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Review Article

Germin, an Oxalate Oxidase, Has a Function in Many Aspects of Plant Life

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Abstract: Germin, a molecular marker of wheat embryo germination, is a remarkably protease-resistant, apoplastic, homopentameric glycoprotein. Different forms of germin found to be related to various aspects of plant development have been discovered. Germins were determined in cereals, dicotyledonous angiosperms, gymnosperms and in a prosit, all these proteins are together called germin-like proteins due to their DNA sequence homology. Cereal germins have oxalate oxidase activity, an activity which generates hydrogen peroxide by degrading oxalic acid. Both of these molecules have been implied in the regulation of cell development, differentiation, signalling and defence systems of plants. The germin-like proteins found in dicotyledonous plants do not appear to have oxalate oxidase activity.

Recently, germin-like proteins were shown to be related to seed storage proteins (vicilins, legumins), SBS (sucrose-binding proteins) and spherulins. On the basis of these similarities, it was proposed that all these proteins are members of a "superfamily" which are structurally related but functionally distinct proteins. Germin and germin-like proteins seem to be involved in the various important processes of plants including development, osmotic regulation, photoperiodic oscillation, defence and apoptosis.

Key Words: Plant development, cell wall, germin, germin-like proteins, oxalate oxidase.

Bir Okzalat Oksidaz Olan Germin Bitki Hayatının Pek Çok İşlevinde Rol Almaktadır

Özet: Buğday embriyo çimlenmesinin bir göstergesi olan germin; proteaz-dirençli, apoplastik, homopentamerik bir glikoproteindir. Bitki gelişiminin değişik işlevleri ile ilgili farklı germin formları mevcuttur. Tahıllarda, dikotiledonlarda, angiospermlerde ve protist de germin proteinleri bulunmuştur. DNA dizilimlerdeki berzerlikten dolayı bunların hepsi germin-benzeri proteinler olarak adlandırılmıştır. Tahıllarda bulunan germin, okzalat oksidaz aktivitesi gösterir ve bu aktivite okzalik asidi parçalayarak hidrojen peroksit üretir. Okzalik asit ve hidrojen peroksitin bitki hücresi gelişiminde, farklılaşmasında, sinyal iletiminde ve savunma sisteminde düzenleyici rollerinin olduğu belirtilmiştir. Dikotiledonlardaki germin-benzeri proteinlerin okzalat oksidaz aktivitesine sahip olmadıkları belirtilmiştir.

Yakın geçmişte, germin-benzeri proteinlerin tohum depo proteinleri (Vicilins, legumins), SBP (Sukroz taşıyıcı proteini) ve spherulins ile bağlantılı oldukları belirtilmiştir. Bu benzerlikten yola çıkarak, bu proteinlerin yapı olarak benzer fakat görev olarak farklı proteinleri içeren bir "Süperaile" üyesi oldukları ileri sürülmüştür. Germin ve germin-benzeri proteinlerin savunma, osmotik regülasyon, fotoperiyodik değişim, apoptosis ve gelişim dahil pek çok önemli bitki mekanizmasına katıldığı ileri sürülmüştür.

Anahtar Sözcükler: Bitki Gelişimi, hücre çeperi, germin, germin-benzeri proteinler, okzalat oksidaz.

Introduction

Plants are organisms which possess unique properties of reproduction, development, physiology and metabolism. During the early development of plants, the zygote which is a product of sexual reproduction turns into a mature embryo in a seed. When the seed is provided with ample water at an appropriate temperature, water is taken up by imbibition to initiate germination. Germination is a critical period in plant development in which the rapid growth of the embryo is driven by water uptake. The water content of a mature wheat embryo in an ungerminated grain is less than 5%; upon germination it rises to about 60% in less than 1 hour. Between 1 and 5 hours of imbibition there is no further increase in fresh mass or water content. A "secondary water uptake" phase then raises the water content from 60% to 85% by 24 hours postimbibition (1). Biochemical analysis of wheat embryo germination indicates that there is only a limited accumulation of new gene products during germination and until recently, just one had been observed to signal the onset of early plant development (2). The synthesis and translation of the mRNA for a soluble protein initially called "g" and later called "germin" (3) was concomitant with the initiation of growth in germinating wheat embryos (4). The appearance of germin mRNA coincided with the secondary water uptake phase and the accumulation of the germin protein reached its highest level 24-48 hours postimbibition (~40ng/embryo in 40 hour germinated embryos (5,6).

