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Expression of Seed Dormancy in Grain Sorghum Lines with Contrasting Pre-Harvest Sprouting Behavior Involves Differential Regulation of Gibberellin Metabolism Genes

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Grain sorghum [*Sorghum bicolor* (L) moench] exhibits intraspecific variability for the rate of dormancy release and pre-harvest sprouting behavior. Two inbred lines with contrasting sprouting response were compared: IS9530 (resistant) and RedlandB2 (susceptible). Precocious dormancy release in RedlandB2 is related to an early loss of embryo sensitivity to ABA and higher levels of gibberellins in imbibed grains as compared with IS9530. With the aim of identifying potential regulatory sites for gibberellin metabolism involved in the expression of dormancy in immature grains of both lines, we carried out a time course analysis of transcript levels of putative gibberellin metabolism genes and hormone content (GA_1, GA_4, GA_8 and GA_{34}). A lower embryonic GA_4 level in dormant IS9530 was related to a sharp and transient induction of two *SbGA2-oxidase* (inactivation) genes. In contrast, these genes were not induced in less dormant RedlandB2, while expression of two *SbGA20-oxidase* (synthesis) genes increased together with active GA_4 levels before radicle protrusion. Embryonic levels of GA_4 and its catabolite GA_{34} correlated negatively. Thus, in addition to the process of gibberellin synthesis, inactivation is also important in regulating GA_4 levels in immature grains. A negative regulation by gibberellins was observed for *SbGA20ox2*, *SbGA2ox1* and *SbGA2ox3* and also for *SbGID1* encoding a gibberellin receptor. We propose that the coordinated regulation at the transcriptional level of several gibberellin metabolism genes identified in this work affects the balance between gibberellin synthesis and inactivation processes, controlling active GA_4 levels during the expression of dormancy in maturing sorghum grains.

Keywords: Germination • Gibberellins • Pre-harvest sprouting • Seed dormancy • *Sorghum bicolor*.

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylate; CPS, *ent*-copalyl diphosphate synthase; DAP, days after pollination; EKAH, *ent*-kaurenoic acid oxidase; EKO, *ent*-kaurene oxidase; GA2ox, gibberellin 2-oxidase; GA3ox, gibberellin 3-oxidase; GA20ox, gibberellin 20-oxidase; GI, germination index; KSB, *ent*-kaurene synthase; PBZ, paclobutrazol; PHD, prohexadione-calcium; PHS, pre-harvest sprouting; PM, physiological maturity.

Introduction

Seed dormancy is of agronomic interest because as a result of its prolonged presence or precocious loss, untimely seed germination can occur and impact negatively on different aspects of crop production. In species with physiological dormancy, abscisic acid (ABA) and gibberellins have an antagonistic role in the control of germination (reviewed by Finch-Savage and Leubner-Metzger 2006, Finkelstein et al. 2008, Nambara et al. 2010). As a general rule, it has been widely accepted that ABA metabolism and/or sensitivity decreases during release of dormancy in parallel with an increased response to gibberellins (Finch-Savage and Leubner-Metzger 2006). Changes in the ABA–gibberellin balance are involved in the expression of dormancy during imbibition in many different species ranging from Arabidopsis to domesticated cereal crops (reviewed by Finch-Savage and Leubner-Metzger 2006, Finkelstein et al. 2008, Nambara et al. 2010). Nevertheless, the regulatory steps that operate in different species seem to be diverse. It is of interest for a particular species to identify those steps that contribute to intraspecific alterations for this balance and, for instance, to different patterns of dormancy release and agronomic behavior.

