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An *alkaline α-galactosidase* transcript is present in maize seeds and cultured embryo cells, and accumulates during stress

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Abstract

Raffinose family oligosaccharides (RFO) accumulate in many developing seeds and are degraded during seed germination. However, acidic α-galactosidase (AGAL) activity and subcellular location do not correlate with raffinose depletion; alkaline α-galactosidases (AGA) may be responsible for RFO hydrolysis in germinating seeds. Three cDNA clones for *AGA/SEED IMBIBITION PROTEIN* were obtained from the Pioneer Hi-Bred maize expressed sequence database. Two of the clones were expressed in *Escherichia coli*, and the recombinant proteins, when incubated with naturally occurring galactosides or *p*-nitrophenyl α-D-galactose, exhibited AGA activity with maximum catalysis at pH 7.5 (ZmAGA1) or pH 8.5 (ZmAGA3). No raffinose biosynthetic capacity was observed with either enzyme. Maximal α-galactosidase activity in mature dehydrated, germinating and germinated maize (*Zea mays*) seeds occurred at pH 7.5. *ZmAGA1* was the sole family member detected in seeds and maize Hi-II, embryo-derived, callus cells. Its transcript accumulated when seed germination was interrupted by heat, cold or dehydration stress, but not in response to NaCl. Tissue prints localized transcripts to the scutellum or the embryo axis, depending on the stress applied. In maize Hi-II callus cells, transcripts accumulated when callus was subjected to heat stress (42 °C), during which *ZmAGA1* transcript accumulation was further induced by sucrose. Galactosides in a variety of forms, including raffinose, partially repressed the sucrose-induced accumulation of transcript in heat-stressed callus.

Keywords: alkaline α-galactosidase, germination, maize, raffinose family oligosaccharides, seed imbibition protein (SIP)

Introduction

The raffinose family oligosaccharides (RFOs) are a group of soluble galactosyl-sucrose carbohydrates thought to play a number of roles in plant development. In seeds, RFOs have been ascribed the following roles: (1) contributing to desiccation tolerance (Blackman *et al.*, 1992; Black *et al.*, 1996; Corbineau *et al.*, 2000; Bailly *et al.*, 2001); (2) increasing the longevity of seeds in the dry state (Koster, 1991; Sun and Leopold, 1993; Sun *et al.*, 1994; Bernal-Lugo and Leopold, 1998; Buitink *et al.*, 1998); and (3) providing a source of rapidly metabolizable carbohydrate during germination (Main *et al.*, 1983; Kuo *et al.*, 1988; Nichols *et al.*, 1993; Buckeridge and Dietrich, 1996; Dirk *et al.*, 1999).

Controversy surrounds the first (Hoekstra *et al.*, 1994; Ooms *et al.*, 1994) and second (Bentsink *et al.*, 2000; Buitink *et al.*, 2000; Gurusinghe and Bradford, 2001) proposed roles, potentially leaving RFOs without any function in seeds, other than as an energy source (Downie and Bewley, 2000). To serve as an energy source, RFOs must be degraded. The hydrolysis of the α-linked galactose moiety(ies) from RFOs in seeds has been attributed to α-galactosidase (AGAL; EC 3.2.1.22) functioning optimally at an acidic pH. However, AGAL activity at acidic pH is not correlated

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with RFO amounts in germinating seeds of soybean (Peterbauer *et al.*, 2003). Some AGAL isoforms present during seed development and germination are localized in protein storage vacuoles (Herman and Shannon, 1985; Bassel *et al.*, 2001; Feurtado *et al.*, 2001), while raffinose likely resides in the cytoplasm (Peterbauer and Richter, 2001).

Gao and Schaffer (1999) have isolated two forms of alkaline α-galactosidase (AGA) from melon (*Cucumis melo*) fruit. The substrate specificities of these enzymes are distinct; form II has a marked preference for stachyose and little activity against raffinose, while the opposite is the case for form I AGA. Further work led to the cloning of *AGA* cDNAs and discovery of their homology to *SEED IMBIBITION PROTEIN* (*SIP*), a group of cDNAs identified in seeds of several species, encoding proteins of a previously unknown function (Heck *et al.*, 1991; Carmi *et al.*, 2003). It is possible that homologues of the AGA enzymes reported by Schaffer's group are responsible for RFO hydrolysis during seed germination in maize, and hence, maize *SIP* expressed sequence tag (EST) clones were identified and sequenced. We report here that maize encodes functional AGA and that at least one *AGA* transcript is expressed during germination, particularly when the seeds are stressed.

Materials and methods

Plant material

Plants of maize (*Zea mays* L.) inbred line B73 were grown at the University of Kentucky Horticulture Experiment Farm in 2000. Developing seeds were harvested from ears that had been bagged prior to presentation of the silks, control-pollinated and tagged with the pollination date. At 12, 24, 36 and 50 d after pollination (DAP; Zhao *et al.*, 2004a), ears were harvested. Kernels were removed immediately from the cob and stored in liquid nitrogen until transported back to the lab, where they were stored at −80°C until use. Mature seeds were harvested from control-pollinated ears left on the plant until dry (*c.* 60 DAP). These seeds were used in germination assays and for stress treatments applied during germination (see below).

Reagents

Galactinol was purchased from Wako Chemicals (Wako BioProducts, Richmond, Virginia, USA). Media and chemicals were purchased from Sigma (St. Louis, Missouri, USA). Restriction enzymes were from New England Biolabs (Beverly, Massachusetts, USA) and radioisotopes were from New England Nuclear (NEN Life Science Products, Boston, Massachusetts, USA).

Germination conditions and treatments

Seeds (10 g) were rinsed with 70% (v/v) ethanol, washed in distilled water and sown on two 8.5 cm diameter blotting paper discs (Grade 628, Stults Scientific, Springfield, Illinois, USA) saturated with distilled, deionized water (15 ml) in a Petri dish. Dishes were placed at 25°C in the dark. Some seeds, initially sown on water at 25°C, were subsequently transferred for 24 h to colder (4°C) or warmer (42°C) temperatures; moved to dishes containing blotting papers saturated with salt solution (138 mM NaCl); or removed from water and placed on dry blotter paper in a germinator for 24 h at 25°C to dry. The moisture content of seeds dried for 24 h was 25% on a fresh weight basis. The first of the maize seeds to complete germination did so 48 h after imbibition (HAI; completion of germination is defined as that point when some portion of the embryo protrudes from the seed). At that time, only seeds that had not completed germination were selected for enzyme assay or Northern blot. At 60 and 72 HAI, only those seeds that had completed germination were assayed for enzyme activity.

Phylogenetic analysis of SIP proteins from dicots and monocots

Three putative *AGA* cDNAs (*ZmAGA1*, *ZmSIP2* and *ZmAGA3*, GenBank Accessions AF497510 through AF497512) were identified in the Pioneer Hi-Bred database. The deduced amino-acid sequences from the cDNAs were used, along with deduced amino-acid sequences of related proteins, to recapitulate the phylogenetic relationship among the various RFO hydrolytic and synthetic enzymes identified in Carmi *et al.* (2003). Including the three maize proteins, there were 12 additions to the 29 proteins used in Carmi's analysis: two SIPs from rice (*Oryza sativa*) that are putatively alkaline α-galactosidases [protein identification numbers (PID numbers) PID BAD72281, OsAGA1; and PID XP_483143, OsAGA2]; one confirmed rice AGA (PID XP_477103, OsAGA3; Lee *et al.*, 2004); a putative rice raffinose synthase (RAF; OsRAF1, EC 2.4.1.82; GenBank Accession NP_909442); a pea (*Pisum sativum*) RAF (PsRAF1; CAD20127); stachyose synthases (STS; EC 2.4.1.67) from artichoke (*Stachys sieboldii*; GenBank Accession CAC86963; SsSTS1) and from mask flower (*Alonsoa meridionalis*; GenBank Accession CAD31704; AmSTS1); an *Arabidopsis* SIP identified by Fujiki *et al.* (2001) AtSIP3; and a putative SIP from an archaeal

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source, *Sulfolobus solfataricus* (GenBank Accession AAK43227; SsAGA1).

These 41 full-length, deduced amino-acid sequences were aligned using ClustalW multiple-sequence alignment software (Thompson *et al.*, 1994). The Clustal aligned proteins were arranged in an unrooted phylogenetic tree using a beta version of PAUP v4.0b10 (Swofford, 1998). Accession numbers for sequences used to construct the tree are listed (see Fig. 1). Details of how the phylogenetic tree was determined are in Zhao *et al.* (2004a).

Hi-II callus production and manipulation

Maize Hi-II callus cultures were initiated according to Armstrong and Green (1985) from immature embryos isolated from Hi-II maize plants provided by Pioneer Hi-Bred. Cultures were incubated in the dark at 25°C on solid N6 medium, containing 2% (w/v) sucrose (Chu *et al.*, 1975) amended with 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 25 mM L-proline, and 10 μM $AgNO_3$, pH 5.8 (N6S2 medium) and sub-cultured every 2 weeks onto fresh media. Approximately 2 g of cells (fresh weight) in 100 mm diameter × 15 mm deep Petri dishes were used for each treatment. Callus was cultured on N6 media with various supplements and grown at 25 °C in the dark (control), exposed to 42 °C for 24 h (heated), exposed to 4 °C for 24 h (chilled) or exposed to a current of air at 25 °C in a Microvoid IIC laminar flow hood (Air Control, Huntingdon Valley, Pennsylvania, USA) for 4 h, at an average flow rate of 27 m min^{-1} in ambient light (dehydrated). Supplements included glucose, fructose, raffinose, mannitol, *myo*-inositol, galactose, or melibiose, or *cis*, *trans*-abscisic acid, alone or in combination. All supplements were sterilized by filtration and added to autoclaved media cooled to 65 °C.

