Journal of Experimental Botany, Vol. 54, No. 390, pp. 2071–2079, September 2003
DOI: 10.1093/jxb/erg233

Journal of Experimental Botany

RESEARCH PAPER

Expression analysis of a GA 20-oxidase in embryos from two sorghum lines with contrasting dormancy: possible participation of this gene in the hormonal control of germination

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Received 14 January 2003; Accepted 29 May 2003

Abstract

The role of GAs in promoting seed germination is well known and experiments with seeds from different species have suggested the requirement of *de novo* synthesis of GAs upon imbibition for germination. There are also strong indications that the enhancement of GA synthesis is part of the mechanism through which environmental signals (i.e. light) induce germination. Since along the GA biosynthetic pathway, oxidation at C-20 carried out by GA 20-oxidases is thought to be a site of regulation, a cDNA clone encoding a GA 20-oxidase was isolated from embryos of sorghum (*SbGA 20ox*). Expression analysis of this gene in embryos within imbibed caryopses with low dormancy showed detectable amounts of the specific mRNA early upon incubation, increasing thereafter. In contrast, it remained barely detectable in embryos from dormant caryopses. Changes in endogenous GA_4 levels were in agreement with those of *SbGA 20ox* mRNA, suggesting that GA production might be regulated differentially at the level of transcription of this gene. The expression of *SbGA 20ox* was enhanced in incubated embryos isolated from either type of caryopses, illustrating a physiological control exerted by the surrounding seed tissues on gene expression. The results also show that ABA leads to a suppression of transcription of this gene.

Key words: Dormancy, GA 20-oxidase, germination, gibberellins, *Sorghum*.

Introduction

The central role of gibberellins (GAs) in promoting seed germination was suggested decades ago and clearly confirmed by the identification of GA-deficient mutants of *Arabidopsis* and tomato seeds which will not germinate unless exogenously supplied with GAs (Koornneef and van der Veen, 1980; Groot and Karssen, 1987). It has been proposed that endogenous GAs control germination through two processes: (i) a decrease in the mechanical

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resistance of the tissues surrounding the embryo (Groot and Karssen, 1987) and (ii) promotion of the growth potential of the embryo (Carpita *et al*., 1979; Karssen *et al*., 1989). In seeds where the tissues covering the embryo are weak or split during imbibition, this increase in embryo growth potential may be the only process required for the completion of germination (Schopfer and Plachy, 1985). The fact that inhibitors of GA biosynthesis, such as paclobutrazol and tetcyclacis, prevent germination in some species (Karssen *et al*., 1989; Nambara *et al*., 1991) suggests the requirement of *de novo* synthesis of GAs for germination upon imbibition in at least such species. Moreover, it has been shown for light-requiring lettuce and *Arabidopsis* seeds that the enhancement of GA biosynthesis and changes in sensitivity to GAs are part of the light stimulus of germination (Yang *et al*., 1995; Toyomasu *et al*., 1998; Yamaguchi *et al*., 1998). In the latter species, the genes *GA4* and *GA4H* encoding two 3β-hydroxylases, the enzymes committed in the last step of the biosynthesis of 'active' GAs, are induced by red light through phytochrome.

Developing cereal grains (i.e. wheat, sorghum, barley) from genotypes with differential pre-harvest sprouting behaviour usually have a contrasting degree of dormancy at a particular stage of their development (i.e. at physiological maturity sprouting-susceptible genotypes will germinate, whereas sprouting-resistant will not) (Walker-Simmons, 1987; Steinbach *et al*., 1995). In most cases this different expression of dormancy has been associated with a different embryo sensitivity to the germination-inhibitor abscisic acid (ABA), which is expected to be present in relatively large amounts in immature grains (i.e. embryos from genotypes with low dormancy have low sensitivity to ABA and vice versa) (Walker-Simmons, 1987; Steinbach *et al*., 1995; Benech-Arnold *et al*., 1999). However, it is not clear if this different dormancy level is also expressed through a differential capacity for GA biosynthesis upon grain imbibition. On the basis of the above-mentioned evidence supporting the *de novo* GA synthesis as a requirement for germination, it might be hypothesized that, in addition to the existence of other constraints, dormant grains will not germinate because their capacity to produce GAs *de novo* is blocked. By contrast, the *de novo* synthesis of bioactive GAs should proceed normally during the early stages of imbibition in non-dormant grains. One piece of evidence supporting this hypothesis is the fact that dormant sorghum seeds can be induced to germinate through exogenously supplied GA_{4+7} (Steinbach *et al*., 1997).

Gibberellin biosynthetic pathways are usually dissected into three sequential stages according to the nature of the enzymes involved (Lange, 1998). The last stages involve oxidation and elimination of C-20 to yield the C19-GAs, which include the biologically active plant hormones. This step is catalysed by a GA 20-oxidase activity, thought to be a site of regulation (Lange *et al*., 1994; Phillips *et al*., 1995; Lange, 1998; Yamaguchi and Kamiya, 2000; Eriksson and Moritz, 2002). Recently, a mutation in the gene encoding this enzyme was recognized as the cause of the phenotype displayed by high-yielding semi-dwarf rice varieties (the so called 'green revolution' rice) (Sasaki *et al*., 2002).

