain and bracts). During maturation, there is a striking concentration of OXO-ψ in structures that tightly envelop (epidermis) and loosely surround (bracts) the wheat grain. Pseudogermin (OXO-ψ) is a uniquely stable germin [13] and its emergence in the epidermis presages the transformation of a delicate 'skin' into a tough shell that fuses with the seedcoat to form the protective outer surface in a ripened wheat grain. Implications of the findings for the biochemical importance of gl-OXOs are discussed.

EXPERIMENTAL

Plant materials

Hexaploid wheat grains of the Neepawa and Conway varieties were obtained from the Alberta Wheat Pool (Lethbridge, Alberta, Canada). Diploid wheat grains having the A (PI 119423 TR SD 1277, *Triticum monococcum*), B (CIae 64 AE SD, *Aegilops speltoides*) or D (CIae 4 AE SD 2038, *Aegilops tauschii*) genomes were supplied by Dr Harold Bockelman (Small Grains and Potato Germplasm Research Unit, USDA/ATRS, Aberdeen, ID, U.S.A.). Immature grains were harvested from Vulcan and Cheyenne cultivars of wheat by Dr William J. Hurkman (Plant Research Center, USDA/ARS Albany, CA, U.S.A.). Two varieties of maize grain, one cold tolerant (C0255) and one cold sensitive (C0286), were kindly supplied by Dr Bob Hamilton and Dr Chris Andrews (Agriculture Canada, Plant Research Centre, Ottawa, Canada). Sorghum seeds (genotype CSH-5) were kindly provided by Dr C. S. Pundir (Department of Bio-Sciences, M. D. University, Rohtak, India) through the agency of Nath Seeds Pvt. Ltd (Aurangabad, India). *Arabidopsis thalania* seeds were kindly provided by Dr François Bernier (Institut Botanique, Université de Strasbourg, Strasbourg, France). Transgenic tobacco and canola (*Brassica Napus*) seeds of the T2 generation (progeny of the primary transformants) were obtained by transforming wild-type tobacco and canola (Brutor cultivar) with an approx. 740 bp *Hin*dIII$_1$/*Sph*I$_2$ fragment of the *gf-2.8* germin gene [11], which contains approx. 15 bp of the 5′ untranslated region, the entire signal sequence, the entire structural-protein coding sequence and approx. 50 bp of the 3′ untranslated region. This germin-coding element, inserted between a 35 S cauliflower promoter and a Nos terminator, was ligated into a pBIN19 vector (with kanamycin selection) and the construct was transferred to *Agrobacter tumefaciens* (LBA 4404) for transformations. Beet seeds (var. Early Wonder, Ruby Queen) were purchased from a local seed supplier. Seedlings were grown by culturing mature grains or seeds on water-soaked Whatman no. 3 filter paper for 1–15 days at room temperature in laboratory light (approx. 12 h/day). Embryos were prepared from mature wheat grains (*Triticum aestivum*, var. Neepawa or Conway) [34]. Purified specimens of highly substituted glucuronogalactoarabinoxylan(s) (hsGGAX) and xyloglucan (XG) were generously supplied by Professor Nicholas Carpita (Purdue University, West Lafayette, IN, U.S.A.).

Preparation and immunostaining of thick sections of water-cultured wheat embryos

Pre-isolated embryos (200 mg) were cultured on a disc (8.5 cm) of Whatman no. 3 filter paper that was immersed in sterile water (5 ml) at room temperature in darkness for 40 h For thick-sectioning, single embryos that seemed to be essentially intact (approx. 30 % of the total number and approx. 50 % of the total mass) were first fixed (1 h) in a solution made by mixing 7 ml of 95 % (v/v) ethanol with 2.5 ml of acetic acid, and then dehydrated successively in 70 % (v/v) ethanol (four times, 15 min), graded concentrations (70 %, 85 %, 95 % and 100 %, v/v) of aqueous 2-methylpropan-2-ol (once, for 30 min each), and finally in 100 % 2-methylpropan-2-ol (twice, 1 h), before being embedded in paraffin blocks at 60 °C. Sections (8 mm) were transferred to slides that had been cleaned for 10 min in ethanol and coated for 10 min in poly-(L-lysine) in a Coplin jar. The slides, with sections, were kept at 60 °C for 1 h before they were incubated in Histo-Clear (twice, 10 min), graded concentrations (100 %, 90 %, 70 % and 50 %, v/v) of aqueous ethanol, water, Tris-buffered saline (TBS) (once, for 3 min each), and finally for 1 h in 200 μl of 'blocking solution' [10 % (v/v) goat serum in TBS]. 'Blocked' sections were incubated for 45 min with 150 μl of germin antiserum (1:100 dilution in TBS), with or without prior pre-adsorption to remove glycan-directed antibodies [13], washed with TBS (three times, 5 min), and incubated for 45 min with alkaline-phosphatase-conjugated goat anti-rabbit IgG [1 μl in 1 ml of 1 % (w/v) BSA in TBS], before again being washed in TBS (twice, 5 min). For staining, sections were incubated for 30 min in 200 μl of substrate solution [100 mM Tris/HCl (pH 9.5)/100 mM NaCl/50 mM $MgCl_2$, containing levamisole, 1.65 mg of Nitro Blue Tetrazolium in 22 ml of 70 % (v/v) dimethylformamide and 0.82 mg of 5-bromo-4-chloroindol-3-yl phosphate in 16.5 ml of dimethylformamide], and after being washed in TBS (three times, 5 min) and water (once, 3 min), and being dehydrated in graded concentrations of ethanol (50 %, 70 %, 90 % and 100 %, v/v) (once, for 3 min each), were overlaid with Crystalmount and incubated for 20 min at 80 °C before light microscopy.

Quantitative solution assay and qualitative blot assay of OXO activity

A published method [35] was used to measure the release of $^{14}CO_2$ from [^{14}C]oxalate. Reaction mixtures containing 2 mM oxalic acid were buffered with 0.1 M sodium succinate, pH 3.5, for maximal activity. Buffers used to assess the pH dependence of OXO activity were: 0.1 M sodium acetate, pH 4.5, 0.1 M sodium phosphate, pH 6.7, 0.1 M Tris formate, pH 7.7, and 0.1 M ammonium formate, pH 9.2. The specific radioactivity of the [^{14}C]oxalate that was used in the $^{14}CO_2$ release assays was approx. 10^5 c.p.m./μmol. Soluble and pellet fractions of homogenates were prepared by homogenizing embryos, seedlings or individual organs in 100 mM potassium acetate/3 mM magnesium acetate/20 mM Hepes (pH 7.5)/1 mM dithiothreitol (1.5, 3 or 20 μl of buffer/mg of fresh mass) in a Duall homogenizer and then centrifuging homogenates at 16000 ***g*** for 10 min (all at 0–4 °C). This buffer conserves cell-free protein-synthesizing activity in the 16000 ***g*** supernatant fraction (S16) of homogenates of mature wheat embryos [36]. Pellets (P16) were suspended in the same buffer by vortex-mixing in an Eppendorf tube in the presence of a straight-pin to obtain smooth suspensions, volumes of the P16 extracts being adjusted to equal those of the corresponding S16 extracts. The quantities (20 μl) of S16 or P16 extract used in $^{14}CO_2$ release assays (total volume 0.5 ml), and for application to SDS/PAGE gels for blot assay [5], were derived from 1 mg (20 μl of buffer/mg), 6.4 mg (3 μl of buffer/mg) or 8.5 mg (1.5 μl of buffer/mg) fresh mass. Reactions were terminated by the addition of trichloroacetic acid after 20 min at 37 °C. After trichloroacetic acid-induced release of $^{14}CO_2$, released $^{14}CO_2$ was collected in a bucket of 40 % (w/v) KOH (100 μl) in a reaction vessel supplied by Kontes (cat. no. 882360-0010). The residual reaction mixture, containing H_2O_2, was

neutralized (to approx. pH 4) with KOH and analysed for H_2O_2 as described in [37]. Measurements of radioactivity were made in 10 ml of CytoScint with an LKB Rackbeta liquid-scintillation counter. After separation of proteins by SDS/PAGE and transfer to nitrocellulose, germin and its isoforms were detected by reaction with a polyclonal anti-germin rabbit serum [13] or by staining for OXO activity [5].