To evaluate whether raffinose was present in maize callus, cells were grown on N6 media supplemented with 2 or 5% (w/v) sucrose, with or without 0.1% (w/v) raffinose. Cells were weighed and some were exposed to mild dehydration in a flow hood. Following treatment, the cells were harvested and sugars extracted from them (see below).

DNA and RNA isolation and analysis

Genomic DNA was isolated from maize seedling shoots according to Dellaporta *et al.* (1983). DNA (40 μg per lane) was digested overnight with restriction enzymes, electrophoresed through 0.8% (w/v) agarose in 1 × Tris–Borate–EDTA (TBE) (Sambrook *et al.*, 1989), and transferred to Hybond-N+ membrane (Amersham BioSciences, Piscataway, New Jersey, USA) by alkaline transfer (Reed and Mann, 1985). Membranes were cross-linked using UV-light (Bio-Rad GS Gene Linker, Bio-Rad Laboratories, Hercules, California, USA). They were prehybridized at 65 °C for 12 h in 6 × sodium phosphate–sodium chloride–EDTA (SSPE), 5 × Denhardt's solution (Denhardt, 1966), 0.5% (w/v) sodium dodecyl sulphate (SDS), and 100 μg ml^{-1} boiled sheared salmon sperm DNA, and hybridized with cDNA probes that were randomly labelled with [α-^{32}P]dCTP (deoxycytidine triphosphate) (Feinberg and Vogelstein, 1983). Hybridized membranes were first washed 2 × 15 min at low stringency [2 × sodium citrate–sodium chloride (SSC) , 0.1% (w/v) SDS at 65°C] and exposed to a phosphor screen for 2 d, and then re-washed at high stringency [0.2 × SSC, 0.1% (w/v) SDS, 65 °C 1 × 30 min] and re-exposed to the phosphor screen for 5 d. Images were captured using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, California, USA).

Total RNA was extracted according to Wan and Wilkins (1994). Northern blots were prepared by conventional methods (Sambrook *et al.*, 1989), using 20 μg RNA per lane and Hybond N+ membrane (Amersham). Blots were dried and UV cross-linked as above. They were rinsed for 5 min in 2 × SSC and prehybridized in a solution of 50% (v/v) formamide, 5 × Denhardt's solution, 100 μg ml^{-1} boiled, sheared salmon sperm DNA, 0.2% (w/v) SDS and 6 × SSPE (pH 7.0) (Sambrook *et al.*, 1989) for 6–12 h at 42 °C; blots were then hybridized with cDNA probes randomly labelled with [α-^{32}P]dCTP for at least 12 h at 42 °C. They were washed once in 2 × SSC, 0.1% (w/v) SDS, at 25 °C for 5 min; once at 65 °C for 30 min; and twice in 0.2 × SSC, 0.1% (w/v) SDS at 65 °C for 30 min. Images were recorded by autoradiography using Kodak X-OMAT X-ray film (Eastman-Kodak Ltd., Rochester, New York, USA) or by phosphor-imagery. Tissue prints were made of seeds bisected longitudinally, according to Zhao *et al.* (2004a).

Inverse PCR of ZmSIP2

Inverse polymerase chain reaction (iPCR) was used to obtain the 3′ end of *ZmSIP2*. Genomic DNA from *Bam* HI, *Hin*dIII and *Pst* I digests (50 μg per lane) was electrophoresed through 0.8% (w/v) agarose gels and stained with ethidium bromide. Gel portions known, from Southern blots, to contain *ZmSIP2*-hybridizing fragments were excised, and DNA was recovered using a Qiaquick kit (Qiagen, Valencia, California, USA). DNA was diluted to a range of concentrations, and 3 to 0.03 ng was self-ligated overnight and used as a template for iPCR. PCR reactions used 2 μM each of the *ZmSIP2*-specific primers (5′-GCATGAAGACCG-ACGTGCTCA-3′, forward; 5′-CATCCACCATAGCTT-GAATCG-3′, reverse), 1 μl template and 10 μl 2 × PCR mix (Sigma) in 20 μl total volume. Cycle conditions

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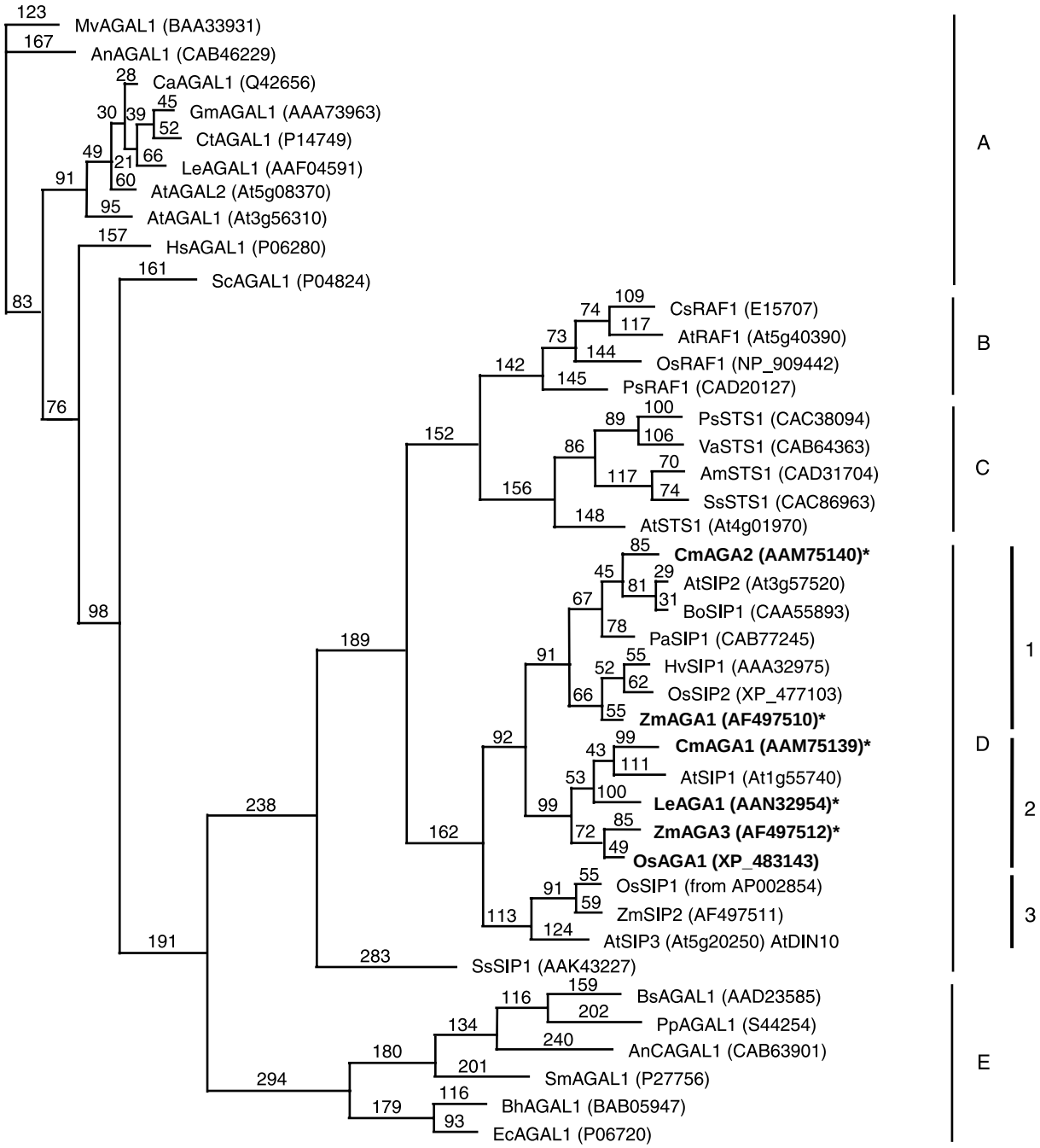

Figure 1. Full-length deduced amino-acid sequences for acid α-galactosidases (AGAL), raffinose synthases (RAFS), stachyose synthases (STS), and alkaline α-galactosidases/seed imbibition proteins (AGA/SIP), were subjected to Clustal analysis. The resulting alignments were used to construct a phylogenetic tree resolving five major clades. Clade A includes eukaryotic α-galactosidases (Family 27; Henrissat and Bairoch, 1993; Carmi *et al.*, 2003), clade B is raffinose synthases, clade C is stachyose synthases, clade D is alkaline α-galactosidases/seed imbibition proteins, and clade E is prokaryotic α-galactosidases (Family 36, Henrissat and Bairoch, 1993). The AGA/SIP clade D was sub-divided into three sub-clades. Consensus branch lengths in the phylogram are proportional to the number of inferred amino-acid changes. Those proteins presented in bold type possess alkaline α-galactosidase activity.

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were: 3 min at 94 °C; 10 × (30 s at 94 °C, 10 min at 65 °C, 4 min at 72°C); 30 × (30 s at 94°C, 30 s at 60°C, 4 min at 72°C); and 10 min at 72°C. When the ligation using 3 ng genomic DNA as a template was amplified, it yielded a 2 kb product. The sequence of this product did not include the stop codon. So new iPCR primers were synthesized to the 3′ end of this amplicon, and an additional round of iPCR was used to obtain a 650 bp product. When this amplicon was sequenced, it included the termination codon of the coding region, permitting the reconstitution of the entire *ZmSIP2* coding sequence.