There is evidence of a feedback functional control by bioactive GAs on levels of GA biosynthetic intermediates (Hedden and Croker, 1992) or on GA 20-oxidase transcript levels (Xu *et al*., 1999). Although recent reports have demonstrated the control exerted by brassinosteroids and auxins on GA 20-oxidase gene expression (Bouquin *et al*., 2001; Ngo *et al*., 2002), the interaction of other hormones with this pathway has not been explored so far and even less in relation to the control of the expression of dormancy. If germination of dormant seeds is indeed restricted through a blockade in GA biosynthesis, one might expect a differential control of these genes at, for example, the transcriptional level. According to the above-mentioned evidence, genes encoding for GA 20-oxidases are attractive targets for expression analysis during imbibition of dormant and non-dormant grains.

In this paper, the isolation of a cDNA clone homologous to GA 20-oxidases [gibberellin, 2-oxoglutarate:oxygen oxidoreductase (20-hydroxylating, oxidizing) EC 1.14.11.-] from sorghum embryos is reported. Expression analysis suggests that the observed GA production in dormant and non-dormant sorghum grains might be regulated differentially at the level of transcription of this gene. Moreover, the first evidence showing that ABA suppresses transcription of this gene is presented.

Materials and methods

Plant material

Sorghum (*Sorghum bicolor* (L.) Moench.) lines IS9530 (sprouting-resistant, high dormancy) and Redland B2 (sprouting-susceptible, low dormancy) were used for the experiments (Steinbach *et al*., 1995). Both inbred lines were sown on the experimental field of the Facultad de Agronomía, Buenos Aires, Argentina, in the first week of December 1997, 1998 and 2001. The two lines were sown in three completely randomized blocks and the density was 8–12 plants m^{-2}. The crop was irrigated when necessary to avoid water stress and fertilized with urea at 250–300 kg ha^{-1}. Each panicle was individually labelled with its flowering date, defined as the day on which pollen had been released in approximately two-thirds of the florets composing the panicle. Flowering started about 64–68 d after sowing for the two lines and was completed 8 d later. To minimize variation, only caryopses coming from the middle third of the panicle were used. Three plants from each line and from each block were harvested on each sampling date. At harvest maturity, seeds were stored at 4 °C.

RT-PCR amplification of Sorghum bicolor *GA 20-oxidase (*SbGA 20ox*) transcript*

Total RNA was extracted from embryos derived from 40 DAP (days after pollination) caryopses by means of the Trizol (Gibco BRL, MD, USA) procedure followed by DNAase treatment. 2.5 μg RNA was converted into cDNA by 300 units M-MLV reverse transcriptase

(Promega, WI, USA) in a 20 µl reaction containing 50 mM TRIS-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 20 U RNAsin (Promega, WI, USA), 400 µM dNTPs, and 3.3 mM polydT(15) (Promega, WI, USA) at 35 °C for 1 h. The reaction was stopped by heat inactivation at 94 °C for 5 min. PCR amplification of *SbGA 20ox* cDNA was carried out at 94 °C for 1 min followed by a varying number of cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min with 0.4 µM of the following primers designed on the basis of published GA 20-oxidase gene from rice (GenBank accession number U50333): upper primer 5′-CTTCGCGTCCAAGCTGCCG-TGG-3′ (which anneals to 538–559 nt positions) and lower primer 5′-GAGAGCGCCATGAAGGTGTCGCCG-3′ (which anneals to 985–1008 nt positions). The length of the expected amplification fragment in rice was 471 bp.

Primers specific for the rice sequence were chosen because that was the species most related to sorghum for which, at the time of beginning this work, there was a GA 20-oxidase sequence available.

Cloning and sequencing of SbGA 20ox *cDNA*

PCR products were electhophoresed in 2% agarose gels. A single amplification band of approximately 500 bp was excised from the gels and then cloned into pBluescript KS^+ vector. Two independent clones were sequenced at the Cornell University DNA sequencing facility. The sequence of *SbGA 20ox* cDNA has been deposited in GenBank (accession number AF249881). The sequences alignments were performed online using the Multalin program (Corpet, 1988).

Southern blot analysis

Genomic DNA (10 µg) of IS9530 and Redland B2 were isolated and Southern blotted as described by Hoisington *et al*. (1994). The blot was hybridized with *SbGA 20ox* amplification fragment labelled with $\alpha[^{32}P]$ by random primer extension (Prime-a-gene kit, Promega, WI, USA). The filter was washed once for 20 min at 65 °C in 2× SSC, 0.1% SDS, once for 20 min at 65 °C in 1× SSC, 0.1% SDS, and finally for 30 min at 65 °C in 0.1× SSC, 0.1% SDS. After washing, the filter was exposed to X-ray film (XOMAT, Kodak, USA) for 3–5 d.