Quantitative assay of oxalate

Fresh or frozen biological material (50–200 mg) was homogenized (1 min) in a round-bottomed 2051 Falcon tube with a Polytron blender (Brinkmann Instruments), at a speed setting of 7 (Kinematica rheostat) with 1 ml of 3 M HCl that had been supplemented with 10 μl (0.01 μM, approx. 10^5 c.p.m.) of [^{14}C]oxalic acid. Tracer oxalic acid was included to assess the percentage recovery of oxalic acid from tissue on the assumption that all tissue oxalate was equilibrated with the tracer in 3 M HCl; the tracer solution was prepared by dissolving 5.2 mg (250 mCi) of [^{14}C]oxalic acid from Sigma (cat. no. 31,391-2) in 100 μl of sterile water (solubility 9.5 g/100 ml at 15 °C). When wheat embryos had been homogenized for 1 min and the homogenate had been left at room temperature for 30 min before again being blended for 1 min, the measured amount of oxalate in the embryos was the same as that found after a single 1 min homogenization, confirming that the tracer was well equilibrated with tissue oxalate. The pH of the homogenate was adjusted to pH 7.0 with 10 M NaOH (approx. 220 μl) and the neutralized homogenate was transferred to a Microfuge tube for centrifugation (10 min, 14000 ***g***, room temperature). The resulting supernatant (approx. 1.5 ml) was transferred to a larger tube for mixing with an equal volume of satd $CaSO_4$ and ethanol (10 ml/1.5 ml satd $CaSO_4$) and allowed to precipitate at room temperature for 24–48 h. The precipitate was suspended in 2 ml of the 1:1 diluent in the oxalate analysis kit supplied by Sigma (cat. no. 591-4) and the suspension was clarified by transfer to an Eppendorf tube for centrifugation in an Eppendorf 5415 centrifuge (14000 rev./min, 10 min, room temperature). A 50 μl aliquot of the clear supernatant solution was used to assess the recovery of [^{14}C]oxalic acid (25–40 %); separate aliquots (approx. 100–200 μl), containing the amounts of oxalate recovered from 5 and 10 mg of fresh mass, were assayed for oxalate. Potassium oxalate standards (0.18, 0.45 and 0.90 μg) and experimental samples, in a total volume of 200 μl of Sigma diluent, were mixed with 1 ml of reagent A and 100 μl of reagent B and incubated at 37 °C for 20–30 min before measurement of A_{590}. Reagent B, containing soluble barley germin and horseradish peroxidase, was obtained from Sigma (cat. no. 591-2); reagent A was prepared by dissolving 23.7 mg of 3-dimethylaminobenzoic acid (Sigma cat. no. D0787) and 2.13 mg of 3-methyl-2-benzothiazolinone hydrazone (Sigma cat. no. M 8006) in 45 ml of 0.1 M sodium succinate, pH 3.2. For a 'low-oxalate' plant (e.g. wheat), A_{590} was approx. 0.1 above background for the amount of oxalate (approx. 0.4 μg) recovered from 10 mg of fresh mass. Measurements were corrected for tracer recovery (25–40 %) and for the contribution of the tracer itself (approx. 5 %) to the total oxalate measured in the analysis.

Because it has been reported [38] that oxalate is generated when ascorbate is incubated in phosphate buffer at slightly alkaline pH (7.4) for several hours, it is significant that we observed no effect on measured oxalate if extraction with tributylphosphate [39] was used to partition the extracted oxalate at alkaline pH. Measurements were also not significantly changed if, during the neutralization of acid extracts of oxalate, the pH was adjusted to approx. 13 and the alkaline extract was incubated at room temperature for 2–3 h before being adjusted to pH 7. Linear increases were observed for aliquots containing the oxalate recovered from 5 and 10 mg of fresh mass; there was additivity when such aliquots of experimental samples were mixed with pure potassium oxalate, i.e. there was no interference with, or suppression of, OXO activity in the assay when aliquots corresponding to 10 mg of fresh mass (or less) were used for analysis. Acid extracts of tissues were stored at −20 °C for up to 3 weeks without affecting the measurements of oxalate.

RESULTS

Increased amounts of germin in pellet fractions of homogenates depend on germinative development of water-cultured mature wheat embryos

For ease of comparison of OXO activities with the oxalate contents of tissues, both are expressed as mass quantities, as in the oxalate literature [40]. As shown in Table 1, the cell walls of germinated embryos had strong OXO activity. The physical properties of the cell-wall form of germin from wheat embryos were very similar to those of a commercially available preparation of barley OXO (preparation no. 1 in Table 1): both activities were totally insoluble in water or aqueous buffers and were fully sedimented at low speeds. In contrast, it was found that the kind

Table 1 Comparison of the OXO activities in equal masses of wheat embryo cell walls and three commercially available preparations of barley OXO

Purified cell walls were prepared after preisolated embryos from mature wheat grains had been germinated by culturing them in water for 48 h [13]. Preparation no. 1 was purchased as 'oxalate oxidase' from Sigma (cat. no. O 4127) and, as with the activity in purified wheat-embryo cell walls, this activity was totally insoluble in water or aqueous buffers. Preparation no. 2 was purchased as 'oxalate oxidase' from Boehringer Mannheim (cat. no. 567 698) and the activity was totally water-soluble. Preparation no. 3 was purchased from Sigma (reagent B in their oxalate analysis kit, cat. no. 591-2) and the activity was totally water-soluble. Most of the mass in preparations no. 2 and no. 3 was due to the commercial suppliers' additions of 'stabilizers' (probably borate) to the proteins. When analysed in SDS/PAGE gels, preparation no. 2 was free of protein impurities but preparation no. 3 had precisely the same contaminants previously found in earlier lots of preparation no. 2 [5]. Protein was estimated by comparing the degree of Coomassie staining of germin oligomers and monomers with the staining of a known amount (0.5 μg) of BSA in SDS/PAGE gels. Allowance was made for the (approx. 50%) greater staining intensity of BSA relative to known amounts (by amino acid analysis) of germin, on a mass basis. Protein was extracted from the cell-wall preparation [13] and the amount of extractable germin monomer was measured in SDS/PAGE as described (Figure 6 in [13]). These results suggest implicitly that the germin in cell walls has much greater specific activity (per mol of germin) than the more highly purified soluble germin preparations (see the discussion of GGAX 'activation' in Table 7).

Specimen	Dry mass (μg)	Germin content (μg)	OXO activity (μg of oxalate degraded in 10 min)
Wheat embryo walls (water-insoluble)	150	0.1	7.8
OXO preparation no. 1 (water-insoluble)	150	0.5	36.2
OXO preparation no. 2 (water-soluble)	150	1.7	3.7
OXO preparation no. 3 (water-soluble)	150	3.3	9.2

Table 2 Temporal emergence of OXO activity in water-cultured mature embryos prepared in bulk from two varieties of hexaploid wheat

After being purged of $^{14}CO_2$, liquid phases from reaction flasks containing insoluble P16 extract were shown to contain an amount of H_2O_2 that was equimolar to the amount of degraded [^{14}C]oxalate, e.g. for the Neepawa insoluble (P16) extract at 15 h the amount of H_2O_2, as measured by a solution assay [35], was equal to the molar amount (3.4/90 = 0.038 μmol) of [^{14}C]oxalate that was degraded (see the Experimental section). The molar equivalence between H_2O_2 release and [^{14}C]oxalate degradation was established for all reaction mixtures whenever the amount of H_2O_2 was sufficient for reliable measurement in the colorimetric assay. For flasks containing soluble (S16) extracts, the amount of H_2O_2 was too small for reliable estimation but quasi-quantitative results were compatible with expectations. Reaction mixtures (0.5 ml) contained 1.5 mM oxalate/oxalic acid (approx. 10^5 c.p.m.) in 0.1 M sodium succinate, pH 3.5, and were supplemented with 25 μl of soluble (S16) or insoluble (P16) extract from 8.5 mg (fresh mass) of embryos (see the Experimental section for further details).