Enzyme assay

The coding regions of *ZmAGA1* or *ZmAGA3* were cloned in-frame into pET43.1 (Novagen, Madison, Wisconsin, USA) and expressed heterologously in *Escherichia coli* strains BL21(DE3)RIL (Stratagene, La Jolla, California, USA) or BL21(DE3)Rosetta (Invitrogen, Carlsbad, California, USA) as amino-terminal N Utilization Substance (NUS)-fusions. An unrelated clone for an *Arabidopsis PROTEIN ISOASPARTYL METHYLTRANSFERASE2* (*PIMT2*, Xu *et al.*, 2004), also expressed as a NUS-fusion from pET43.1, was used as a control. One-litre cultures were grown at 37 °C to $OD_{600} = 0.4$, and then grown overnight at room temperature with (ZmAGA1, AtPIMT2) or without (ZmAGA3) 1 mM isopropyl β-D-thiogalactoside (IPTG) induction. [Preliminary experiments determined that withholding IPTG from cells expressing ZmAGA3 provided the greatest amount of soluble protein from leaky expression (data not shown).] Pelleted cells were resuspended in 10 ml 10 mM Tris, pH 7.5, lysed using a French press, and centrifuged at 18,000 *g* for 20 min at 4°C. The supernatant was collected, the presence of soluble recombinant protein confirmed using SDS-PAGE, and aliquots used in the assay. The assay for enzyme activity consisted of a 50 µl aliquot of sample, 50 µl 20 mM *p*-nitrophenyl-α-D-galactopyranoside (PNG) and 150 µl 50 mM triple buffer (pH from 5.0 to 10.5; triple buffer was made by combining equal volumes of 0.5 M stocks of CHES, HEPES and MES buffers, adjusting the pH with 10 N NaOH, and diluting with water to 10 × volume of any one stock buffer). The reaction was incubated 10 min at 37°C, stopped by addition of 2 ml 0.2 M Na_2CO_3, and the amount of product formed determined spectrophotometrically (Shimadzu UV-2101PC, Shimadzu, Tokyo, Japan) at 410 nm. The pH optimum of each enzyme was determined using triple buffer, spanning a pH range from 5 to 10.5. Additionally, recombinant proteins ZmAGA1, ZmAGA3 or AtPIMT2 were incubated for 2 h at 37 °C, pH 7.5 with 1 mM each of galactinol and sucrose, to determine if they possessed raffinose biosynthetic activity. Recombinant protein extracts were also incubated for 2 h with 1 mM raffinose, stachyose, melibiose or lactose. Thereafter, reaction mixtures were diluted with 10 volumes 80% (v/v) ethanol (EtOH), boiled for 5 min, centrifuged at 5000 *g*, the supernatant collected, diluted with 5 volumes water, frozen and lyophilized to dryness. Mixtures were reconstituted in water and aliquots analysed using high performance liquid chromatography with pulsed electrochemical detection (HPLC-PED) as in Zhao *et al.* (2004a).

In separate experiments, to assess raffinose entry into maize Hi-II callus cells, equal fresh weights of maize callus cells, or solid media from the Petri dishes on which the callus was grown, were extracted for sugars as above. Following processing and reconstitution in water, aliquots were diluted 100-fold, and component sugars separated using HPLC. Alternatively, 10 µl undiluted aliquots of samples and standard sugar solutions (1 mM) were spotted 2 cm from the bottom of dry silica gel 60 plates (Sigma), which had been run in resolving buffer (chloroform:methanol: acetic acid, 70:30:15 v/v/v; Haer, 1969) and dried prior to sample spotting. The extracts were developed for 4 h in resolving buffer, the plate dried in a fume hood and then placed back in the thin-layer chromatography (TLC) tank to develop for an additional 4 h. Plates were then dried, sprayed with orcinol ferric chloride (Bial's reagent) and dried, and stained sugars developed at 100 °C in a convection oven for 15 min.

Seeds or callus tissue for enzyme assay were pulverized in liquid nitrogen with a mortar and pestle, and homogenized in 1 ml ice-cold extraction buffer (50 mM potassium phosphate, pH 5.8). Following centrifugation at 10,000 *g* for 20 min, the supernatant was assayed for AGA activity directly, using a 50 µl aliquot of sample, 50 µl 20 mM PNG in water, and 150 µl 50 mM triple buffer (pH 5.5–8.5 in 1 pH unit increments). The reactions were incubated for 10 min at 37 °C, stopped by addition of 2 ml 0.2 M Na_2CO_3 and A_{410} values were converted to nmol *p*-nitrophenyl released, using the extinction coefficient for *p*-nitrophenyl of 18,300 and Beer's law. Protein concentrations in all samples were determined by the Bradford (1976) assay, with bovine serum albumin as a standard.

Statistics

All comparisons of the effect of stress or media supplement treatments on seed or callus AGA activity were performed using a general linear model (GLM; Statistical Analysis Systems, 1999). The effects of the treatments (hours after imbibition, stress type and pH) were tested, along with their interactions. If the GLM indicated significant differences among means,

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Tukey's mean separation test was used to distinguish among them at an experiment-wide error rate of $\alpha = 0.05$.

Results

Identification and sequence characterization of maize AGA *cDNAs*

Three putative *ALKALINE α-GALACTOSIDASE* (*AGA*) sequences were identified in the Pioneer Hi-Bred database. Full-length cDNA clones were obtained for *ZmAGA1* and *ZmAGA3*. *ZmSIP2* was 3′-truncated, and the full coding region was deduced from the sequence of this region of the gene obtained from two rounds of iPCR. The deduced ZmAGA1 amino-acid sequence was most similar to the SIP translated from the rice cDNA clone (AK065100), sharing 79% identity and 84% similarity. The ZmSIP2 deduced amino-acid sequence was most similar to that of the SIP translated from a *Triticum aestivum* cDNA (BT009384), sharing 76% identity and 83% similarity. ZmAGA3 was most similar to a *bona fide* AGA from rice (Lee *et al.*, 2004; XP_483143; also AAL65392); 79% identical and 88% similar.

A phylogenetic analysis (Carmi *et al.*, 2003) was capable of distinguishing among AGAs, raffinose- and stachyose-synthases (RAFs, STSs) and acidic α-galactosidases (AGALs). A recapitulation of this analysis, including nine additional proteins and the three full-length maize proteins, resolved five major clades (Fig. 1). Clade B is comprised of RAF, clade C of STS, clade D of AGA, clade E of microbial AGALs (Family 36 α-galactosidases; Henrissat and Bairoch, 1993), and clade A of eukaryotic AGAL (Family 27 α-galactosidases; Henrissat and Bairoch, 1993; Fig. 1). The maize AGAs are allied with the AGA/SIP proteins (clade D in Fig. 1) that Carmi *et al.* (2003) identified. The sub-clades within clade D are demarcated as D1, D2 and D3. ZmAGA1 is associated with sub-clade D1 containing CmAGA2, a form II AGA with greatest substrate affinity for stachyose and less affinity for raffinose (Gao and Schaffer, 1999; Carmi *et al.*, 2003). ZmAGA3 is most similar to proteins residing in sub-clade D2, including CmAGA1, a form I AGA with greater affinity for raffinose than stachyose (Gao and Schaffer, 1999). ZmSIP2 along with OsSIP1 from rice and AtSIP3 – a dark-induced, sucrose-repressed *Arabidopsis* transcript (Fujiki *et al.*, 2001; *D*ark *IN*duced 10) – forms sub-clade D3.

Recombinant ZmAGA1 (not shown) and ZmAGA3 (Fig. 2C) and a crude, soluble protein extract from 24-h-imbibed barley grains (Fig. 2D) were not capable of synthesizing raffinose from sucrose and galactinol. Instead, the two recombinant proteins hydrolysed galactinol to *myo*-inositol and galactose (Fig. 2C and data not shown). Barley caryopses also hydrolysed galactinol to *myo*-inositol and galactose, and contained considerable invertase activity (Fig. 2D). Galactinol, sucrose and raffinose were all stable in solution, and were not modified by bacterially derived protein or by the NUS-fusion protein at the amino terminus of pET43.1-derived proteins (Figs 2A, B, E, F). ZmAGA1 (data not shown) and ZmAGA3 were also capable of hydrolysing raffinose (Fig. 2G) and stachyose (data not shown) to galactose and sucrose, as was the 24-h-imbibed barley seed extract that also contained an invertase activity, resulting in melibiose, glucose and fructose accumulation rather than sucrose (Fig. 2H). ZmAGA1 had a pH optimum of 7.5, while ZmAGA3 had optimal activity at pH 8.5 (Fig. 2I).

The stringency with which Southern blots were washed did not influence the number of bands present for any of the *ZmAGAs*, and most bands were unique to a specific *ZmAGA* (Fig. 3). Hence, only images from blots washed at high stringency are presented (Fig. 3). Based on banding patterns, *ZmAGA1* and *ZmSIP2* appear to be single-copy genes, but *ZmAGA3* has at least one other, highly homologous gene residing in the genome. This possibility was explored by cloning multiple amplicons generated from genomic DNA from the maize B73 inbred line with primers scanning the *ZmAGA3* cDNA. A reconstruction of an approximately 1100 bp gene fragment, including five introns, revealed that there is a gene with very high homology to *ZmAGA3* present in the maize genome. The two genes differ only by four bases, scattered throughout the coding region. They are more readily distin-

Figure 2. Recombinant ZmAGA3 and an unrelated protein, protein isoaspartyl methyltransferase2, were produced in *E. coli* and tested, along with appropriate controls, for a raffinose synthase (A–D) or alkaline α-galactosidase activity (E–H). Buffer (A) and (E); crude bacterial lysate from a clone expressing the full coding region of protein isoaspartyl methyltransferase2 as an amino-terminal NUS-fusion (B) and (F); NUS-fused *ZmAGA1* (C) and (G); or a crude protein homogenate extracted from 24-h-imbibed barley seeds (D) and (H) were assayed using 100 μM naturally occurring sugars (e.g. raffinose). Retention time (minutes) is on each *x*-axis and detector response in μCoulombs is on the *y*-axis. Galol, galactinol; Suc, sucrose; *myo*-I, *myo*-inositol; Gal, galactose; Glc, glucose; Frc, fructose; Raf, raffinose; Meli, melibiose. Once an α-galactosidase activity was established, the pH optimum of the enzyme was determined (I) using *p*-nitrophenyl α-D-galactose as substrate. Activity was monitored spectrophotometrically at 410 nm.