Expression analysis of SbGA 20ox *by quantitative RT-PCR*

Expression analysis of *SbGA 20ox* was carried out with the following material: (i) IS9530 and Redland B2 embryos excised from 40 DAP caryopses that had been incubated for 0, 18, 40, 72, and 96 h; (ii) isolated embryos dissected from 35 DAP caryopses and then incubated in distilled water or 50 µM ABA (Sigma Co., MO, USA) for 0, 0.5, 2, 4, 6, 8, and 16 h. In all cases about 200 mg of embryos were used for RNA extraction. Between 0.8 and 2.5 µg were used for RT-PCR reactions. To detect changes in levels of cDNA present before amplification, the number of cycles was calibrated in order to ensure linearity of output (amplification product) in relation to input (template amounts). All RT-PCR data obtained for GA 20ox were normalized against those for actin transcript. RT-PCR was repeated at least five times in every replicated (three to five times) physiological experiment.

The band intensity of the PCR products was analysed by the NIH-image program. In this way the optimum cycle number was determined for each experiment. Amplification of the actin sequence was used as a control and was performed in a similar way except for the annealing temperature (55 °C) with the following primers: upper primer 5′-TGGCATCATACCTTTTACAA-3′ and lower primer 5′-TCCGGGCATCTGAACCTCTC-3′.

Germination assays and sampling

To assess for seed germinability at different stages of development, grains from both lines were harvested at 23, 30, 37, 44, 51, and 60 DAP and incubated in Petri dishes with 6 ml of distilled water at 25°C. Germination was counted daily along a 12 d period.

For most of the experiments carried out in this study, caryopses at 40 DAP were used. In all experiments, caryopses were incubated in Petri dishes with 6 ml distilled water at 25 °C for different periods and germination percentages were scored daily.

GA extraction, purification and quantification

Gibberellin analysis was carried out with IS9530 and Redland B2 grains (40 DAP) from plants sown in 2001 and harvested in 2002. Intact caryopses were incubated in distilled water for various times. After each incubation period, two samples from 100 embryos from grains of each variety were excised and frozen in liquid nitrogen. The samples were freeze-dried, powdered and GA extraction performed for 2 h with methanol:water:acetic acid (79:20:1, by vol.). Twenty ng of each one of stable-isotope labelled [17,17-$^2H_2$]GA_1, [17,17-$^2H_2$]GA_3 and [17,17-$^2H_2$]GA_4 (L Mander, Australian National University, Canberra, Australia) were added as internal standards and allowed 30 min for isotope equilibration. After filtration and methanol evaporation under reduced atmospheric pressure, the sample was adjusted to pH 8.0 and partitioned once with equal volume of petroleum ether. The aqueous phase was then partitioned three times at pH 3.0 with water-saturated (1% acetic acid) ethyl acetate. After solvent evaporation, the ethyl acetate fraction was sequentially derivatized with fresh diazomethane and a mixture (1:1, v/v) of dry pyridine and *N,O-bis* trimethylsilyl trifluroacetamide (1% trimethylchlorosilane, Sigma Chemical Co., MO, USA). Samples were dissolved in hexane and aliquots were injected splitless in a HP-1 cross-linked methyl silicone capillary column (25 m length × 0.25 mm internal diameter × 0.22 µm film thickness) fitted in a HP5890 Series II Gas Chromatographer with a capillary direct interface to a 5970B mass selective detector. The GC temperature program was 100–195 °C at 15 °C min^{-1}, then to 260 °C at 4 °C min^{-1}. In each case, characteristic ions of protio and [17,17-$^2H_2$]GA_1, [17,17-$^2H_2$]GA_3 and [17,17-$^2H_2$]GA_4 were monitored. Identification was carried out by similarities of retention times and relative intensities of characteristic ions for protio and [17,17-$^2H_2$]GA_1, [17,17-$^2H_2$]GA_3 and [17,17-$^2H_2$]GA_4 (e.g. m/z 508/506, 493/491, 450/448, 506/504, 491/489, 447/445, 420/418, 403/401, 286/284, respectively). By comparing the peak area of the parent ion for the purposed GA versus that of [^2H]-GA standard, the amount of endogenous GA was calculated. GA assessment was performed by triplicate.

Results

To confirm that the germination behaviour of the IS9530 and Redland B2 lines was as reported in previous experiments, germination indices of intact caryopses were determined throughout ontogeny.