Germin was first detected in germinating cereals (5), but subsequently, germin- like proteins were also identified in protists (7), dicotyledonous angiosperms (8-11) and gymnosperms (12). Wheat germin is a relatively rare water-soluble glycoprotein (less than 0.1% of the mass of soluble proteins in germinating wheat embryos) which in homogenates exists as an oligomeric complex (~130 KDal) and does not dissociate when analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) unless boiled in the presence of a detergent (2,13).

Germin isoforms

Three isoforms of germin have been defined. These are G, G' and ΨG (pseudogermin). The G and G' oligomers are water soluble, resistant to digestion by pepsin and to dissociation in aqueous SDS solutions at room temperature (2,14). Amino acid composition and sequencing indicate that G and G' have the same apoprotein and differ only in that G has two further N-acetylglucosamine units attached to the basic core of its N-glycans. (15). Both forms of germin were defined as glycoproteins. The carbohydrate content of germin is about 10% by weight (15).

In addition to the germination related germin isoforms (G and G'), and antigenically related homotetrameric form of germin (pseudogermin-ΨG) has also been detected, particularly in the cell walls of immature wheat embryos at a time when the maximum cellular enlargement associated with embryogenesis when maturation is occurring (20-25 dpa). Unlike germin, pseudogermin is thermostable; the oligomer remains undissociated even when boiled in the presence of SDS, so long as non-reducing conditions e.g. in the absence of mercaptoethanol) are maintained (16).

A virtually full length germin cDNA was isolated (4) and its polynucleotide sequence was determined (17). The cDNA was used as a probe to indicate that germin is encoded by a multigene family which has ~5 copies on chromosome 4A, ~3 copies on chromosome 4B and 9 copies on chromosome 4D in hexaploid wheat. The probe was also used to screen a genomic wheat DNA library and the nucleotide sequences of a 2.8 kbp fragment (gf-2.8) from one genomic clone, and of a 3.8 kbp fragment (gf-3.8) from another clone were determined. The protein coding-regions of these two genes are intronless and 87% identical (7).

The role of germin in plant development

It was observed that even if germin was purified free of other proteins, there was a selective association between germin and the highly substituted glucuronogalactoarabinoxylans (HS-GGAX) (15) whose synthesis was reported to be closely associated with cell wall extension in cereals and grasses (18). Upon this observation, it was suggested that germin-like oxalate oxidase might play a crucial role in early plant development by controlling integration of cell wall extension, for example by transporting extending wall material (e.g. HS-GGAX) into the cell wall to support extension and at the same time promoting cross-linkage between wall polymers to restrict extension (19).

Isolation and examination of the two germin genomic clones (4) and the determination of the predicted amino acid sequences have revealed high homology with spherulin 1a/1b proteins of the slime mould *Physarum polycephalum* 57). The synthesis of these proteins occurs during spherulation: a transition leading to developmental arrest imposed by environmental conditions such as osmotic stress and plant nutrient deficiency (20). This similarity led to the suggestion that another possible function for germin might be related to teh changing osmotic properties of cells. In support of this notion, synthesis of germin-like proteins was discovered to be altered upon salt stress in barley (21) and in the halophytic "ice plant", *Mesembryanthemum crystallinum*, (8). In salt-stressed barley, these proteins, like wheat germin, were resistant to protease and were glycosylated and heat stable. They were detected in barley roots (but not in root tips) and coleoptiles but not leaves of 6 day old seedlings. Their synthesis increased in roots upont salt stress, but decreased in coleoptiles. On the other hand, it was observed that the synthesis of a germin-like protein in the ice plant declined after salt stress (8). Thus, these different studies implied that germin might represent a family of proteins of which individual members may have different biochemical functions related to changes in the osmotic properties of the cell.