	OXO activity (μg of oxalate degraded in 10 min)			
	Neepawa embryos		Conway embryos	
Time (h)	Soluble (S16)	Insoluble (P16)	Soluble (S16)	Insoluble (P16)
0	0.01	1.7	0.00	1.9
15	0.03	3.4	0.05	3.4
25	0.34	11.7	0.18	7.1
35	0.20	13.5	0.16	16.8
45	0.32	12.0	0.27	13.9

of commercially available preparations included as part of 'reagent B' in oxalate analysis kits (preparations no. 2 and no. 3 in Table 1) were freely soluble in water and they had specific activities (per μg of protein) that approached the OXO activity reported for soluble wheat germin [5].

Compatible with a prominent association of germin with wheat cell walls [13], germin activity became increasingly concentrated in the P16 fractions of homogenates made from embryos that were pre-isolated from field-ripened grains and cultured in water [41] (Table 2). Strong OXO activity in the soluble (S16) fractions of wheat homogenates was largely repressed and was undetected when OXO activity was measured by $^{14}CO_2$ release from [^{14}C]oxalate (Table 2) (see below). The striking increase in OXO activity in embryo homogenates during the temporal development of water-cultured, pre-isolated wheat embryos (Table 2) paralleled previously demonstrated increases in levels of germin, its translatable mRNA [10] and its gene expression [42] under the same conditions. The burgeoning amounts of germin made during germinative development were confirmed (results not shown) in Western (antibody) and activity blots after SDS/PAGE separation of proteins in the different extracts [5].

Localization of germin in different parts of wheat and maize seedlings

As previously defined [10], the 'stem' is that part of the plant that remains after excision of the roots and shoots from embryos or seedlings, there being little stem growth when grains are cultured on water-soaked filter-paper. Immunocytology (see below) showed that 'stem' germin was mostly localized to enveloping tissues (coleorhiza or coleoptile) [33], not the axis proper. OXO activities in fractionated homogenates of roots, 'stems', coleoptiles and leaves from hexaploid and diploid seedlings are compared in Table 3. Notably, when grown on filter paper, hexaploid roots had more OXO activity than diploid roots. Hexaploid, A-diploid and D-diploid seedlings (and parts) generally had much greater germin activity than the B diploid. It is of incidental interest that unfractionated homgenates of the A and D diploids, and of the B diploids, had approx. 30% and approx. 5% respectively of the OXO activity measured in a homogenate of hexaploid (cv. Conway) wheat. Similar large differences existed between the germin activities found in cold-sensitive and cold-tolerant maize cultivars (Table 4). The germin character [5] of the OXO activities listed in Tables 3 and 4 was confirmed in companion (Western and activity) nitrocellulose blots of SDS/PAGE separations (results not shown).

Oxalate and OXO levels during the early growth of selected plants

There have been no previously reported systematic comparisons of OXO-to-oxalate ratios in higher plants, all of which contain oxalate. To explore factors that might mitigate and/or enhance OXO activity in plants, it was of interest to know whether OXO activity and oxalate levels in wheat seedlings were related to those in (1) another cereal (sorghum) that contained an OXO that was not a gl-OXO, i.e. did not have germin-like stability [8,9], (2) a dicotyledon (*A. thalania*) that contained GLPs, i.e. non-OXO proteins encoded by germin-like coding elements, and (3) a dicotyledon (beet) that contained an OXO but was, unlike wheat, a 'high-oxalate' plant [40].

As shown in Table 5, the oxalate content (approx. 0.1 μg per mg of hydrated mass) of wheat kept pace with the approx. 100-fold increase in (hydrated) fresh mass that accompanied germinative development of a mature embryo, *in situ* (in the grain), into a 6-day seedling. Although there were only modest differ-

Table 3 Comparison of OXO activities in intact seedlings and individual organs from hexaploid and diploid wheat varieties

Mean fresh masses of seedlings at excision, after grains had been cultured in water for 9 days, were 110 mg for hexaploid (Conway), 107 mg for A diploid, 25 mg for B diploid and 24 mg for D diploid (see the Experimental section for additional details). Values of 0.14, 0.10 and 0.14 μg of oxalate/mg of fresh mass were measured for single A, B and D diploids, values similar to those found for hexaploids (see Table 5). Limited availability of materials dictated the preparation of more dilute homogenates (20 μl of S16 buffer/mg of fresh mass) than were made in the experiments shown in Table 2 (3 μl of S16 buffer/mg of fresh mass). Reaction mixtures (0.5 ml) contained 20 μl of soluble or insoluble fraction from approx. 1 mg of fresh mass.

		OXO activity (μg of oxalate degraded in 10 min)							
		Root		'Stem'		Coleoptile		Leaf	
Wheat variety	Mass distribution (%) between root, 'stem', coleoptile and leaf	S16	P16	S16	P16	S16	P16	S16	P16
Hexaploid	30, 10, 26, 34	0.23	2.0	0.53	14.9	0.11	4.0	0.01	0.25
A diploid	33, 3, 18, 46	0.01	0.05	0.42	16.0	0.02	0.91	0.01	0.004
B diploid	16, 4, 18, 58	0.01	0.09	0.05	0.88	0.004	0.11	0.01	0.01
D diploid	12, 6, 48, 34	0.01	0.33	0.71	15.4	0.11	0.40	0.01	0.01

Table 4 Comparison of OXO activities in the organs of cold-tolerant and cold-sensitive cultivars of maize

Distributions of mass between the organs of single cold-sensitive C0286 (380 mg) and cold-tolerant C0255 (431 mg) seedlings were significantly different, owing largely to limited leaf development in the C0286 seedling when grains had been cultured in water for 11 days; significantly, however, similar differences in OXO activity were obtained with pot-grown (vermiculite) cultivars, in which case leaf growth was equally luxuriant for cv. C0255 and cv. C0286. These results, with freshly harvested grain, are similar to the observations made with seedlings grown from 5-year-old grains. Assays were performed at 20 °C but similar large (10–20-fold) differences were observed between the two cultivars when extracts were assayed at 37 °C. Assays were otherwise performed as in the legend to Table 2, except that these reaction mixtures (0.5 ml) contained 20 μl (rather than 25 μl) of the soluble (S16) or insoluble (P16) fractions of homogenates prepared from 6.4 mg (not 8.5 mg) of fresh mass. Germin-like properties (oligomer stability during SDS/PAGE and nitrocellulose transfer) for these OXO activities were demonstrated: SDS/PAGE mobilities of the maize germin activities were similar to that of OXO-ψ but their thermal stabilities were similar to that of germin.

		OXO activity (μg of oxalate degraded in 10 min)							
		Root		'Stem'		Coleoptile		Leaf	
Maize cultivar	Mass distribution (%) between root, 'stem', coleoptile and leaf	S16	P16	S16	P16	S16	P16	S16	P16
Cold-sensitive	27, 28, 28, 17	0.01	0.03	0.02	0.46	0.02	0.27	0.01	0.02
Cold-tolerant	12, 23, 27, 38	0.01	1.1	1.8	8.3	0.20	2.8	0.01	0.01

Table 5 Oxalate contents and OXO activities of equal fresh masses of intact seedlings and individual organs from selected higher plants

The oxalate measurement given in parentheses for the ungerminated wheat embryo allows for a degree of hydration equivalent to a germinated embryo (85%), to illustrate that there was little or no net change in oxalate content (μg/mg of hydrated mass) in the embryo when a mature embryo was germinated. As with wheat homogenates, most of the OXO activity in homogenates of sorghum and beet, as measured by $^{14}CO_2$ release from [^{14}C]oxalate, was confined to the insoluble (P16) fractions when homogenates were made in the 'S16 buffer' that was used to prepare all extracts made in this study (see the Experimental section). Reaction mixtures (0.5 ml) contained 1.5 mM oxalate/oxalic acid (approx. 10^5 c.p.m.) in 0.1 M sodium succinate, pH 3.5, and 20 μl of insoluble (P16) extract from 6.4 mg (fresh mass) of plant material (see the Experimental section for further details).