Grains from the sprouting-susceptible genotype Redland B2 were released from dormancy much earlier throughout ontogeny than those from the sprouting-resistant line IS9530 (data not shown). For example, at 37 DAP (i.e. prior to physiological maturity which took place at 42 DAP), germination indices where 20 for Redland B2 grains against almost zero for IS9530 ones. The higher dormancy observed in IS9530 seeds was maintained until late stages of maturation (51 DAP). At harvest (60 DAP), the germination index attained by IS9530 and Redland B2 were 20 and 80, respectively. These patterns of dormancy release are similar to those reported in previous works with

A

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  SbGA20-ox<  .......... .......... .......... .......... ..........
       rice<  NHGIEAALLE EAHRCMDAFF TLPLGEKQRR SGARGRT.AY ASSFTGRFAS 126
     tomato<  NHGVDANLIS NAHRYMDTFF ELPLLEKQKA QRKIGEHCGY ASSFTGRFSS 141
        pea<  NHGIDTKLIE HAHSYMNDFF EVPLSQKQRC QRKTGEHCGY ASSFTGRFSS 148
Arabidopsis<  NHGVDESLLS RAYLHMDSFF KAPACEKQKA QRKWGESSGY ASSFVGRFSS 144
       bean<  NHGVDKKLVE KAHKLIDTFF CMELPEKQKL QRKLGEHCGY ANSFIGRFSS 141
 watermelon<  NHGVDMELIK GVHECMDEFF TLPFDVKQKS QRKFGENYGY ANSFIGRFSN 139
                                                  * **********

  SbGA20-ox<  ....KETLSF RYS..DDQGD GDVVVDYFVD KLGDAY.RHH GEVYGRYCSE
       rice<  KLPWKETLSF RYSSAGDEEG EEGVGEYLVR KLGAEHGRRL GEVYSRYCHE 176
     tomato<  KLPWKETLSF RYS..AKKES SHIVEEYFQS TLGESF.NHL GNVYQEYCNS 188
        pea<  NLPWKETLSF QFS..DEKNS SNIVKDYLSN TLGEDF.QQF GEVYQEYCEA 195
Arabidopsis<  KLPWKETLSF KFS.PEEKIH SQTVKDFVSK KMGDGY.EDF GKVYQEYAEA 192
       bean<  KLPWKETLSF HYA.PDTK.. ..TVEDYFLN SMGEEF.REF GSFFQEYCEV 185
 watermelon<  NLPWKETLSL PYV.ANHN.. STSVQDFVSK CVGPEL.SHQ GKVYQECGKA 185
              ********** *

  SbGA20-ox<  MSRLSLELME VLGESLGV.. .GRRHFRRFF QGNGSIMRLN YYPPCQRPYD
       rice<  MSRLSLELME VLGESLGIVG DRRHYFRRFF QRNDSIMRLN YYPACQRPLD 226
     tomato<  MNTLSLGIME LLGMSLGV.. .EKSHFKEFF EENDSIMRLN YYPPCQKPEL 235
        pea<  MSKLSLGIME LLGMSLGV.. .GKECFRDFF EENKSIMRLN YYPPCQKPDL 242
Arabidopsis<  MNTLSLKIME LLGMSLGV.. .ERRYFKEFF EDSDSIFRLN YYPQCKQPEL 239
       bean<  MSNLSLEIME LLGMSLGV.. .SRECFRDFF KNNESVMRLN YYPPCHKPEL 232
 watermelon<  LSDLSLKIVE LLGLSLGV.. .PKEKFKKFY EDNDSIIRLN YYPPCEKPEL 232
                                          ******* ***

  SbGA20-ox<  TLGTGPHCDP TSLTILHQDD VGGLQVFDGX TGPGTGRWRS IRPHPGAFVV
       rice<  TLGTGPHCDP TSLTILHQDH VGGLEVW... ...AEGRWRA IRPRPGALVV 270
     tomato<  ALGTGPHCDP TSLTILHQDC VGGLQVF... ...VDDEWRS ISPNFNAFVV 289
        pea<  TLGTGPHCDP TSLTILHQDQ VGGLQVF... ...VDNEWHS IRPNFNAFVV 286
Arabidopsis<  ALGTGPHCDP TSLTILHQDQ VGGLQVF... ...VDNKWQS IPPNPHAFVV 283
       bean<  ALGTGPHCDP TSLTVLHQDQ VEGLQVF... ...VDGKWCS VAPKEDAFVV 276
 watermelon<  TLGTGPHCDP TSITILHQDH VSGLQVY... ...VDDEWHS IPPTKDSFVI 276
               ********* ********** ******

  SbGA20-ox<  NI........
       rice<  NVGDTFMALS 280
     tomato<  NIGDTFMALS 299
        pea<  NIGDTFMALS 296
Arabidopsis<  NIGDTFMALT 293
       bean<  NIGDTFMALS 286
 watermelon<  NIGDTFMALT 286
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B