In gymnosperms, three germin-like proteins were discovered among the extracellular proteins produced by cells grown in liquid tissue culture. These proteins were found to be present in cultures which retained embryogenic potential, but to be absent in non-embryogenic cell lines. These proteins had high N-terminal amino acid sequence homology with other germins and were immunologically cross-reactive with an antiserum raised against the *apo*-protein component of cereal germin. Their molecular weight was ~26 KDal (12).

Recently, photoperiodic treatments of the short-day plant (SDP) Pharbitis nil seedling resulted in synthesis of a germin-like protein during darkness-induced flowering. This germin-

like protein had a molecular mass of 22 KDal in SDS-PAGE analyses, and it reached the highest level of accumulation after the critical length of the dark period (~10 hours after the light was turned off). The cotyledons and leaves, known to be the two major organs that perceive the photoperiod and produce the floral stimulus, were the only expression sites for this protein. Sequence analysis showed that the *Pharbitis nil* germin-like protein shared the highest homology with a germin-like protein in another dicotyledonous plant, *Sinapus alba* (22).

Germin has oxalate oxidase activity

Since 1993 (23,24), it has been known that germin has an oxalate oxidase activity (EC 1.2.3.4), an activity which degrades oxalic acid into H_2O_2 and CO_2. The substrate of the enzyme, oxalic acid, is thought to have significant implications int he metabolism of animals, plants and fungi (25). The identification of cereal germins as oxalate oxidases instantly suggested several specific ways in which germin might function. Specifically, linkage of the developmental appearance of cell wall bound germin (16) to oxalate degradation suggests that germin might have a role in cell wall reinforcement by producing Ca^{++} and H_2O_2 for pectic cross-lingking and peroxidase mediated cros-linking of cell wall polymers respectively (26). Proteins could be cross-linked through tyrosine side-chains, lignin via -OH groups and carbohydrates via -COOH groups (27,28). The anchorage of at least a proportion of the germin-like oxalate oxidase in the cell wall with GGAX oligomers also implicates it in the cross-linking of these components within the cell wall matrix; notably, arabinoxylan comprises a substantial fraction of monocotyledonous cell walls, in which the oxalate oxidaes activity of germin-like proteins is characteristic. Although germin-like proteins have been identified in dicotyledonous plants, these, for the most part, have not been shown to possess oxalate oxidase activity. An exception to this is the oxalate oxidase of *Beta vulgaris*, which has been found to be germin-like in its properties (a protease-resistant SDS-stable oligomer): notably, the cell walls of this species, and other members of the *Chenopodiaceae*, are more "cereal-like" than "typically dicotyledonous", being enriched in arabinoxylan content (29). Germin synthesis also appears to be auxin responsive (26,30). Typically, auxins stimulate cell wall loosening and bring about cell wall expansion. On the other hand, germin (oxalate oxidase activity) produces hydrogen peroxide which is believed to be required for the peroxidase mediated cross-linking reactions in the cell wall. Thus, it was suggested that germin synthesis might be associated with both initiation and termination of cell wall expansion in early development (19).

Germin is a pathogen and stress inducible gene

Germin-like oxalate oxidase is a pathogen inducible enzyme (31-33). The production of H_2O_2 (the "oxidative burst") is the primary response of some higher plants to pathogen (*Erisyphe graminis*) infection. H_2O_2 can be implicated inthe development, differentiation, wvascularization, defence and signalling processes of higher plants. The action of oxalate oxidase, in generating H_2O_2, could be an especially otent defence mechanism. In a parallel study of transcripts induced in response to *Erisyphe* infection, two novel germin-like sequences were identified as pathogen-responsive (31).Dumas et al. (32) demonstrated an increase in the activity of germin-like oxalate oxidase in association with the response of barley to Erisyphe graminis. In normally growing seedlings, oxalate oxidase activity was detected in a tissue specific manner in seed germinated