Specimen	Sample	Fresh mass (mg)	Oxalate (μg/mg)	OXO (μg/10 min per mg)	Ratio OXO to oxalate
Pre-isolated wheat embryo	Ungerminated	0.35	0.67 (0.10)	0.17	2
	Germinated (water)	1.9	0.10	1.1	11
	Germinated (5% sucrose)	2.9	0.059	0.51	9
Wheat seedling (3 days)	Overall	51	0.10	1.8	18
	Root	25	0.080	0.34	4
	'Stem'	6.4	0.085	4.6	54
	Coleoptile	19	0.12	0.65	5
	Leaf	–	–	–	–
Wheat seedling (6 days)	Overall	90	0.10	1.4	14
	Root	43	0.071	0.57	8
	'Stem'	6.2	0.073	6.2	85
	Coleoptile	22	0.11	0.65	6
	Leaf	19	0.11	0.01	0.09
Sorghum seedling (12 days)	Overall	45	0.16	0.035	0.22
	Root	10	–	0.10	–
	'Stem'	6.3	–	0.072	–
	Coleoptile	11	–	0.002	–
	Leaf	18	–	0.003	–
Beet seedling (17 days)	Overall	64	1.2	0.20	0.2
	Root	5.8	2.0	0.025	0.01
	Stem	38	0.98	0.34	0.4
	Leaf	20	1.4	0.003	0.002

ences between their oxalate contents, there were gross differences between OXO activities of different parts of the seedling; e.g. the OXO-to-oxalate ratio, as depicted in Table 5, varies more than 500-fold between leaf and 'stem' in a 6-day wheat seedling: 'stem' germin was concentrated in enveloping tissues (coleorhiza or coleoptile) in embryos or seedlings.

Sorghum analyses (Table 5) showed that the oxalate content (approx. 0.16 μg/mg) was similar but the OXO activity was approx. one-tenth of that found in hexaploid wheat. The sorghum OXO activity was not detected (results not shown) if soluble or insoluble extracts were subjected to SDS/PAGE and blotting on nitrocellulose, either at the pH optimum of the sorghum enzyme [15] or at the pH optimum (3.5) of the wheat enzyme, which was used for the assays reported in Table 5. Accordingly, although sorghum OXO is oligomeric [15], the protein and its OXO activity are not germin-like in character: they do not, as do the germin oligomer [8] and its OXO activity [5] in soluble extracts of wheat, survive SDS/PAGE and nitrocellulose blotting. Interestingly, however, a band with the expected mobility of sorghum OXO (molecular mass approx. 60 kDa) [15] was weakly antigenic to wheat germin apoprotein antiserum after SDS/PAGE and Western blotting (results not shown).

Analyses of beet (Table 5) showed that the oxalate content of this dicotyledon (approx. 1.2 μg/mg) was approx. 10-fold greater but the OXO/oxalate ratio was approx. 100-fold smaller than in hexaploid wheat. The beet OXO activity [16] had the same pH optimum as the wheat enzyme (results not shown); when beet extracts were subjected to SDS/PAGE and blotting on nitro-

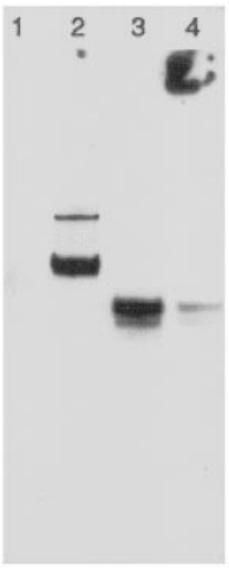

Figure 1 Beet and wheat OXO activities are detected after blotting an SDS/PAGE gel

This is an 'activity' blot [5]. Proteins (approx. 65 μg) in the soluble (S16) and insoluble (P16) fractions of wheat embryo [4] and beet homogenates were derived from 6.4 mg of fresh mass (approx. two embryos) and were separated by SDS/PAGE; they were then electroblotted on nitrocellulose and assayed for gl-OXO activity [5]. The base of each numeral marks the top of the well of the parent SDS/PAGE gel. Lanes 1, 2, 3 and 4 contained proteins derived from beet S16, beet P16, hexaploid wheat S16 and hexaploid wheat P16 fractions respectively. The gl-OXO activity bands in lanes 3 and 4 correspond to the G (the stronger, slower band; molecular mass approx. 125 kDa) and G′ glycoforms of germin [67]. The beet OXOs have not yet been similarly characterized but they were concentrated in the pellet fraction of the beet homogenate (lane 2); unlike the wheat OXOs in the pellet fraction, which were trapped in the position of the well of the parent SDS/PAGE gel, the beet OXOs entered freely into the SDS/PAGE gel.

cellulose (Figure 1), the beet OXO activity was detected by SDS/PAGE after nitrocellulose blotting but antigenicity toward wheat germin antiserum could not be detected in Western blots (results not shown). Although not explored by electrophoretic analysis, the beet OXO shared at least some of the protease resistance properties of germin in the oxalate-dependent $^{14}CO_2$ release assay.

Other selected dicotyledon seedlings that we have studied (*A. thalania*, canola, lettuce, radish and tobacco) are 'low-oxalate' plants (0.1–0.2 μg/mg). Unlike the (low-oxalate) cereals (wheat, maize and sorghum) we have studied, extracts of these dicotyledon seedlings did not have either OXO activity by $^{14}CO_2$ release assay, or gl-OXO activity by H_2O_2 release assay after SDS/PAGE and nitrocellulose blotting (results not shown).

Methodological study of the cryptic germin activity present in soluble wheat extracts

The large disparity between the OXO activities of soluble and insoluble fractions of wheat homogenates, found with the oxalate-dependent $^{14}CO_2$ release assay (Tables 2–4), was very surprising. Not only had similar amounts of the germin monomer been detected in soluble extracts [10] and (insoluble) cell-wall fractions of wheat embryos [13] before the OXO activity of germin was known [5], but, afterwards, gel-blot assays, by direct blotting on nitrocellulose or by electrophoresis in SDS/PAGE gels [5], had indicated similar amounts of OXO activity in the soluble and pellet fractions of wheat homogenates (Figure 1). However, evidence of its gl-OXO character, by mobilizing most of the putative germin oligomer in the pellet fraction in SDS/PAGE, was not obtained. Thus, although the putative germin in the wells (gel-slots) of SDS/PAGE gels (Figure 1, lane 4) could be released and mobilized as the monomer by being heated before SDS/PAGE [13], the monomer was devoid of OXO activity in SDS/PAGE blots.

In totality, our findings had suggested that, when wheat extracts were made with a buffer designed to conserve the physiological integrity of protein-synthesizing activity (see the Experimental section), the OXO activity in soluble wheat extracts was inhibited by a factor that was removed, destroyed or separated from germin during SDS/PAGE and/or nitrocellulose blotting. Because gl-OXO enzymes are refractory to treatment with pepsin, soluble fractions of wheat homogenates can be treated with pepsin to determine whether the putative inhibition might be caused by a protein. Compatible with this possibility was the observation that treatment of a soluble wheat extract with pepsin caused a marked increase in OXO activity in the $^{14}CO_2$ release assay (Table 6). A similar large increase in OXO activity was achieved when untreated soluble wheat extracts were blotted on nitrocellulose (Table 6). Although still unproved, these results were consistent with the possible existence of a protein inhibitor of OXO in soluble wheat extracts.