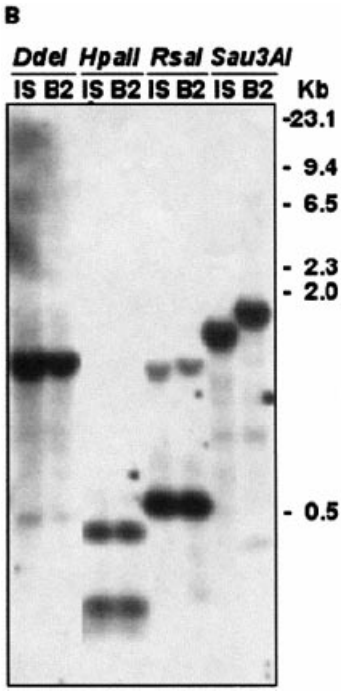

Fig. 1. (A) Alignment between predicted polypeptide from the *Sorghum bicolor* GA 20-oxidase (*SbGA 20ox*) homologous sequence and other known GA 20-oxidases. Conserved amino acid residues are in bold. Asterisks indicate specifically conserved regions in GA 20-oxidases, but not in other oxygenases. Dots indicate either unknown sequence regions or alignment gaps. (B) Southern blot of genomic DNA from IS9530 (IS) and Redland B2 (B2) lines digested with the indicated restriction enzymes and probed with the cloned *SbGA 20ox* fragment.

these genotypes (Steinbach *et al*., 1995; Pagano *et al*., 1997; Steinbach *et al*., 1997; Benech-Arnold *et al*., 2000; Carrari *et al*., 2001).

Cloning and characterization of SbGA 20ox *cDNA*

The PCR product obtained with primers based on the published sequence of the GA 20 oxidase gene from rice (U50333; Toyomasu *et al*., 1997) were cloned and characterized. The sequence analysis of the cDNA indicated that part (465 bp) of the coding region had been amplified. At the amino acid level, the predicted polypeptide shows 69% identity to watermelon (AF074709-1, Kang *et al*., 1999), 70% to bean (U70532, García-Martínez *et al*., 1997), 68% to *Arabidopsis* (X83381, Phillips *et al*., 1995), 76% to tomato (AF049898, Rebers *et al*., 1999), 81% to rice (U50333, Toyomasu *et al*., 1997), and 82% to wheat (Y14008) GA20-oxidase proteins. The alignment with GA 20-oxidase proteins from some plant species is shown in Fig. 1A. Peptide regions ^{116}Tyr-^{137}Arg, ^{209}Asn-^{219}Pro and ^{228}Leu-^{252}Val, which are particularly well conserved in previously reported GA 20-oxidases (Toyomasu *et al*., 1997), are also present in *SbGA 20oxidase.*

To confirm that the PCR-amplified sequence is actually part of the sorghum genome, a Southern blot was performed with different restriction enzymes. Figure 1B shows that the expected PCR-generated probe appears to recognize a single genomic region in both lines. An RFLP was revealed when genomic DNA from IS9530 and Redland B2 was digested with Sau 3AI, indicating the existence of two different allelic forms.

SbGA 20ox *expression during seed incubation*

To investigate the possibility of a differential control of the genes encoding for GA synthesis enzymes, the expression of the *SbGA 20ox* gene was analysed in embryos excised

Table 1. *Gibberellin A_4 and A_1 content and germination percentages in embryos isolated from incubated 40-DAP caryopses from the 2002 harvest*

Each measurement is based on two independent 100 embryo samples. Germinated embryos were excluded from the samples used for GA quantification. ND, not determined.

Incubation time (h)	GA_4 (ng g^{-1})		GA_1 (ng g^{-1})		% Germination	
	Redland B2	IS9530	Redland B2	IS9530	Redland B2	IS9530
0	378±275	181±47	428±182.4	288±85	0±0	0±0
12	654±64	211±180	410±203	189±11.4	2±0.5	0±0
24	1092±207	83±39	405±9.7	133±27.7	7.1±1.4	1±0.5
96	ND	ND	ND	NDα	26.4±1.8	1±0.8