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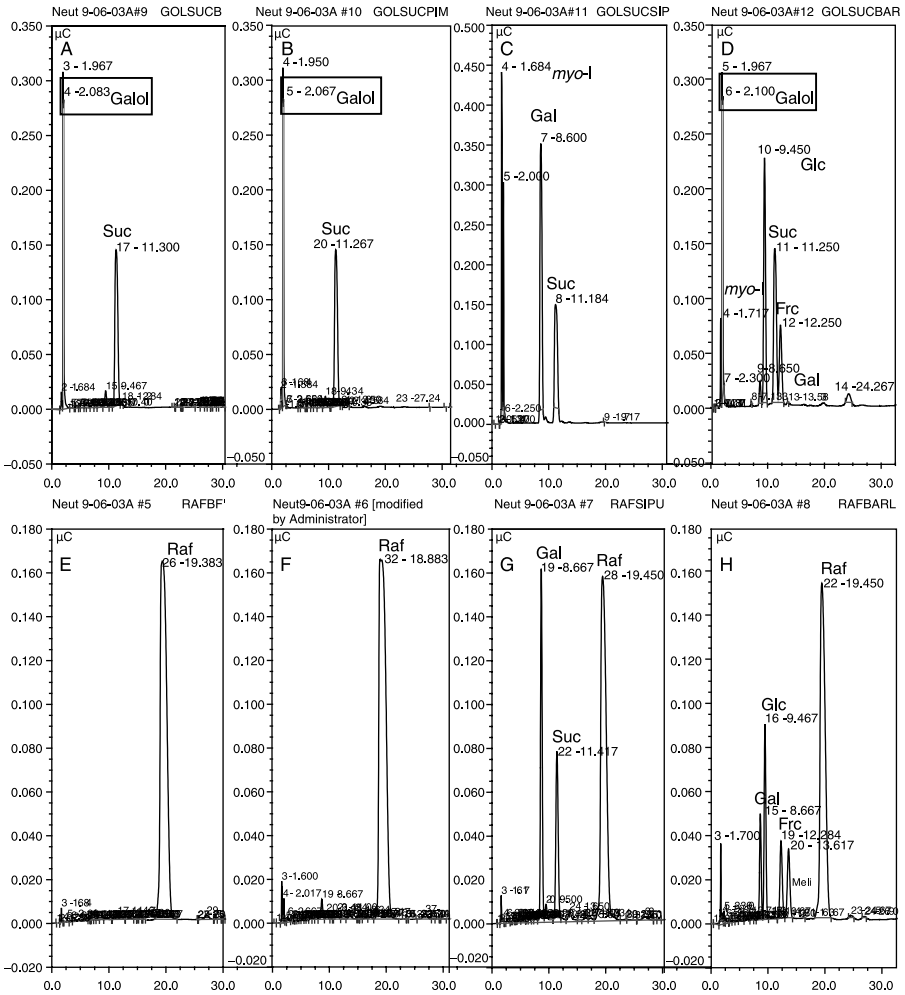

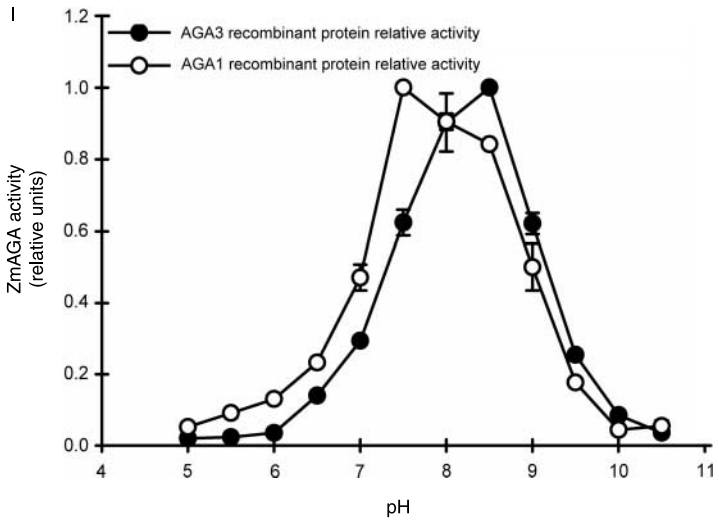

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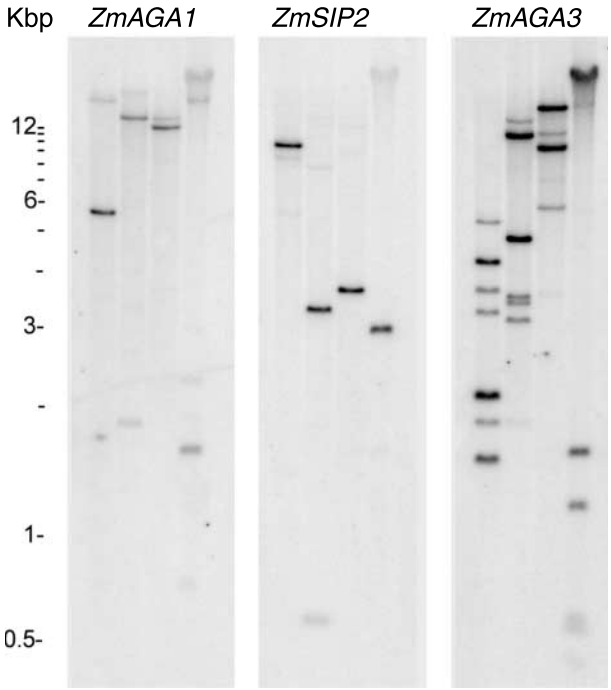

Figure 3. Southern blots of maize genomic DNA were probed with [α-^{32}P]dCTP-labelled *ZmAGA/SIP* cDNA clones and were developed by phosphorimagery. No differences were detected between blots washed at low or high (shown) stringency. Each lane contained 40 μg DNA cut with *Ase* I, *Bam* HI, *Hin*dIII or *Pst* I, respectively.

guished by multiple, contiguous-base inclusions/deletions occurring in the first, second and third introns present in the cloned fragments. Introns four and five are identical between the two genes. This portion of the gene encoding *ZmAGA3* has been given the GenBank accession number AY705440, and that of the homologue, AY705441.

ZmAGA1 *gene expression and total α-galactosidase activity in maize seeds*

Expression of *ZmSIP2* or *ZmAGA3* was not detected using Northern blots of seed RNA. *ZmAGA1* transcript amounts were low in developing seeds 12–50 DAP (data not shown) and in mature, dry seeds (Fig. 4A). Transcript abundance in seeds imbibed on water increased slightly at 24 and 36 HAI (Fig. 4A). All abiotic stresses imposed on germinating seeds at 24 HAI, with the exception of salt stress, increased *ZmAGA1* transcript amounts, particularly heat shock and dehydration (Fig. 4A). α-Galactosidase activity was not detected in developing seeds (data not shown), but was present in mature, dehydrated seeds (Fig. 4B). There was no interaction between the hours after imbibition and the pH of the assay. Activity was greatest at 36 and 60 HAI (Fig. 4B). Activity in dehydrated and germinating/germinated seeds was greatest at pH values of 6.5 and 7.5. Activity at pH 6.5 was statistically equal to those at pH 5.5 and 8.5 (Fig. 4B). Activity in 24-h-imbibed seeds, subsequently stressed for 24 h (48 HAI in total), was not significantly influenced by pH, and the interaction between pH and stress was also not significant. Activity was lowest for those seeds stressed at 42 °C for 24 h, while the other stress treatments were indistinguishable from each other (Fig. 4B). Tissue prints indicated that transcripts were present predominately in the scutellum at 48 HAI and in 24-h-imbibed seeds subsequently salt stressed for an additional 24 h, but in the axis after 24 h of heat shock that was applied from 24 to 48 HAI (Fig. 4C). Seeds subjected to dehydration stress were too dry to permit RNA transfer in tissue prints.

ZmAGA1 *gene expression and total α-galactosidase activity in callus tissue*

Other than an intriguing publication documenting the repression of an *Arabidopsis SIP* by sucrose (*DIN10*, a putative *AGA* based on phylogenetic analysis, see Fig. 1; Fujiki *et al.*, 2001), there are few published data on the potential biochemical regulation of *AGA* gene expression. To elucidate potential roles that carbohydrates exert on the regulation of transcription of *ZmAGA* genes, transcript amounts, enzyme activity and sugar quantities were examined in maize Hi-II callus cells grown on a variety of media with or without stress. Callus cells are a uniform cell assemblage able to take up exogenous carbohydrate. Information from these embryonic callus tissues may provide an insight into the regulation of *AGA* gene expression in more complex systems.