Table 6 Effects of treatment with pepsin and/or blotting to nitrocellulose on the cryptic OXO activity present in a soluble extract of germinated (48 h) wheat embryos

For treatment with pepsin, soluble (S16) extracts (1.2 ml) were adjusted to pH 2 by adding approx. 0.16 ml of 1 M HCl and then mixed with approx. 0.16 ml of pig pepsin (0.5 mg/ml in 0.01 M HCl) and incubated at 37 °C for 90 min before being neutralized to pH 7.5 with approx. 0.08 ml of 2.5 M Tris [10]. The pepsin treatment destroyed most proteins in the extracts, except germin [9]. Treatment of the corresponding insoluble (P16) fraction of the homogenate with pepsin led to a comparable absolute increase in OXO activity but this constituted a relatively small (50–100%) proportional increase owing to a much higher activity in the insoluble (relative to the soluble) fraction before treatment with pepsin. Reproducible results were obtained consistently if extracts of different cultivars and batches of embryos were made by homogenization in 1.5–3 μl of S16 buffer/mg of fresh mass (Table 2), but results could be capricious with more dilute homogenates, e.g. homogenization in 20 μl of S16 buffer/mg of fresh mass.

Fraction	OXO activity (μg of oxalate degraded in 10 min)	
	In solution	Dot-blotted on nitrocellulose
Untreated soluble (S16)	0.2	56.7
Untreated insoluble (P16)	59.2	–
Adjusted to pH 2.0 and readjusted to pH 7.5	0.4	41.0
Adjusted to pH 2.0, incubated at 37 °C for 90 min without pepsin and readjusted to pH 7.5	0.4	48.6
Adjusted to pH 2.0, incubated at 37 °C for 90 min with pepsin and readjusted to pH 7.5	46.6	50.4

Table 7 Effects of preincubating soluble germin and germin-containing soluble cell extracts with soluble or particulate fractions of homogenates or cell-wall polysaccharides

Assays were performed at 20 °C after test solutions or suspensions (20 μl) had been incubated, separately or mixed, for 10 min at 20 °C, then added to buffered substrate solution. The S16 homogenizing buffer, polysaccharides and all soluble and insoluble fractions of homogenates of beet, wild-type canola and maize were virtually devoid of OXO activity. For further details, see the Experimental section and the legend to Table 2.

Fractions	OXO activity (μg of oxalate degraded in 10 min)
Soluble fraction of a transgenic tobacco homogenate	3.5
+ Soluble fraction of a wheat homogenate	0.2
Soluble fraction of a transgenic canola homogenate	2.2
+ Soluble fraction of a wheat homogenate	0.1
Soluble germin (dialysed, salt-free)	42.5
+ S16 homogenizing buffer	38.2
+ Soluble fraction of a beet homogenate	55.7
+ Soluble fraction of a cold-sensitive maize homogenate	52.8
+ Soluble fraction of a wheat homogenate	7.3
Soluble fraction of a wheat homogenate (with pepsin)	37.1
+ Soluble fraction of a wheat homogenate (without pepsin)	0.3
Soluble fraction of a wheat homogenate	0.3
+ Soluble fraction of homogenate of a wild-type canola	0.2
+ Insoluble fraction of homogenate of a wild-type canola	42.9
+ hsGGAX (100 μg)	6.8
+ XG (100 μg)	0.4

'Mixing experiments' were done to determine whether the OXO activities of soluble extracts of transgenic dicotyledons might be inhibited by preincubating them with soluble wheat extract. As shown in Table 7, there were notable decreases in OXO activity if (1) a soluble extract of transgenic dicotyledons, (2) a pepsin-treated soluble extract of wheat or (3) germin itself was preincubated (10 min at room temperature) with soluble wheat (but not beet or maize) extract. Moreover, the existence of a cryptic germin activity was manifested when a soluble extract of wheat was preincubated with particulate fractions of wild-type rape (devoid of germin) or with some (hsGGAX) but not other (XG) types [43,44] of cell-wall polysaccharide (Table 7).

It is germane to note that failure to detect OXO activity by $^{14}CO_2$ release assay of crude extracts is distinct from failure to detect OXO activity by H_2O_2 release in solution assays of crude extracts. In the latter instance it is common for the assay not to detect the product of OXO action on oxalate (H_2O_2) because the H_2O_2 formed from oxalate is often, if not usually fully, degraded by peroxidases and catalases in crude cell extracts. Failure to detect OXO activity by the $^{14}CO_2$ release assay of crude extracts is, in contrast, a hitherto unknown process that is reversible either by pretreatment with proteases or with (cell-wall) polysaccharide-containing specimens.

It is worth mentioning that the putative inhibitor might be (1) diffusible (dialysable), (2) able to bind and repress the activity of a pepsin-susceptible activator of OXO activity (e.g. GGAX) or (3) a pepsin-susceptible oxygen-consuming activity that deprives the OXO enzyme of an essential reactant (oxygen) needed to oxidize oxalate (see also the Discussion section).

Germin is concentrated in surface structures when 'pre-isolated' wheat embryos are germinated

In the now classical system of Marcus [41], mature embryos, pre-isolated from dry, field-ripened wheat grains, undergo a triphasic uptake of water. First, rapid hydration (0–1 h) increases the water content of the embryos (from approx. 5% to approx. 65%) and more than doubles their mass. Secondly, there is a germination period (1–5 h) during which fresh mass does not change but notable changes occur in the translatable mRNA population. Finally, there is a resumption of water uptake (5–48 h) in singular association with the conspicuous nascent synthesis of germin and its mRNA [2,3]. This latter growth process increases the water content of the embryos (to approx. 85%) and increases their mass 3–4-fold [4] (Table 5). To obtain biochemical insight into the critical changes that occur in this system, in alliance with a surge of cell-wall germin activity (Table 2), the gross anatomical distribution of germin in the germinated embryos was determined.

For this purpose, thick sections of water-cultured embryos were immunostained with an anti-germin serum [13]. Sections were virtually transparent with preimmune serum, but with anti-germin serum there was, at 40 h after imbibition, differential staining of support tissues surrounding the embryo. Immunostaining of the envelope (coleorhiza) that surrounds the embryonic roots was particularly extensive in all sections (e.g. Figures 2A and 2B) but blue staining in the sheath (coleoptile) surrounding the embryonic leaves was extensive in some (Figure 2A) and superficial in other (Figure 2B) sections, and staining near the mesocotyl was strong in some (Figure 2B) but absent from other (Figure 2A) sections. Although the cause is unknown, non-specific staining of nuclei, observed with immune serum but not preimmune serum, was useful because it provided, without counterstaining, clear rust-coloured delineation of structures (nuclei) also observed when sections were counterstained with Nuclear Fast Red.

At higher magnification it was apparent that there was, in addition to non-specific staining of nuclei in the embryonic apex, primordial roots, leaves and mesocotyl (Figure 2), some weak (blue) staining in the cytoplasm of these non-vacuolated tissues (e.g. Figure 2C, a). The higher magnification emphasizes that there was much stronger, selective staining of vacuolated cells in the enveloping tissues that were adjacent to the apex (Figure 2C, b and c) and primordial roots (Figure 2C, d–f) in Figure 2(A). Immunostaining in the region of the mesocotyl (Figure 2B) was, at higher magnification (Figure 2C, g), comparable to the intense staining seen in enveloping tissues (Figure 2C, h) and also in superficial layers of the coleoptile (Figure 2C, i) of the same