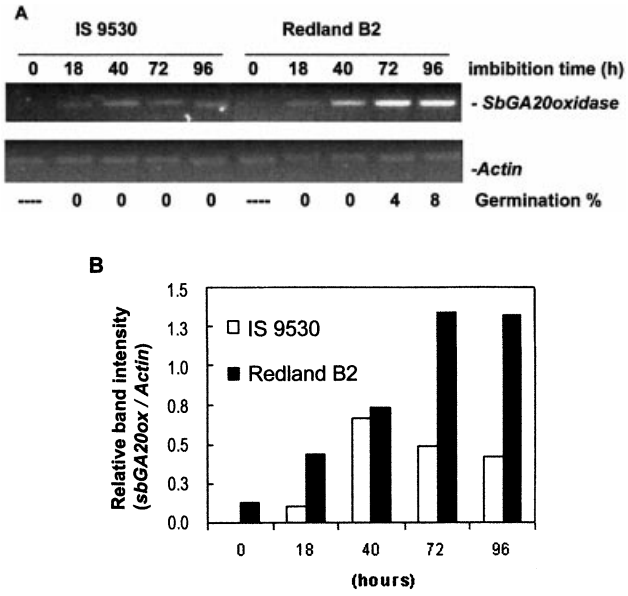

Fig. 2. (A) Expression analysis of *SbGA2-ox* in embryos isolated from incubated caryopses (40 DAP) during the indicated times. RT-PCR was performed in a linear range as described in the Materials and methods and the number of cycles were 28 and 30 for *SbGA 20ox* and *actin*, respectively. One of five repeats is shown. Germination percentages are indicated below each lane. Mean values from three repeats of 50 caryopses are shown. Bar indicates SE. (B) *SbGA 20ox* mRNA band intensities relative to those of actin mRNA .

from caryopses that had been incubated for different periods (Fig. 2A, B). In Redland B2 embryos *SbGA 20ox* mRNA was detected from 18 h after grain imbibition. From there onwards the amount of the specific transcript increased steadily until 96 h after imbibition, when radicle protrusion started to be visible. By contrast, in IS9530 embryos the amount of *SbGA 20ox* mRNA remained barely detectable throughout the incubation period. The differences in phenotype also increased steadily, reaching 65% and 5% germination for the less and more dormant genotypes, respectively, at day 12 of incubation (data not shown). To see if this differential regulation of the gene expression indeed controls the rate of synthesis of at least some of the active gibberellin products, its endogenous levels were measured in embryos from matching caryopses samples. The results (Table 1) show genotype-dependent trends in GA_4 accumulation, which also preceded overt germination events. In agreement with results observed for the *SbGA 20ox* transcript (Fig. 2A, B), GA_4 displayed a clear accumulation pattern during imbibition in Redland B2 embryos, while it remained constant or even decreased in IS9530 ones (Table 1). By contrast, no differences in GA_1 were found between varieties. Its content remained almost unmodified throughout the period considered and at lower values than those observed for GA_4 in non-dormant embryos (Table 1).

*S*bGA 20ox *expression in isolated embryos: the effect of ABA*

Unlike the entire grain, embryos from both varieties are known to germinate readily when isolated from the rest of the seed and incubated in water (Steinbach *et al*., 1995). Therefore, an investigation was undertaken of the extent to which the *SbGA 20ox* expression pattern in isolated embryos could be different from that observed in embryos that had been within the incubated grain until excised for mRNA extraction. In isolated embryos, the specific transcript was detected early in both varieties (Fig. 3A, B) preceding, again, the start of germination (data not shown). Overall, the differential expression pattern was lost, suggesting that the expression of this gene in the embryo is down-regulated by the presence of the tissues surrounding it (namely, endosperm plus pericarp).

The possibility was also evaluated that ABA can interfere with the expression of *SbGA 20ox*. Therefore, *SbGA 20ox* gene expression was assessed in embryos incubated in the presence of 50 μM ABA. At this concentration, ABA suppressed *SbGA 20ox* expression in embryos of both lines, at least until 16 h after imbibition (Fig. 3A, B).

Discussion

The two sorghum lines used in this study are known to have a contrasting pattern of exit from dormancy during grain development (Steinbach *et al*., 1995): Redland B2 caryopses start losing dormancy well before physiological