Total RNA was isolated from maize Hi-II callus subjected to various stress treatments and media supplements, and probed for *ZmAGA* transcript abundance (Fig. 5). As in seeds, *ZmAGA1* was the only *AGA* transcript detected in the embryonic callus cells regardless of treatment, but only heat shock stimulated a marked accumulation of this transcript (Fig. 5A), and some media supplements were more efficacious than others in enhancing this increase. Within a stress treatment, the inclusion of 5% (w/v) sucrose in the media resulted in more transcript than any other media supplement (Fig. 5A, lanes 1, 6, 11 and 16), including 7.8% (w/v) mannitol plus 2% (w/v) sucrose (Fig. 5A, lanes 5, 10, 15 and 20), which had a more negative osmotic potential.

Because of the efficacy of sucrose in stimulating *ZmAGA1* transcript accumulation, the effects of adding its component monosaccharides, glucose and fructose, to the media during heat stress at 42 °C were

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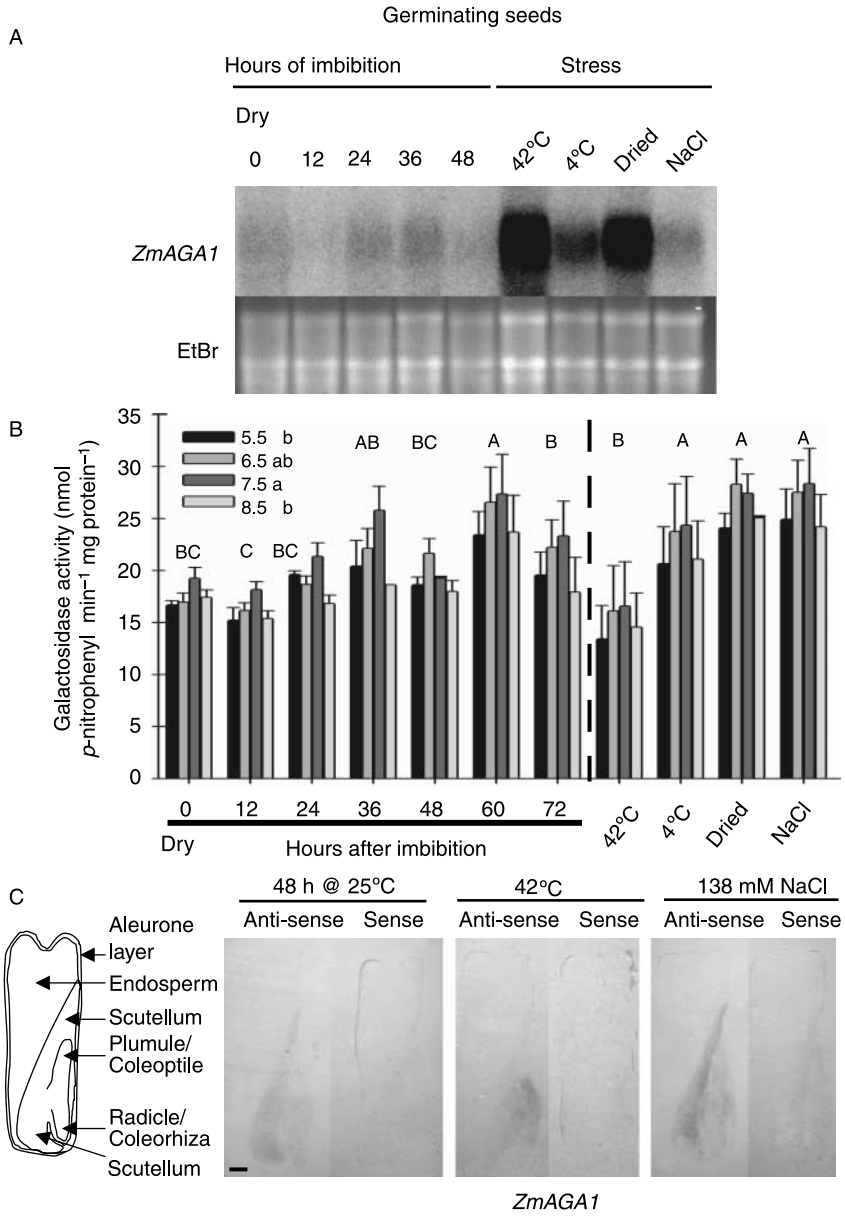

Figure 4. (A) Northern blots were used to monitor *ZmAGA1* transcript abundance during germination and stresses applied during germination. Stressed seeds were imbibed on water at 25°C for the first 24 h, and then transferred for the next 24 h to warmer (42°C) or colder (4°C) germination temperatures; removed from water and dried at 25°C to 25% moisture content; or moved to dishes containing blotting papers saturated with salt solution (138 mM NaCl). *ZmSIP2* and *ZmAGA3* transcripts were not detected. (B) α-Galactosidase activity, using *p*-nitrophenyl α-D-galactopyranoside as a substrate, was assessed at four different pH values in replicate samples of maize seeds during germination or stress during germination. There was no significant interaction between pH of the enzyme assay and the hours after imbibition (HAI) at which the seeds were sampled, or the stress during germination. α-Galactosidase activity did not vary significantly due to pH for seeds stressed during germination but, during the germination time course, the pH at which the assay was conducted was significant. Significant differences in the average activity at each pH over all HAI are depicted by different lower-case letters to the right of the pH legend. Significant differences in average activity due to imbibition time over all pH values are indicated by different upper-case letters over the bars. The heavy broken line between the bars for the germination time course and the stress during germination indicates that the two experiments were analysed separately. (C) Tissue prints determined the localization of *ZmAGA1* transcript in maize seeds during the later period of germination, or during heat or salt stress during germination. Tissue prints of bisected seed were probed with sense or antisense digoxigenin (DIG)-labelled probes for *ZmAGA1*. The bar in the first panel represents 1 mm. *ZmSIP2* and *ZmAGA3* transcripts were not detected in such tissue prints.

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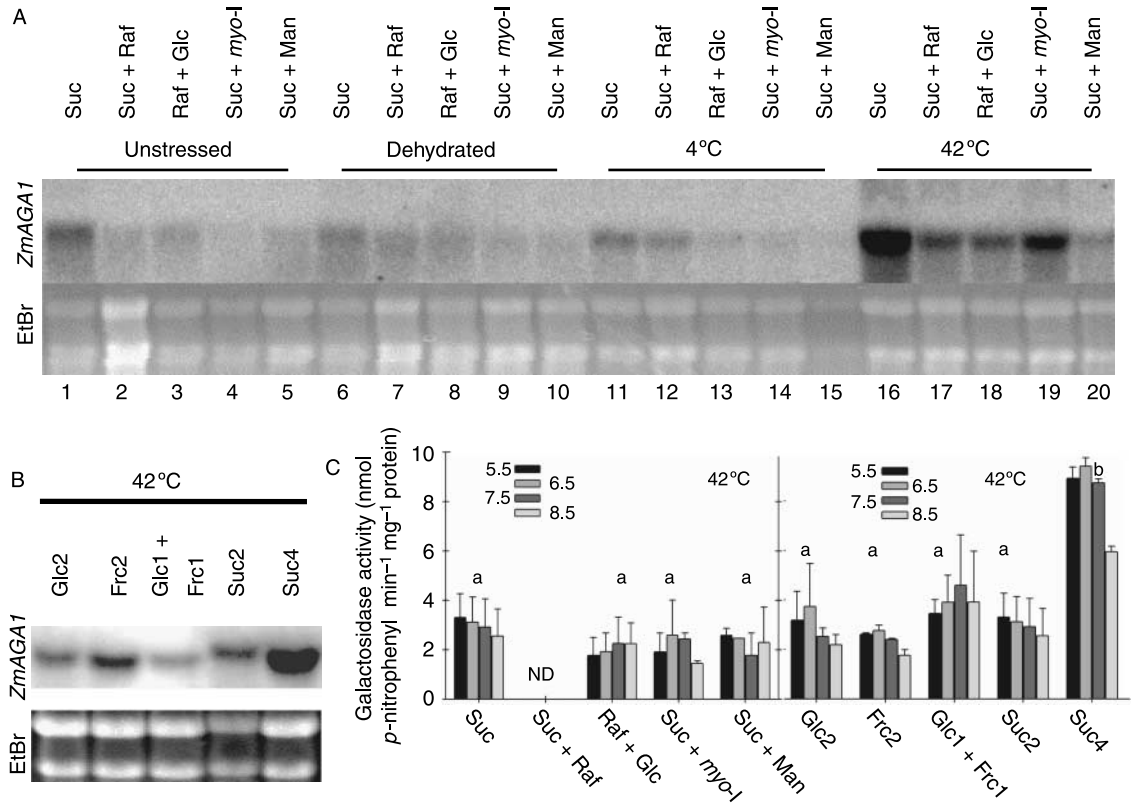