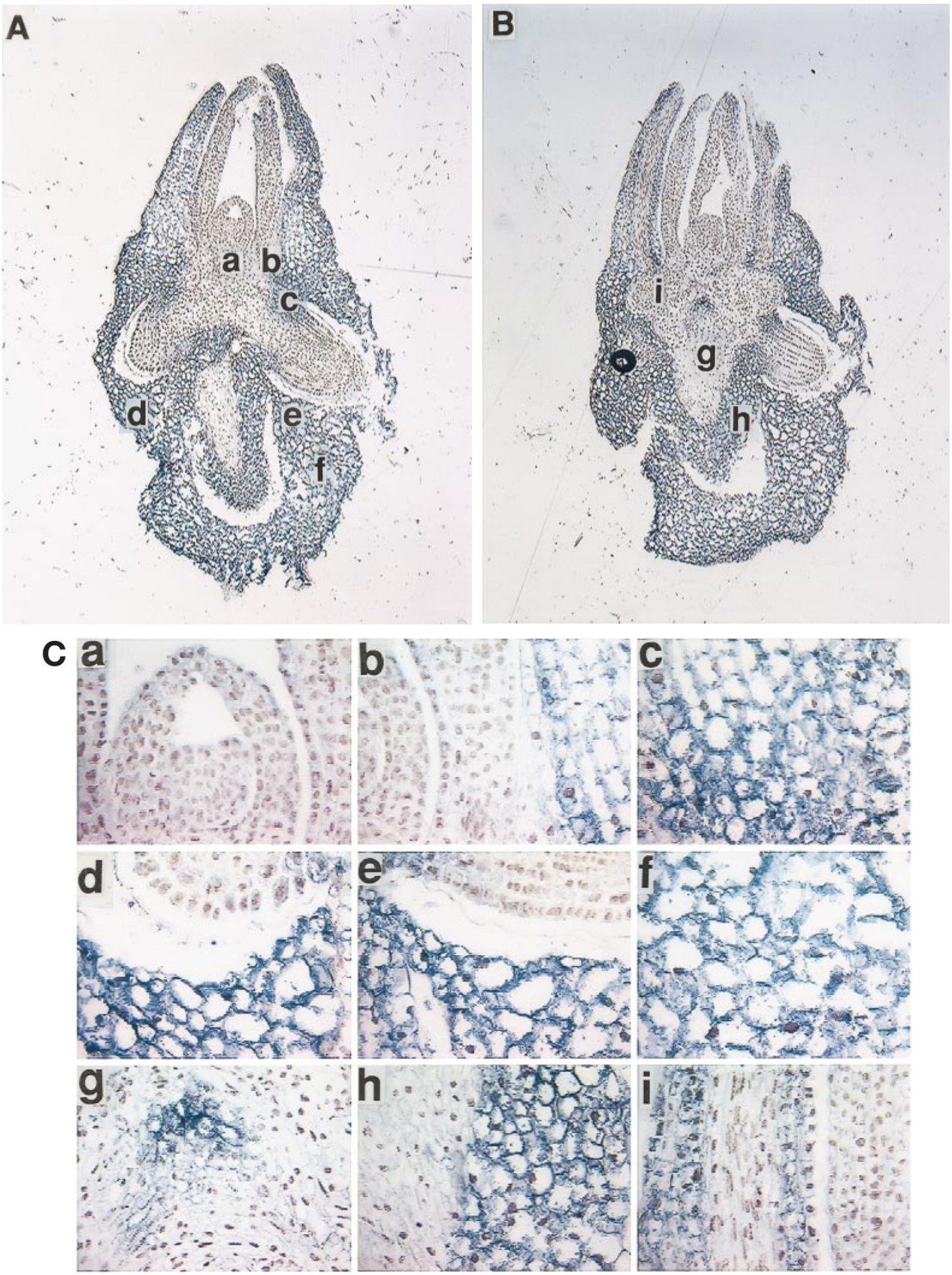

Figure 2 For legend see facing page.

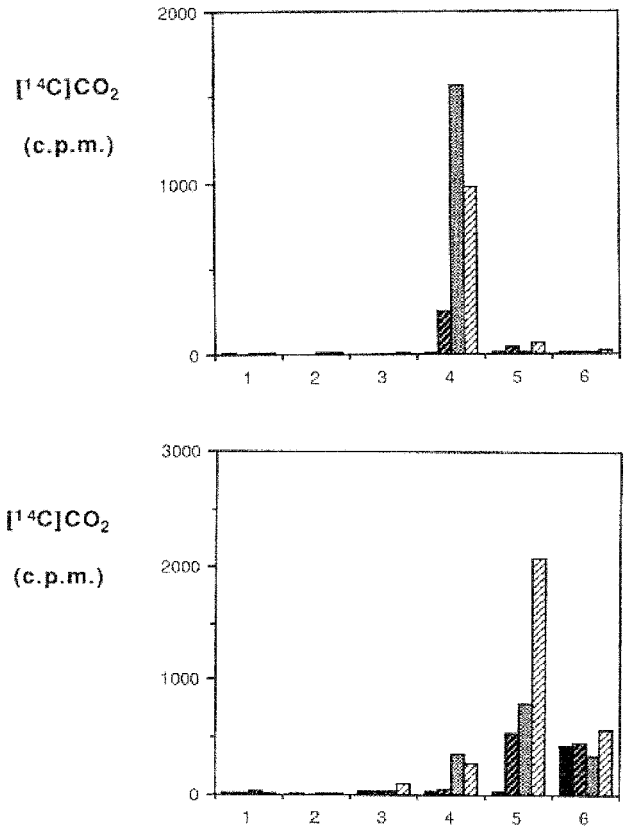

Figure 3 Temporal and spatial distributions of OXO activity in maturing wheat grains

The bar graphs show the relative release of $^{14}CO_2$ from [^{14}C]oxalate at 10 d.p.a. (black), 15 d.p.a. (dark hatching), 20 d.p.a. (stippling) and 25 d.p.a. (light hatching) when aliquots of homogenates of approx. 1 mg fresh mass of embryos, seedcoats, epidermes and bracts from developing wheat grains (cv. Vulcan) were assayed in solution/suspension [35]. Upper panel: soluble fractions of homogenates: 1, embryo; 2, endosperm; 3, seedcoat; 4, epidermis; 5, palea/lemma; 6, glumes. Lower panel: pellet fractions of homogenates: 1, embryo; 2, endosperm; 3, seedcoat; 4, epidermis; 5, palea/lemma; 6, glumes.

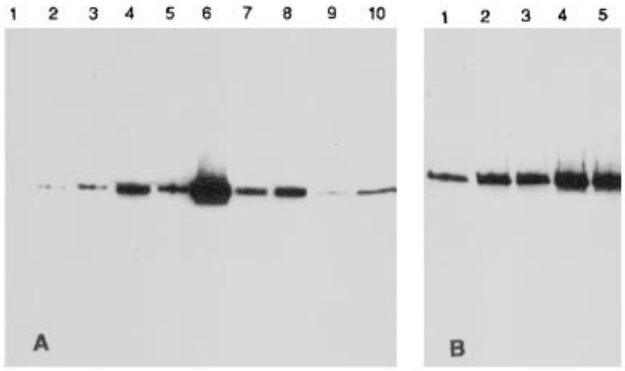

Figure 4 Temporal and spatial distributions of gl-OXO in selected parts of maturing wheat grains

'Activity' blots [5] are shown. Proteins in soluble (S16) fractions of homogenates from different parts (approx. 1 mg of fresh tissue) of developing wheat grains (cv. Vulcan) were separated by SDS/PAGE, electroblotted on nitrocellulose and assayed for gl-OXO activity [5]. (**A**) Lanes contain embryo proteins extracted at 10 (lane 1) and 20 (lane 2) d.p.a., seedcoat proteins extracted at 10 (lane 3) and 20 (lane 4) d.p.a., epidermis proteins extracted at 10 (lane 5) and 20 (lane 6) d.p.a., palea/lemma proteins extracted at 10 (lane 7) and 20 (lane 8) d.p.a. and glume proteins extracted at 10 (lane 9) and 20 (lane 10) d.p.a. (**B**) Lanes contain glume proteins extracted at 10 (lane 1), 15 (lane 2), 20 (lane 3), 25 (lane 4) and 30 (lane 5) d.p.a.

section. Neighbouring tracheid-like structures (arrowhead in Figure 2C, g) suggest that the selectively stained region near the mesocotyl might be a part of the scutellar node [45].