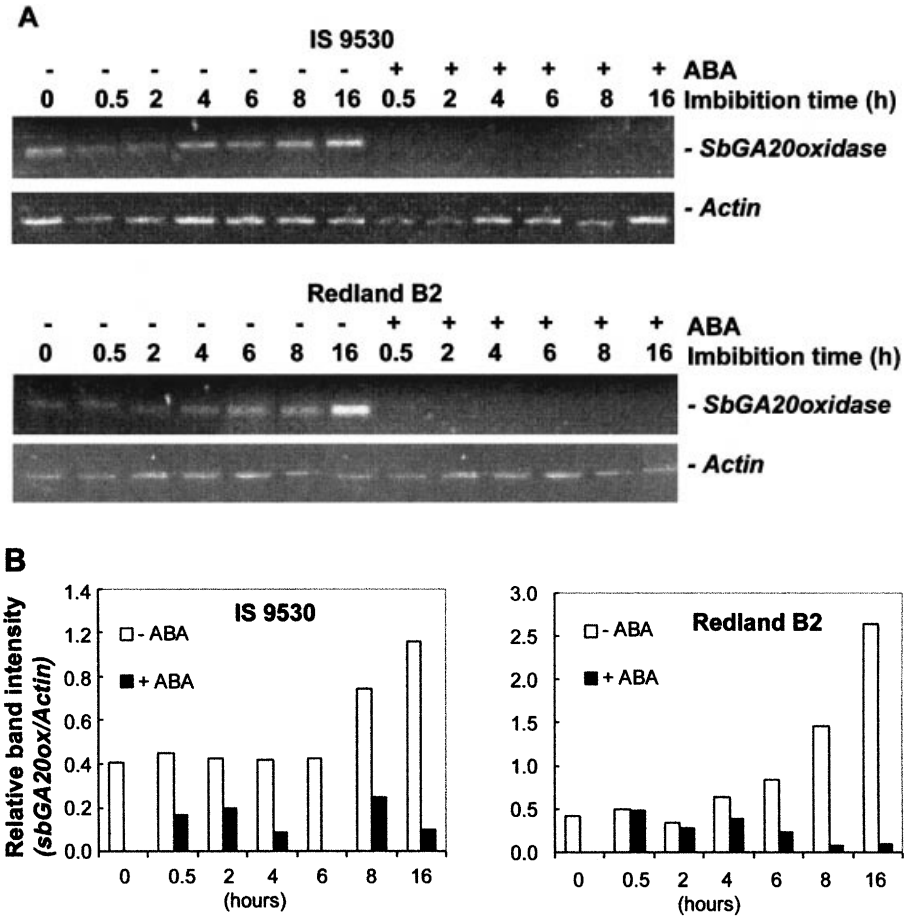

Fig. 3. (A) Expression analysis of *SbGA 20ox* in 35 DPA isolated embryos incubated in H_2O or ABA (50 μM) for the indicated times. RT-PCR was performed in a linear range as described in the Materials and methods and the number of cycles were 29 and 30 for *SbGA 20ox* and *actin*, respectively. One of four repeats is shown. (B) *sbGA 20ox* mRNA band intensities relative to those of actin mRNA.

maturity while IS9530 caryopses remain dormant for some time after full maturity. The mechanisms controlling the pattern of exit from dormancy in each line are largely unknown. However, it has been shown that modifications of the endogenous level of the growth regulators ABA and GAs during seed development (i.e. by means of the application of inhibitors of their synthesis almost immediately after anthesis) can modulate the characteristic pattern of exit from dormancy for each line (Steinbach *et al*., 1997). Thus, it appears that, as in the case of mechanisms behind the expression of dormancy, dormancy-controlling mechanisms are, in turn, under hormonal control.

For most of the experiments presented in this paper developing sorghum caryopses at 35–40 DAP were used, a stage at which differences in dormancy level between lines are conveniently contrasting. The different dormancy level is expressed through a distinct germination capacity and also through a differential embryo sensitivity to ABA. Since caryopses of that age still have large amounts of ABA (Steinbach *et al*., 1995), a different embryo sensitivity to ABA is likely to be instrumental for the different germination capacity. Based on the fact that dormant IS9530 grains can be induced to germinate if supplied with GA_{4+7} (Steinbach *et al*., 1997) and other works stating that GA *de novo* synthesis upon seed imbibition is required for germination (Karssen *et al*., 1989; Nambara *et al*., 1991), it was hypothesized that a different dormancy level should also result from the contrasting capacity of the embryos to synthesize GAs upon imbibition. Although the possibility of de-conjugation as a source of bioactive GAs cannot be ruled out, the results of these experiments suggest that *de novo* synthesis of at least GA_4 took place upon imbibition in embryos from Redland B2 caryopses with low dormancy. This increase in measurable GA_4 clearly preceded the initiation of visible germination, thus suggesting that the increase in GA content is possibly a requirement for germination and not merely the result of it. By contrast, no increase in the content of either GA_4 or other bioactive GAs appeared to have occurred in embryos from dormant IS9530 caryopses during the first 48 h of imbibition. On the contrary, GA_4 content steadily went down, suggesting that breakdown or conjugation predominated over synthesis. Although 13-hydroxy GAs have

been reported to be biologically significant in sorghum (Lee *et al*., 1998), no correlation was found between GA_1 levels and germination capacity of the caryopses. These results allow the proposal that a high dormancy level could also be expressed as a blockade on the production of some bioactive GAs upon imbibition. There are strong indications that enhancement of GA synthesis is part of the mechanism through which environmental signals (i.e. light) induce germination (Hilhorst and Karssen, 1988; Derkx and Karssen, 1993; Yang *et al*., 1995; Toyomasu *et al*., 1998; Yamaguchi *et al*., 1998). On the other hand, there is evidence showing that changes in dormancy level might be accompanied by modifications in sensitivity to GAs (Derkx and Karssen, 1993; Benech-Arnold *et al*., 2000). However, the authors are not aware of any previous work showing that a different dormancy level could be correlated with a differential capacity to produce *de novo* GA synthesis upon seed imbibition.

The step that involves the action of GA 20-oxidase is considered to be a site of regulation for the synthesis of bioactive GAs (Phillips *et al*., 1995; Phillips, 1998). Therefore, a PCR-based approach was used for cloning the sorghum GA 20-oxidase gene. The deduced amino acid sequence of *SbGA 20ox* revealed a high degree of identity (62–75%) compared with GA 20-oxidases from other species, which are mainly expressed in seeds (Phillips *et al*., 1995; García-Martínez *et al*., 1997; Toyomasu *et al*., 1997; Kang *et al*., 1999; Rebers *et al*., 1999). Moreover, three regions were identified that are specifically conserved between GA 20-oxidases, but not in other dioxygenases (Toyomasu *et al*., 1997).