Figure 5. Northern blots of RNA (20 μg per lane) from embryonic callus cells were hybridized with [α-^{32}P]dCTP-labelled probes of *ZmAGA* cDNAs. Only the *ZmAGA1* transcript was detected. (A) Maize cells were subcultured from N6 media [2% (w/v) sucrose] to N6 media amended with: sucrose (Suc) [5% (w/v)]; sucrose and raffinose (Raf) [2% (w/v each)]; glucose (Glc) and raffinose [2% (w/v) each]; sucrose [2% (w/v)] and *myo*-inositol (*myo*-I) [0.01% (w/v)]; or sucrose [2% (w/v)] and mannitol (Man) [7.8% (w/v)]. After 9 d on these media, cells were harvested (lanes 1–5; Unstressed); or after 8 d 20 h, maize cells were dehydrated in a flow hood for 4 h (lanes 6–10; Dehydrated); or after 8 d, maize cells were moved to 4 °C (lanes 11–15) or 42°C (lanes 16–20) for 1 d. (B) Maize cells were grown on N6 media supplemented with glucose [2% (w/v)] (Glc2); fructose [2% (w/v)] (Frc2); glucose and fructose (Glc1 + Frc1) [1% (w/v) each], or [2% (w/v); Suc2] or [4% (w/v); Suc4] sucrose, and then stressed for 1 d at 42°C prior to harvest and RNA extraction. The osmotic potential of 2% (w/v) monosaccharides is equal to that of 4% (w/v) sucrose. (C) α-Galactosidase activity at four different pH values of maize callus tissue exposed to heat stress on a variety of media. The pH at which the assay was conducted was not significant, nor did it interact with the media composition. Significant differences in average enzyme activity over all pH values due to media composition are indicated by different lower-case letters over the bars. In some instances, the standard error bars are not evident at the scale depicted on the ordinate. ND, not determined. Ethidium bromide (EtBr)-stained gel images of rRNA bands are presented below the Northern blots.

tested (Fig. 5B). No remarkable differences in *ZmAGA1* transcript abundance were detected in callus grown on 2% (w/v) glucose, 2% (w/v) fructose, 1% (w/v) each of glucose and fructose, or 2% (w/v) sucrose. The abundance of transcript detected in callus grown on 4% (w/v) sucrose was not an osmotic effect, because concentrations of glucose and fructose at 2% (w/v) created the same osmotic potential as sucrose at 4% (w/v). Indeed, even media containing 2% (w/v) sucrose, which had half the osmotic strength of 1% (w/v) each of glucose and fructose, resulted in greater *ZmAGA1* accumulation (Fig. 5B). α-Galactosidase activity was not well-correlated with *ZmAGA1* transcript abundance, possibly excepting media compositions with greater sucrose amounts, which, at least in one experiment, had greater α-galactosidase activity (Fig. 5C). The pH at which the α-galactosidase assays of callus tissue were conducted had no significant influence on enzyme activity, and the interaction between pH and media composition was also not significant.

In a further test of the ability of sucrose to stimulate the accumulation of the *ZmAGA1* transcript, the amount of sucrose in the media was increased, while maintaining a similar medium osmotic potential. Neither 2% (w/v) fructose nor 2% (w/v) glucose was as effective at increasing the amount of *ZmAGA1* message in Hi-II callus as 2% (w/v) (half the osmotic

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potential) or 4% (w/v) (similar osmotic potential) sucrose (compare lanes 1 and 2 with lanes 5 and 6; Fig. 6). Media containing predominately glucose (lanes 3 and 4), including small amounts of sucrose, and generating similar (lane 3) or less (lane 4) osmoticum relative to 2% (w/v) glucose or fructose, resulted in similar or slightly greater *ZmAGA1* transcript amounts (Fig. 6). Again, substituting 2% (w/v) sucrose for 2% (w/v) glucose increased *ZmAGA1* transcript abundance, although media osmotic potential declined, and 4% (w/v) sucrose nearly doubled transcript abundance at an osmotic potential similar to that of 2% (w/v) glucose or fructose (Fig. 6).

The *ZmAGA1* transcript abundance in callus on 4% (w/v) sucrose tended to decrease if a source of galactose was also provided to the cells (raffinose, galactose or melibiose), but not if fructose was supplied (Fig. 6). With the exception of 0.1% (w/v) raffinose, as raffinose amounts (and osmotic potential) increased in the 4% (w/v) sucrose plates, *ZmAGA1* transcript abundance declined (Fig. 6, lanes 6–9). Using different galactosides at concentrations designed to hold the amount of galactose present equal to that in 1% (w/v) raffinose [0.33% (w/v)], a decline in *ZmAGA1* transcript abundance was observed, relative to 4% (w/v) sucrose, in all cases (Fig. 6, lanes 9–11). On the other hand, the amount of fructose represented in 1% (w/v) raffinose, when added to 4% (w/v) sucrose, had no effect on *ZmAGA1* transcript abundance relative to 4% (w/v) sucrose (Fig. 6, lane 12).

Because it was difficult to reconcile the fact that the AGA substrate raffinose, when added to 4% (w/v) sucrose media, tended to decrease *ZmAGA1* transcript abundance, it was necessary to test whether raffinose was accumulating in cells grown on raffinose-containing media. Sucrose concentration increased in the cells as the sucrose concentration of the media increased. However, using TLC analysis, the cells did not contain detectable amounts of raffinose, whether or not they were dehydration stressed (data not shown), although raffinose was detected in these cells using HPLC-PED (Zhao *et al.*, 2004b).

Discussion

AGA phylogeny

The AGA branch of the phylogenetic tree is divided into three distinct sub-clades, all of which contain a maize AGA/SIP. Sub-clade D1 (ZmAGA1) contains the CmAGA2 protein, a form II AGA with marked

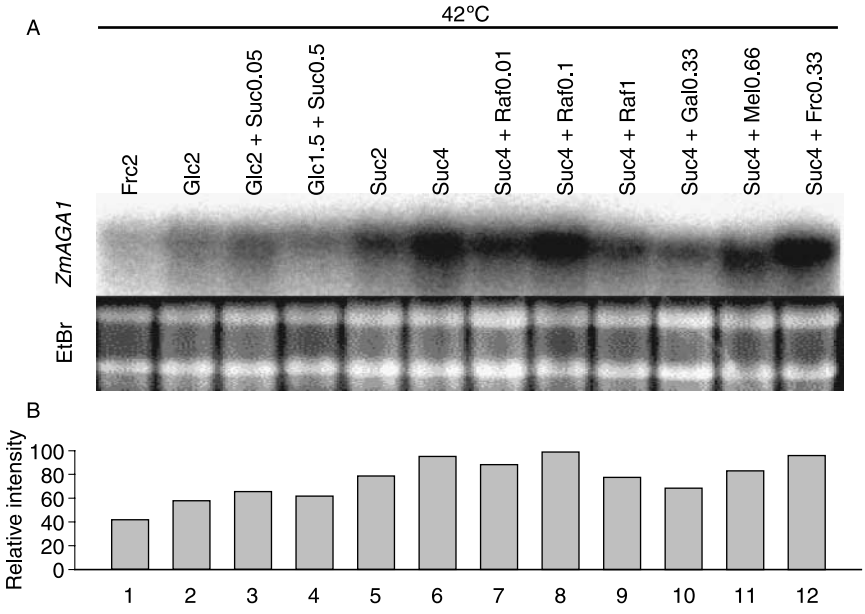

Figure 6. Maize cells were grown on N6 media supplemented with fructose [2% (w/v)] (Frc2); glucose [2% (w/v)] (Glc2); glucose and sucrose [1.5 or 2% (w/v) Glc; 0.05 or 0.5% (w/v) Suc]; sucrose [2 or 4% (w/v)]; 4% (w/v) sucrose with increasing amounts of raffinose [from 0.01 to 1% (w/v)] (Raf), galactose [0.33% (w/v)] (Gal), melibiose [0.66% (w/v)] (Mel) or fructose [0.33% (w/v)]. Galactose, melibiose and fructose amounts added to 4% (w/v) sucrose were chosen to represent the amount of each sugar present in 1% (w/v) raffinose. Cells were stressed for 1 d at 42 °C prior to harvest and RNA extraction. Northern blots of RNA (20 μg per lane) from callus cells were hybridized with [α-^{32}P]dCTP-labelled probes of *ZmAGA* cDNA. Ethidium bromide (EtBr)-stained gel images of rRNA bands are presented below the Northern blots.

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substrate specificity for stachyose. Sub-clade D2 (ZmAGA3) contains CmAGA1, a form I AGA with greatest affinity for raffinose and a rice AGA (OsAGA1) that is dark-induced and associated with leaf senescence by preferentially attacking the galactose moieties of digalactosyl diacylglycerol, an important component of the chloroplast thylakoid and envelope membrane lipids (Lee *et al.*, 2004). ZmSIP2 is assigned to sub-clade D3, which also contains an *Arabidopsis* AGA that is up-regulated in the dark and upon sucrose starvation (Fujiki *et al.*, 2000). A putative SIP from an archaeal source (AAK43227) is situated at a branch point on the tree between the AGAs and the RAF and STS proteins they resemble (Carmi *et al.*, 2003). Whether this protein is capable of raffinose or stachyose degradation/synthesis remains an intriguing possibility. Its position between highly similar plant enzymes, synthesizing or degrading members of the raffinose family oligosaccharides (RFOs), may provide insights into the evolution of the RFOs as important soluble carbohydrates in plants and the enzymes responsible for their metabolism.

Based on the activity of recombinant ZmAGA1 and ZmAGA3 with naturally occurring sugars (raffinose, etc.) and an artificial substrate (*p*-nitrophenyl α-D-galactopyranoside), their pH optima, the similarity of the ZmAGAs to other proteins with known AGA activity, the inclusion of the 'WWM(T/A)QR' motif [notably absent from RFO synthetic enzymes but present in CmAGA1 (Carmi *et al.*, 2003)] in the maize sequences, and their placement in the AGA/SIP phylogenetic group (Fig. 1; Carmi *et al.*, 2003), it is likely that all three maize cDNAs encode alkaline α-galactosidases, and a nomenclature has been chosen to reflect this. In addition, based on Southern blot analysis, the maize AGA family includes at least one other member highly homologous to *ZmAGA3*. Peterbauer *et al.* (2002) demonstrated that raffinose synthase, with considerable sequence homology to AGAs (Carmi *et al.*, 2003), possesses α-galactosidase activity. However, recombinant ZmAGA1 and ZmAGA3 clearly do not possess any raffinose synthetic capacity, but can cleave galactinol to *myo*-inositol and galactose.