The dominant features of these immunostained sections of water-cultured mature wheat embryos at 48 h after imbibition were the differential immunostaining of germin in the vacuolated enveloping tissues (Figure 2C, h) and in terminally differentiated vascular tissue (Figure 2C, g). These thick sections revealed, primarily, tissue rather than subcellular distributions of germin, but there is little doubt that much of the total germin, as previously shown by electron-scanning microscopy [13], was allied with cell walls. At 10 h after imbibition, just before the onset of secondary uptake of water in concert with germin accumulation (results not shown), vacuolation was absent from the enveloping tissues of water-cultured mature embryos and there was only very weak (but still selective) immunostaining of the enveloping tissues (results not shown). Likewise, in immature [20–30 days post-anthesis (d.p.a.)] wheat embryos (results not shown) there was weak but selective immunostaining of enveloping tissues, comparable to that seen in the axial tissues of water-cultured mature embryos (Figure 2C, a).

OXO-ψ is concentrated in surface structures of the naturally maturing and mature wheat grain

The $^{14}CO_2$ release assays in Figure 3 illustrate the differential temporal accumulations of OXO-ψ that occurred in various parts of a developing wheat grain. Accumulations of soluble OXO-ψ in the epidermis (Figure 3, upper panel) and of insoluble OXO-ψ in the palea/lemma (Figure 3, lower panel) are most noteworthy. Limited availability, friability and tractability of structures enveloping the caryopsis dictated the preparation of relatively dilute extracts (i.e. 20 μl rather than 3 μl of buffer/mg of fresh mass).

Blot comparisons of release of H_2O_2 in Figure 4(A) illustrate the special character (stable to SDS/PAGE and blot transfer), temporal emergence and relative magnitudes of OXO activities

Figure 2 Germin is immunostained in thick sections of 'pre-isolated' germinated wheat embryos

(**A**) A longitudinal section of a water-cultured (40 h), mature wheat embryo was immunostained with an anti-germin rabbit serum as a source of primary antibodies, and alkaline-phosphatase conjugated goat anti-rabbit IgG as a source of secondary antibodies. A serial section from the same embryo was almost transparent when it was processed as described (see the Experimental section) with pre-immune serum in place of the anti-germin serum. The reason why all nuclei stained non-specifically is unknown. Lettering (a–f) is placed below areas shown at higher magnification in the nine-panel composite (**C**). Magn. $\times$37. (**B**) As in (**A**), but this longitudinal section contains a centrally located, immunostained structure that could be the scutellar node (above g). Lettering (g–i) is placed below areas that are shown at higher magnification in the nine-panel composite (**C**). Magn. $\times$37. (**C**) The lettered areas indicated in (**A**) and (**B**) are shown at higher magnification ($\times$184) The apex is shown in (**a**), and regions adjacent to the apex, including primary leaves and vacuolated enveloping tissue, are shown in (**b**) and (**c**). Primary root and adjacent enveloping tissue (coleorhiza) are shown in (**d**) and (**e**), and vacuolated enveloping tissue is again shown in (**f**). A region that might correspond to the scutellar node, with allied tracheids, is shown in (**g**), and the apposed tissues of the primary root and vacuolated envelope are shown in (**h**). The coleoptile and vacuolated enveloping tissue, possibly including the coleoptile epidermis, are shown next to primary leaf tissue in (**i**).

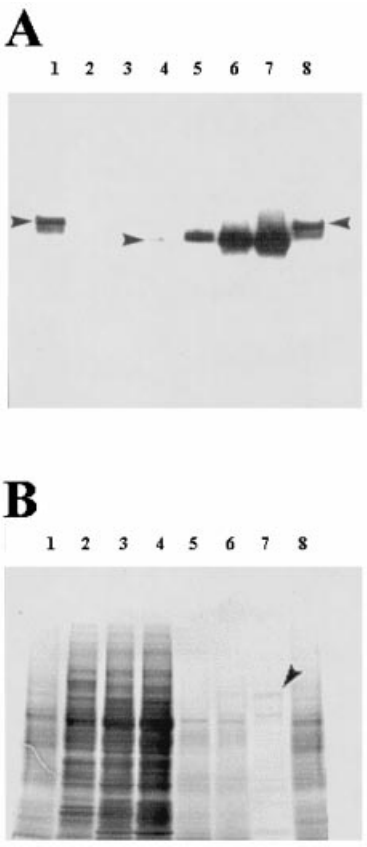

Figure 5 Comparison of gl-OXO activities and dye-stainable proteins in the embryos and epidermes of maturing wheat grains

(**A**) This shows an 'activity' blot [5]. Proteins in the soluble fractions of homogenates were prepared from approx. 1 mg of fresh tissue, separated by SDS/PAGE, electroblotted to nitrocellulose and assayed for gl-OXO activity [5]. The fresh tissues used for these analyses derived from the embryos and epidermes of developing wheat grains (cv. Cheyenne), and from the same 'pre-isolated' wheat embryos (cv. Neepawa) that had been germinated (40 h) and used for the histological studies of Figure 2. Lane 1, proteins from germinated (40 h after imbibition) wheat embryos; lane 2, proteins from developing (15 d.p.a.) wheat embryos; lane 3, proteins from developing (25 d.p.a.) wheat embryos; lane 4, proteins from developing (35 d.p.a.) wheat embryos; lane 5, proteins from developing (15 d.p.a.) wheat epidermes; lane 6, proteins from developing (25 d.p.a.) wheat epidermes; lane 7, proteins from developing (35 d.p.a.) wheat epidermes; lane 8, proteins from germinated (40 h after imbibition) wheat embryos. Arrowheads indicate the G glycoform of germin (molecular mass approx. 130 kDa) [61] in lanes 1 and 8, and OXO-ψ (molecular mass approx. 100 kDa) [67] in lane 4. (**B**) A dye-stained SDS/PAGE gel is shown. The samples analysed on this gel were identical with those on the gel that was used to obtain the activity blot in (**A**), but in (**B**) the gel was stained directly with Coomassie Blue without being blotted to nitrocellulose. Comparison of the corresponding lanes (1–8) in (**B**) with those in (**A**) illustrates the vast differences in protein content between the embryo and epidermis extracts that were assayed for gl-OXO activity in (**A**). The arrowhead indicates OXO-ψ in lane 7; i.e. OXO-ψ (molecular mass approx. 100 kDa) is visible by dye-staining and constitutes a considerable proportion of the total protein in epidermal extracts at 35 d.p.a.

in soluble extracts made from different parts of the caryopsis at 10 and 20 d.p.a. At all stages of development, the endosperm was virtually devoid of detectable OXO activity in either $^{14}CO_2$ release or H_2O_2 release assays (results not shown). The blot comparison in Figure 4(B) shows that, in spite of their toughness and resistance to disintegration, developing glumes yielded easily detected and increasing amounts of OXO in soluble extracts made between 10 and 30 d.p.a.

Further blot comparisons of H_2O_2 release (Figure 5) show the temporal emergence and relative magnitudes of OXO activities in the embryo and epidermis of the immature wheat caryopsis at 15, 25 and 35 d.p.a. Compatible with the results in Figure 3 for a more rapidly developing wheat cultivar was the observation that the soluble and insoluble fractions of homogenates of the epidermis had much greater activity than the corresponding embryo fractions at each time point (Figure 5A). Extracts applied to each gel slot in Figures 3–5 were derived from approx. 1 mg (fresh mass) of tissue. The replica dye-stained gel (Figure 5B) was not subjected to blot transfer and showed that embryo extracts contained vastly more protein than did extracts of epidermis, which tissue accumulates large amounts of 'pentosans' during this period of development [46].

The accumulation of OXO-ψ, in relation to total protein, was very much greater in the epidermis than in the embryo; it seems likely that this has a significant bearing on the profound physical change that occurred in the epidermis of this cultivar between 25 and 35 d.p.a. The epidermis of the pericarp had a pliable, rubbery consistency, similar to the peeled human-skin epidermis in appearance and texture, and it readily separated from underlying tissue at 25 d.p.a.; however, at 35 d.p.a. the epidermis had been transformed into a tough, horny layer that was difficult to separate from underlying tissue.