Differential expression of *SbGA 20ox* in embryos from high- and low-dormancy caryopses during the incubation period was highly consistent with these observations in terms of genotype-dependent germination behaviour. Whereas almost no expression was detected in embryos from the high-dormancy genotype in agreement with the apparent absence of *de novo* synthesis of bioactive GAs, detectable amounts of the corresponding mRNA were found in embryos from the low-dormancy genotype shortly after imbibition and reaching high levels well before radicle emergence. Taken together, these results support the proposition that the regulation of GA synthesis upon imbibition in sorghum seeds with different dormancy is exerted, at least in part, at the level of transcription of the gene that encodes the enzyme GA 20-oxidase. The role of this enzyme as a regulator of GA synthesis has been shown in relation to a number of physiological processes (Gilmour *et al*., 1986; Hedden and Croker, 1992; Appleford and Lenton, 1997). While Appleford and Lenton (1997) showed a correlation between GA 20-oxidase expression and α-amylase in germinating wheat seeds, the results of this work show that a different dormancy level could be expressed as a differential regulation of this gene. A recurring feature of GA biosynthesis is a feedback mechanism operating on the expression of genes encoding GA 20-oxidases to modulate the levels of bioactive GAs (Lange, 1998; Phillips, 1998; Toyomasu *et al*., 1998; Xu *et al*., 1999). However, the results of the present work suggest a lack of this type of regulation in line Redland B2 or, alternatively, that GA levels had not reached the necessary threshold for such an inhibition.

Embryos from the sorghum lines used in this study and also from other cereals are known to germinate readily in water from early stages of development (i.e. 15 DAP) if isolated from the rest of the seed (Walker-Simmons, 1987; Steinbach *et al*., 1995; Benech-Arnold *et al*., 1999). Similarly, no differential pattern of the early expression of *SbGA 20ox* in isolated embryos was observed. These results suggest the existence of a genotype-dependent physiological control by the tissue surrounding the embryos on inhibition of both germination and expression of this gene.

In addition, it was observed that the expression of *SbGA 20ox* was suppressed in embryos incubated in ABA. This hormone is known to act antagonistically to GAs in the control of a number of processes. For example, in the cereal aleurone layer, ABA inhibits the expression of GA-inducible genes including those for α-amylase and proteases required for post-germinative growth (Bethke *et al*., 1997; Lovegrove and Hooley, 2000). Moreover, it has recently been reported that an ABA-responsive protein kinase mediates suppression by ABA of GA induction of α-amylase and protease genes in barley aleurone tissue (Gómez-Cárdenas *et al*., 1999). In this context, the inhibition of *SbGA 20ox* expression in ABA-incubated embryos found in this work indicates that ABA could interfere directly with GA biosynthesis and hence with overall GA-induced gene expression. Although the role of ABA as a germination inhibitor has been known for decades, there are noticeably few examples of germination-related genes that are down-regulated by this plant hormone. In a comprehensive analysis of gene expression during tomato germination, Bradford *et al*. (2000) reported only three genes that are down-regulated by ABA: a β-1, 3-glucanase, a vacuolar H^+-ATPase and a GA-stimulated transcript with unknown function. Therefore, the observed suppression of GA 20-oxidase gene expression is among the first cases of a germination-related gene that is down-regulated by ABA.

As mentioned before, the control exerted by the seed environment on *SbGA 20ox* expression appears to be qualitatively different between opposing genotypes (i.e. the presence of pericarp plus endosperm is able to block expression of the gene in grains from the genotype with high dormancy, but only delays it in the low dormancy seeds). In the light of the finding that ABA inhibits *SbGA 20ox* expression, it might be speculated that the well-known differences in embryo sensitivity to ABA between

these varieties (higher for IS9530) might be involved in the determination of this differential expression pattern (Steinbach *et al*., 1995). Therefore, a higher sensitivity to ABA might lead to a larger interference with GA synthesis, despite similar endogenous ABA levels. Experiments are currently in progress to test this possibility

Overall, the results of this work allow a model to be postulated that poses ABA as a regulator of sorghum seed germination, modulating the expression of the GA 20-oxidase gene. Further research is required to assess the validity of this model.

Acknowledgements

The authors want to thank Mr Luis Hercun for assistance with the field experiments and Mr Oscar Masciarelli for his help with GAs quantification. This research was funded by the National Agency for Science and Technology Promotion (ANPCyT) (PICT 99-No. 08-06609) and by the University of Buenos Aires (IX-06). RO-F and FC hold fellowships from CONACYT (Mexico) and the University of Buenos Aires, respectively.

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