for 3 days (in roots) and seedlings germinated for 10 days (in the residual coleorhiza). These activities were demonstrated to be associated with a germin-like protein by immunoblotting using anti-germin antibody. In 10 day old seedling coleoptiles a 26 KDal polypeptide reactiod with anti-germin serum but appeared to lack oxalate oxidase activity. This suggested that an inactive form of oxalate oxidase could accumulate. On infection by the fungus, oxalate oxidase was induced in barley leaves (after 5 days), especially along the vascular bundles. This suggested that oxalate oxidase belonged to a class of proteins that responds to pathogen attack. Similarly, in wheat, germin mRNA, germin and oxalate oxidase activity were induced in leaves of wheat upon infection with Erisyphe (6 day old seedlings + 2, 4, 6, 8 days after inoculation). The control leaves at the same age gave negative results. An increase in expression of peroxidase was also detected. These results reinforce the suggestions that germin activity has a role in plant defence through the local production of H_2O_2 for the hypersensitive defence response (33). This suggests that the genes encoding germin-like oxalate oxidase might have potential in transgenic approaches to plant defence. Another peroxide generating enzyme, a fungal glucose oxidase, has been shown to enhance the resistance of transformed plants to fungal infection, when introduced as a transgene (34). In experiments designed to protect *Brassica napus* plants from the oxalate secreting fungus, *Sclerotinia* transgenic oilseed rape plants, transformed with barley oxalate oxidase, were found to express as 25 a KDal protein reactive with anti-germin antiserum and to express oxalate oxidase activity which protected plants against potentially toxic applications of oxalic acid (35).

Experiments based on the extraction of mRNA from different organs suggest that wheat and barley have different spatial distributions of germin mRNA expression. The vascular transition region was reported to contain the highest levels of germin mRNA in wheat, whereas roots displayed the highest germin expression levels in barley seedlings. It was additionally observed that salt stress caused an increase in germin mRNA in roots at an early developmental stage (on the 3rd day in 0.2M NaCl), whereas, in he whole seedling, salt stress causes the "normal" expression of germin to be prolonged, relative to that in control seedlings (26). Overall, it was concluded that germin gene expression in barlye seedlings was developmentally regulated in a tissue-specific manner, and also, potentially, by various plant hormones (IAA, ABA). Interestingly, it was discovered that a decline in germin expression occurred after 3 days in control seedlings and after 4 days in stressed seedlings-in both case seedlings were at a similar developmental stage, as assessed by seedling weight (approximately 25 mg in each treatment). Thus, one interpretation of the experimental data is that NaCl treatment prolongs gene expression for an additional 1 day indirectly, through a slowing of seedling growth (36).

Germin is a member of superfamily

It was discovered that both germins and spherulins had statistically significant similarity with plant seed storage globulin domains. The germins were clearly related both to seed globulins (37) and spherulins (7). On the basis of these similarities, it has been proposed there is a superfamily (groups of gene families encoding structurally related but functionally distinct proteins) of related genes encoding vicilins, legumins, SBPs (sucrose-binding proteins), germins and spherulins. SBPs are proteins associated with the plasma membrane and have a role in

sucrose transport, and it is known that spherulins have a function in the cellular desiccation process, including osmotic regulation. It has been suggested that sucrose may serve as one of the principal agents in the acquisition of desiccation tolerance in seeds and other tissues, where the role of disaccharides in the assumption of a "glassy state" by the cytosol has been inferred (38). The legumin-like 11S and the vicilin-like 7S seed proteins are synthesized and accumulated during seed maturation, and are stored in protein bodies in mature seeds. In the timing of their accumulation, and their regulation by agents such as abscisic acid, they are at least associated with the acquisition of desiccation tolerance by seeds, which occurs during the maturation phase of seed development (39).

Initially, germins were defined as a marker of early plant development. Today, germin and germin-like proteins have been implied in numerous aspects of plant life including development, defence, signalling, differentation and apoptosis. Germins are under under extensive study in many laboratories to elucidate the mechanisms in which germins and germin-like oxalate oxidase act.

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