ZmAGA1 *transcription in seeds*

Developing and desiccated kernels at 50 DAP and maturity, respectively, contain similar, low but detectable amounts of *ZmAGA1* transcript (Fig. 4A). A very slight increase in *ZmAGA1* transcript abundance was discernable 24 and 36 HAI in maize seeds (Fig. 4A). In previous work, Zhao *et al.* (2004a) found that 48 h imbibition on water at 25 °C was necessary for raffinose to decline to low amounts, which is in agreement with other accounts (Koster and Leopold, 1988). *ZmAGA1* transcripts also declined at 48 HAI (Fig. 4A). However, they did not increase substantially in germinating maize seeds without stress imposition during germination (stress was imposed 24 HAI in this study). The induction of *ZmAGA1* transcript accumulation in seeds stressed during germination was different from rice *OsAGA1* in vegetative tissues (sub-clade D2), which is not induced by heat, drought, salt or osmotic stress (Lee *et al.*, 2004).

Dehydration was the only stress that did not cause raffinose to decline below the amount present in the 24-h-imbibed seed (Zhao *et al.*, 2004a); if an active ZmAGA1 was produced from the transcript that accumulated due to dehydration (Fig. 4A), it (and any other galactoside-hydrolysing enzyme) must be incapable of hydrolysis in the increasingly dry seed environment. Certainly a detectable α-galactosidase enzyme activity was present in the seeds regardless of stress treatment (Fig. 4B). α-Galactosidase activity of any type (acid, neutral, alkaline) in germinating or stressed seeds was not well correlated with the amount of transcript present in the seeds (Figs 4A, B). However, *ZmAGA1* transcript abundance (but not enzyme activity) was well correlated with the amount of raffinose in seeds stressed during germination, with the exception of dehydrated seeds (compare Fig. 4A and Zhao *et al.*, 2004a).

In tissue prints of mature seeds during germination, or of those stressed during germination, *ZmAGA1* transcripts were detectable only in the maize embryo. Because of the small amount of tissue represented by the aleurone layer, it is impossible to determine if transcript was absent from, or undetectable in, the prints. However, results for dry and 24-h-imbibed barley embryos and endosperms (Carmi *et al.*, 2003) suggest that AGA is not synthesized in the aleurone layer.

The location of detectable transcript in the embryo was affected by the stress applied during germination. The shift in expression of *ZmAGA1* transcript from the scutellum of the germinating seed to the embryonic axis of the heat-stressed seed is perhaps of physiological significance (Fig. 4C). Cells of the embryonic axis are more sensitive to stress than cotyledon or aleurone layer cells (Koster and Leopold, 1988; Golovina *et al.*, 1997), perhaps due to mobilization of RFOs by the preferential accumulation of AGAs in the axis. The putative dual roles of raffinose in providing a rapidly metabolizable source of energy during germination (Kuo *et al.*, 1988; Downie and Bewley, 2000), as well as providing protection against a variety of stresses (Liu *et al.*, 1998; Taji *et al.*, 2002), may be regulated *in planta* through differential localization of transcription and hence, hydrolytic enzyme. However, in this study, transcript abundance and enzyme activity were not well correlated.

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Metabolite control of ZmAGA *transcription in heat-stressed callus cells*

Based on a prior report of metabolite control of *AtSIP3* expression (Fujiki *et al.*, 2001), an examination of metabolite control of *ZmAGA1* accumulation was undertaken using embryonic callus (represented by Hi-II cells). An increase occurred in *ZmAGA1* transcripts in undifferentiated heat-stressed callus cells due to increasing sucrose in the medium. This is in marked contrast to the repression of *AtSIP3* (presumably an *AGA*) by sucrose in *Arabidopsis* leaves (Fujiki *et al.*, 2001). The obvious conclusion, that the response of *AGA* transcription to sucrose behaves differently depending on whether the tissue is autotrophic (leaf) or not (callus), may not be valid, because the transcript is only stimulated in leaves in the dark (Fujiki *et al.*, 2000). Neither is the discrepancy due to differences in tissue type, because *Arabidopsis* cell cultures, like leaves, also exhibit a decrease in *AtSIP3* transcript in response to added sucrose (Fujiki *et al.*, 2000). The two proteins fall into distinctly different subclades (Fig. 1, clades D1 and D3), possibly indicating divergent roles in the cell. Unfortunately, *ZmSIP2* message was not detectable in maize seeds or Hi-II callus cells, regardless of treatment, when its cDNA was used as a probe (the ZmSIP2 protein is assigned to the same sub-clade as AtSIP3). The difference may be due to species because, while *Arabidopsis* transports RFOs, maize does not (Ohshima *et al.*, 1990; Weiner *et al.*, 1991), which may necessitate different transcriptional responses to supplied sucrose. Sucrose is both a product of AGA action on raffinose and a potent signalling molecule in its own right, exerting influence over the transcription of many genes associated with sugar metabolism (Chiou and Bush, 1998; Farrar *et al.*, 2000). Why sucrose should serve to stimulate transcription of *ZmAGA1* (or repress *AtSIP3*) is not evident, but the underlying conclusion is that *AGA* gene expression is attuned to the presence of carbohydrate in both species. It is also possible that heat stress, necessary for obvious *ZmAGA1* transcript accumulation, reverses sucrose repression.

Sucrose-stimulated *ZmAGA1* transcription in heat-stressed callus cells was somewhat antagonized by galactosides (Fig. 6A,B), most obviously by galactose, another product of the enzyme's action on RFOs. Galactose is also capable of decreasing the enzymatic activity of form II AGA (and form I to a lesser degree) from melon (Gao and Schaffer, 1999), and slightly decreasing activity of OsAGA1 from rice (Lee *et al.*, 2004). That galactose may also exert a limited, negative influence on *ZmAGA1* transcript accumulation is interesting, and has been shown previously for a gene from asparagus encoding β-galactosidase (Irving *et al.*, 2000). Why raffinose or melibiose, both substrates of ZmAGA, decreased *ZmAGA1* transcript accumulation is not obvious. However, most of the exogenous raffinose was apparently not entering the callus cells intact, or is hydrolysed shortly after it enters. One or more α-galactosidases presumably cleaved α-galactosides into galactose and a component sugar prior to, or just after, uptake. Considerably more galactose is detected by HPLC in heat-stressed callus cells on media including raffinose, than on media containing only sucrose (data not shown; Zhao *et al.*, 2004b). It is presumably the influx or generation of galactose by galactoside hydrolysis that repressed *ZmAGA1* transcript abundance in cells on galactoside-containing media. Nevertheless, galactose-induced repression of *ZmAGA1* accumulation was relatively minor. Additionally, the presence of both *ZmAGA1* transcript and α-galactosidase activity in heat-stressed callus cells correlated well with the absence of detectable raffinose from maize callus exposed to 42 °C, and the presence of detectable galactose from these same cells (Zhao *et al.*, 2004b). In contrast, *ZmAGA1* transcripts in dehydration-stressed maize callus were in lower abundance than in heat-stressed callus, while raffinose was present in these cells, and galactose is below the detection limit of HPLC-PED (Zhao *et al.*, 2004b). This indicated that maize callus produced raffinose in response to some, perhaps all, stresses, but that raffinose amounts may be controlled by hydrolytic activity. This conclusion is at odds with that of Peterbauer *et al.* (2003), who found no relationship between acidic α-galactosidase activity and RFOs in seeds of several pea lines that contain widely divergent amounts of RFOs. This discrepancy could be due to the difference in species or tissue type between their study and this one; however, whether the acidic α-galactosidases are solely responsible for RFO hydrolysis in any tissue is in question (Bassel *et al.*, 2001; this report).

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References

Armstrong, C.L. and Green, C.E. (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. *Planta* **164**, 207–214.

Bailly, C., Audigier, C., Ladonne, F., Wagner, M.H., Coste, F., Corbineau, F. and Côme, D. (2001) Changes in oligosaccharide content and antioxidant enzyme activities in developing bean seeds as related to acquisition of drying tolerance and seed quality. *Journal of Experimental Botany* **52**, 701–708.

Bassel, G.W., Mullen, R.T. and Bewley, J.D. (2001) α-Galactosidase is synthesized in tomato seeds during development and is localized in the protein storage vacuoles. *Canadian Journal of Botany* **79**, 1417–1424.

Bentsink, L., Alonso-Blanco, C., Vreugdenhil, D., Tesnier, K., Groot, S.P.C. and Koornneef, M. (2000) Genetic analysis of seed-soluble oligosaccharides in relation to seed storability of *Arabidopsis*. *Plant Physiology* **124**, 1595–1604.

Bernal-Lugo, I. and Leopold, A.C. (1998) The dynamics of seed mortality. *Journal of Experimental Botany* **49**, 1455–1461.

Black, M., Corbineau, F., Grzensik, M., Guy, P. and Côme, D. (1996) Carbohydrate metabolism in the developing and maturing wheat embryo in relation to its desiccation tolerance. *Journal of Experimental Botany* **47**, 161–169.

Blackman, S.A., Obendorf, R.L. and Leopold, A.C. (1992) Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. *Plant Physiology* **100**, 225–230.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Buckeridge, M.S. and Dietrich, S.M.C. (1996) Mobilisation of the raffinose family oligosaccharides and galactomannan in germinating seeds of *Sesbania marginata* Benth. (Leguminosae-Faboideae). *Plant Science* **117**, 33–43.

Buitink, J., Claessens, M.M.A.E., Hemminga, M.A. and Hoekstra, F.A. (1998) Influence of water content and temperature on molecular mobility and intracellular glasses in seed and pollen. *Plant Physiology* **118**, 531–541.