The OXO activities in extracts made from parts of the immature caryopsis, when assayed by $^{14}CO_2$ release from [^{14}C]oxalate or by H_2O_2 release in activity blots of SDS/PAGE gels, all had the same special properties, i.e. the OXO-ψ oligomer and its activity were both refractory to heating, diminishing only marginally after 1 min at 100 °C in aqueous SDS-containing solution [13]; however, the activity of germin was fully eradicated in less than 20 s.

DISCUSSION

Biological considerations

It is remarkable that the source of H_2O_2 used to form covalent cross-links in cell walls generally [47–49], and in the cell walls of seedcoats and pericarps in particular [50], remains unknown. The present study was initiated to address and perhaps redress some of this deficit in our understanding. Temporal and spatial correlation between the extensive vacuolation of, and the emergence of OXO enzymes in, root and leaf sheaths of the germinated embryo (germin) (Figure 2), and in epidermis and bracts of the maturing grain (OXO-ψ) (Figures 3 and 5), lend compelling support to the idea [3,33] that oxalate is a source of the H_2O_2 used to form cell-wall cross-links in the surface tissues of developing wheat.

The oxalate contents of cereals (0.1–0.2 μg/mg of fresh mass) (Table 5) correspond to mean concentrations of 1–2 mM in normally hydrated tissues. Without compartmentation of substrate and enzyme, it would be expected from the data in Table 5 that there would be rapid and complete oxidation of oxalate by germin. This could occur *in vivo* if, during the formation of the hard surface structures of ripened grains or during pathogen invasion, there were a collapse of cellular permeability barriers [51] and broad penetration of the OXO-containing extracellular matrix by oxygen and vacuolar [52] soluble oxalate at appropriately acidic pH values (see [3]). In this context, programmed cell death (apoptosis) is seen as a constitutive as well as an adaptive form of differentiation [53,54] or tissue remodelling. For example, OXO-ψ can decrease the toxic levels of oxalate secreted by predator organisms such as *Sclerotinia sclerotiorum* [55] and unlike the predator, which degrades oxalate without forming H_2O_2, OXO-ψ converts oxalate into H_2O_2, which is toxic to the predator and promotes the formation, in the host, of cross-linked wall barriers against predator penetration.

It was reported, on the basis of their superoxide dismutase and peroxidase activities, that hexaploid wheats are conspicuously deficient in their antioxidant capacities [56]. From the present study (Table 5), it seems that this 'antioxidant deficiency' of hexaploids might reflect elevated pro-oxidant capacity, to which the A and D diploid progenitors contribute most heavily (Table 3). Compatible with this is the observation that the *gf-2.8* OXO promoter occurs, minimally, in one or two, none or one, and three or four copies respectively in the A, B and D genomes, and maximally at 2–3-fold these frequencies [11]. There have been reports that oxalate might also have a role in creating pro-oxidant environments in animal cells. This seems unlikely in the case of neutrophils, in which reportedly elevated levels of oxalate [57] were probably due to a procedural artifact: the conversion of ascorbate into oxalate in blood specimens ([38,58], and B. G. Lane, unpublished work). In contrast, wheat products in the human diet could be a source of H_2O_2 in the gastrointestinal tract, where oxalate degradation ordinarily proceeds without H_2O_2 formation [59]. For example, wheat brans used to manufacture breakfast cereals are a rich source of OXO-ψ, and robust OXO-ψ-like activity has been found in several commercial products, including 'shredded wheat' (B. G. Lane, unpublished work). In view of its formidable stability, some of this OXO-ψ activity might survive in the gut and have as yet unknown but possibly significant consequences.

Molecular considerations

The stability of OXO-ψ is unusual, even for germins. Indeed, it is characteristic of the stability of enzymes in hyperthermophilic organisms [60]. Accordingly, pepsin digestion at pH 2 and extended periods (30 min) of boiling are steps used to isolate OXO-ψ from bulk, commercially accessible wheat bran and whole grains (B. G. Lane, unpublished work). It is of special interest to know what causes germin to lose its OXO activity completely after a few seconds in boiling water, whereas OXO-ψ, so closely related to germin antigenically and in its primary structure [13], is vastly more stable at 100 °C. Differences in quaternary structure and/or molecular mass (approx. 130 kDa for wheat germin; approx. 100 kDa for wheat OXO-ψ, by SDS/PAGE) might be important factors. Historically, these properties of the germins have presented knotty problems. On the basis of our comprehensive physicochemical studies, which first revealed extensive β-structure in germins, it had seemed that the 130 kDa wheat germin oligomer was homopentameric [61]; however, our subsequent X-ray diffraction study of barley germin (E. F. Pai and B. G. Lane, cited in [62]) clearly showed that the germin oligomer lacked a fivefold axis of symmetry and that it was likely to be a tetramer or hexamer, most probably a hexamer because a pentamer had been detected by cross-linking with dimethylsuberimidate [61]. This was confirmed by more complete X-ray diffraction study [63].

Transformation of oilseed rape by a full-length barley germin-coding element was first reported by Thompson et al. [64]. Transformations of dicotyledons (sunflower, tobacco, canola, soybean and potato) and a monocotyledon (maize), with a full-length wheat germin-coding element supplied by this laboratory, have also been achieved. Field trials, now under way to assess any improved host-resistance to microbial predators in the transgenics, have shown promise.

Study of the classical 'pre-isolated wheat-embryo system' [41,65] led to the discovery of germin [2,3] and later to the discovery of OXO-ψ [13]. Growth in this system is 'unnatural'. Unlike 'natural' embryos, *in situ*, in wheat grains, which vigorously accumulate DNA (2-fold increase), protein (3-fold increase) and carbohydrate (5-fold increase) during the 48 h that follow the onset of germination, 'pre-isolated embryos', after their mass isolation from grains, accumulate no DNA or protein and only a modest amount of carbohydrate during the same period [66]. Growth in the 'pre-isolated wheat embryo' is due largely to water uptake. To account for a conspicuous correlation between the bulk of the water uptake in this system [65] and the nascent synthesis of germin, which was shown to be adventitiously associated with GGAX [13,67], it was proposed, after germin was found to be an OXO, that the co-transport of germin with GGAX from the Golgi network to cell walls might initiate vacuolation by enhancing wall plasticity and that, later, vacuolation might be terminated by germin-generated induction by H_2O_2 of cross-linking in cell walls [3].

The discovery that there is a concerted accumulation of OXO-ψ (this study) and 'pentosans' [46] in the maturing epidermis of the wheat grain has provided an unprecedented opportunity to study the relation between OXO accumulation and GGAX [44,67] in a natural environment, in which differentiation is allied with profound physical transformation, perhaps the H_2O_2-mediated hardening/lignification of surface structure. As we noted when the OXO activity of germins was discovered [5], the fact that peroxidases, with their reliance on H_2O_2, are a parameter of metabolic activity during growth alterations [68] gives testimony to the importance of H_2O_2 production during growth. This concept was recently well articulated [33] in the context of embryo growth during wheat germination. It is therefore tempting to speculate that hsGGAX-induced activation of OXO activity in the soluble fraction of wheat homogenates (Table 7) might reflect intimate linkage *in vivo* between H_2O_2 generation and peroxidatic activity. If, as suspected, GGAX is a natural target of the H_2O_2 generated by gl-OXO *in vivo*, perhaps in a coupled reaction sequence that involves the peroxidase-dependent cross-linking of GGAX to ferulate and hydroxycinnamate [43,44,69–71], then hsGGAX might be expected to have an idiosyncratic capacity to activate gl-OXO *in vitro*, and thereby, to contribute to order-of-magnitude variations in the OXO activities of different 'soluble' and 'insoluble' fractions of 'pre-isolated wheat embryos' (Tables 2, 5–7).

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