Buitink, J., Hemminga, M.A. and Hoekstra, F.A. (2000) Is there a role for oligosaccharides in seed longevity? An assessment of intracellular glass stability. *Plant Physiology* **122**, 1217–1224.

Carmi, N., Zhang, G., Petreikov, M., Gao, Z., Eyal, Y., Granot, D. and Schaffer, A.A. (2003) Cloning and functional expression of alkaline α-galactosidase from melon fruit: Similarity to plant SIP proteins uncovers a novel family of plant glycosyl hydrolases. *Plant Journal* **33**, 97–106.

Chiou, T.-J. and Bush, D.R. (1998) Sucrose is a signal molecule in assimilate partitioning. *Proceedings of the National Academy of Sciences, USA* **95**, 4784–4788.

Chu, C.C., Wang, C.C., Sun, C.S., Hsu, C., Yin, K.C., Chu, C.Y. and Bi, F.Y. (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Scientia Sinica* **18**, 659–668.

Corbineau, F., Picard, M.A., Fougereux, J.-A., Ladonne, F. and Côme, D. (2000) Effects of dehydration conditions on desiccation tolerance of developing pea seeds as related to oligosaccharide content and cell membrane properties. *Seed Science Research* **10**, 329–339.

Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter* **1**, 19–21.

Denhardt, D.T. (1966) A membrane-filter technique for the detection of complementary DNA. *Biochemical and Biophysical Research Communications* **23**, 641–646.

Dirk, L.M.A., van der Krol, A.R., Vreugdenhil, D., Hilhorst, H.W.M. and Bewley, J.D. (1999) Galactomannan, soluble sugar, and starch mobilization following germination of *Trigonella foenum-graecum* L. seeds. *Plant Physiology and Biochemistry* **37**, 41–50.

Downie, B. and Bewley, J.D. (2000) Soluble sugar content of white spruce (*Picea glauca*) seeds during and after germination. *Physiologia Plantarum* **110**, 1–12.

Farrar, J., Pollock, C. and Gallagher, J. (2000) Sucrose and the integration of metabolism in vascular plants. *Plant Science* **154**, 1–11.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13.

Feurtado, J.A., Banik, M. and Bewley, J.D. (2001) The cloning and characterization of α-galactosidase present during and following germination of tomato (*Lycopersicon esculentum* Mill.) seeds. *Journal of Experimental Botany* **52**, 1239–1249.

Fujiki, Y., Ito, M., Nishida, I. and Watanabe, A. (2000) Multiple signaling pathways in gene expression during sugar starvation. Pharmacological analysis of *din* gene expression in suspension-cultured cells of *Arabidopsis*. *Plant Physiology* **124**, 1139–1147.

Fujiki, Y., Yoshikawa, Y., Sato, T., Inada, N., Ito, M., Nishida, I. and Watanabe, A. (2001) Dark-inducible genes from *Arabidopsis thaliana* are associated with leaf senescence and repressed by sugars. *Physiologia Plantarum* **111**, 345–352.

Gao, Z. and Schaffer, A.A. (1999) A novel alkaline α-galactosidase from melon fruit with a substrate preference for raffinose. *Plant Physiology* **119**, 979–987.

Golovina, E.A., Tikhonov, A.N. and Hoekstra, F.A. (1997) An electron paramagnetic resonance spin-probe study of membrane-permeability changes with seed aging. *Plant Physiology* **114**, 383–389.

Gurusinghe, S. and Bradford, K.J. (2001) Galactosyl-sucrose oligosaccharides and potential longevity of primed seeds. *Seed Science Research* **11**, 121–133.

Haer, F.C. (1969) *An introduction to chromatography on impregnated glass fiber*. Ann Arbor, Michigan, Ann Arbor Science Publishers.

Heck, G.R, Dorsett, C., Ho, T.-H.D. (1991). Cloning and characterization of a gene, *Sip1*, associated with seed imbibition in barley. Accession M77475. GenBank Direct submission.

Henrissat, B. and Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **293**, 781–788.

Herman, E.M. and Shannon, L.M. (1985) Accumulation and subcellular localization of α-galactosidase-hemagglutinin in developing soybean cotyledons. *Plant Physiology* **77**, 886–890.

Hoekstra, F.A., Haigh, A.M., Tetteroo, F.A.A. and Van Roekel, T. (1994) Changes in soluble sugars in relation to desiccation tolerance in cauliflower seeds. *Seed Science Research* **4**, 143–147.

Irving, D.E., Shingleton, G.J., Hurst, P.L., Seelye, J.F. and Sinclair, B.K. (2000) Inhibition of hexokinase and expression of *asparagine synthetase* and *α-galactosidase* genes during sugar feeding and starvation of asparagus (*Asparagus officinalis*) callus cultures. *New Zealand Journal of Crop and Horticultural Science* **28**, 81–88.

Koster, K.L. (1991) Glass formation and desiccation tolerance in seeds. *Plant Physiology* **96**, 302–304.

Koster, K.L. and Leopold, A.C. (1988) Sugars and desiccation tolerance in seeds. *Plant Physiology* **88**, 829–832.

Kuo, T.M., Van Middlesworth, J.F. and Wolf, W.J. (1988) Content of raffinose oligosaccharides and sucrose in various plant seeds. *Journal of Agricultural and Food Chemistry* **36**, 32–36.

Lee, R.-H., Lin, M.-C. and Chen, S.-C.G. (2004) A novel alkaline α-galactosidase gene is involved in rice leaf senescence. *Plant Molecular Biology* **55**, 281–295.

Liu, J.J., Krenz, D.C., Galvez, A.F. and de Lumen, B.O. (1998) Galactinol synthase (GS): increased enzyme activity and levels of mRNA due to cold and desiccation. *Plant Science* **134**, 11–20.

Main, E.L., Pharr, D.M., Huber, S.C. and Moreland, D.E. (1983) Control of galactosyl-sugar metabolism in relation to rate of germination. *Physiologia Plantarum* **59**, 387–392.

Nichols, M.B., Bancal, M.-O., Foley, M.E. and Volenec, J.J. (1993) Non-structural carbohydrates in dormant and afterripened wild oat caryopses. *Physiologia Plantarum* **88**, 221–228.

Ohshima, T., Hayashi, H. and Chino, M. (1990) Collection and chemical composition of pure phloem sap from *Zea mays* L. *Plant and Cell Physiology* **31**, 735–737.

Ooms, J.J.J., Wilmer, J.A. and Karssen, C.M. (1994) Carbohydrates are not the sole factor determining desiccation tolerance in seeds of *Arabidopsis thaliana*. *Physiologia Plantarum* **90**, 431–436.

Peterbauer, T. and Richter, A. (2001) Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. *Seed Science Research* **11**, 185–197.

Peterbauer, T., Mach, L., Mucha, J. and Richter, A. (2002) Functional expression of a cDNA encoding pea (*Pisum sativum* L.) raffinose synthase, partial purification of the enzyme from maturing seeds, and steady-state kinetic analysis of raffinose synthesis. *Planta* **215**, 839–846.

Peterbauer, T., Karner, U., Mucha, J., Mach, L., Jones, D.A., Hedley, C.L. and Richter, A. (2003) Enzymatic control of the accumulation of verbascose in pea seeds. *Plant, Cell and Environment* **26**, 1385–1391.

Reed, K.C. and Mann, D.A. (1985) Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Research* **13**, 7207–7221.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning. A laboratory manual* (2nd edition). Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.

Statistical Analysis Systems (SAS) (1999) *Statistical analysis systems Version 8*. Cary, North Carolina, SAS Institute Inc.

Sun, W.Q. and Leopold, A.C. (1993) The glassy state and accelerated aging of soybeans. *Physiologia Plantarum* **89**, 767–774.

Sun, W.Q., Irving, T.C. and Leopold, A.C. (1994) The role of sugar, vitrification and membrane phase transition in seed desiccation tolerance. *Physiologia Plantarum* **90**, 621–628.

Swofford, D.L. (1998) *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*. Version 4. Sunderland, Massachusetts, Sinauer Associates.

Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant Journal* **29**, 417–426.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.

Wan, C.-Y. and Wilkins, T.A. (1994) A modified hot borate method significantly enhances the yield of high quality RNA from cotton (*Gossypium hirsutum* L.). *Analytical Biochemistry* **223**, 7–12.

Weiner, H., Blechschmidt-Schneider, S., Mohme, H., Eschrich, W. and Heldt, H.W. (1991) Phloem transport of amino acids. Comparison of amino acid contents of maize leaves and of the sieve tube exudate. *Plant Physiology and Biochemistry* **29**, 19–23.

Xu, Q., Belcastro, M.P., Villa, S.T., Dinkins, R.D., Clarke, S.G. and Downie, A.B. (2004) A second protein L-isoaspartyl methyltransferase gene in *Arabidopsis* produces two transcripts whose products are sequestered in the nucleus. *Plant Physiology* **136**, 2652–2664.

Zhao, T.-Y., Thacker, R., Corum, J.W., Snyder, J.C., Meeley, R.B., Obendorf, R.L. and Downie, B. (2004a) Expression of the maize *GALACTINOL SYNTHASE* gene family: (I) Expression of two different genes during seed development and germination. *Physiologia Plantarum* **121**, 634–646.

Zhao, T.-Y., Martin, D., Meeley, R.B. and Downie, B. (2004b) Expression of the maize *GALACTINOL SYNTHASE* gene family: (II) Kernel abscission, environmental stress and myo-inositol influences accumulation of transcript in developing seeds and callus cells. *Physiologia Plantarum* **121**, 647–655.

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