Typically, in imbibed non-dormant seeds ABA levels decrease and gibberellin levels increase, while the dormant state is usually associated with high ABA and low gibberellin levels. This is the case in Arabidopsis (Ogawa et al. 2003, Ali-Rachedi et al. 2004) and lettuce (Argyris et al. 2008, Sawada et al. 2008a, Sawada et al. 2008b). A requirement for

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gibberellins for normal germination of Arabidopsis is evidenced by the incapacity to germinate of gibberellin-deficient mutants (Ogawa et al. 2003) or wild-type seeds incubated in the presence of a gibberellin biosynthesis inhibitor (Nambara et al. 1991). In imbibed Arabidopsis seeds, an increase in GA_4 content precedes radicle protrusion (Ogawa et al. 2003). Exposure of Arabidopsis seeds to inhibitory environmental signals (i.e. high incubation temperature, far red-enriched light) promotes ABA synthesis and slows down its catabolism (Ali-Rachedi et al. 2004, Seo et al. 2006) while in parallel gibberellin synthesis is blocked and, as a result, dormancy is maintained (Seo et al. 2006). Increased ABA levels during incubation at high temperatures (that inhibit germination) have also been described for lettuce seeds (Argyris et al. 2008).

Regulation of hormone metabolism at the transcriptional level during normal seed germination was first studied in detail in Arabidopsis by Ogawa et al. (2003), who observed that the expression of several gibberellin biosynthesis genes increased a few hours after imbibition. More recently, other groups have addressed the transcriptional regulation of ABA and gibberellin metabolism genes during the release of seed dormancy and/or under repressing environmental conditions. Gubler et al. (2008) studied ABA and gibberellin metabolism genes in imbibed barley grains, and observed that after-ripening had no effect on the expression of ABA biosynthesis genes, but promoted the expression of an ABA catabolism gene (*HvABA8OH1*), a gibberellin biosynthetic gene (*HvGA3ox2*) and a gibberellin catabolic gene (*HvGA2ox3*) following imbibition. Expression of ABA and gibberellin metabolism genes was also shown to be different between imbibed dormant and non-dormant Arabidopsis CVi seeds (Finch-Savage et al. 2007). In this work, the authors observed that dormancy relief of CVi ecotype seeds by after-ripening or exposure to dormancy breaking treatments promoted a *GA3ox1* (gibberellin 3-oxidase 1) gene, while expression of ABA synthesis genes declined. Similarly, Seo et al. (2006) observed that exposure of imbibed Arabidopsis seeds to a far red light pulse (that inhibits germination) repressed *AtGA3ox1* and *AtGA3ox2* (otherwise induced under red light), but in this case a strong promotion of a gibberellin catabolism gene, *AtGA2ox6*, was also observed. The evidence presented by different works mentioned here supports that regulatory genes involved in the control of ABA and gibberellin levels during imbibition can differ not only inter- and intraspecifically but also in response to different environmental factors that affect germination. At the same time, gibberellin synthesis genes encoding GA3oxs appear to have a more 'universal' role as they are regulated consistently with germination response in a variety of species and situations. Nevertheless, it should be noted that most evidence supporting a role for these genes in controlling active gibberellin levels has not been accompanied by determinations of the corresponding gibberellin metabolites.

Both ABA and gibberellins have a decisive role in controlling dormancy in grain sorghum [*Sorghum bicolor* (L.) moench] seeds: spraying of developing sorghum panicles with ABA and gibberellin biosynthesis inhibitors either accelerated or slowed down the pattern of dormancy release, respectively (Steinbach et al. 1997). These hormones are also involved in the expression of dormancy in imbibed grains. Nevertheless, the physiological mechanisms behind the repression of germination in dormant sorghum seeds do not involve the capacity to keep ABA levels high. In previous works we showed that ABA levels decreased similarly in imbibed grains of two sorghum lines with contrasting dormancy (Gualano et al. 2007, Rodríguez et al. 2009). In sorghum, it is embryonic sensitivity to ABA that correlates best with the level of dormancy throughout development and when comparing genotypes with contrasting sprouting behavior (Steinbach et al. 1995, Gualano et al. 2007, Rodríguez et al. 2009). An early loss of embryo sensitivity to ABA in sprouting-susceptible genotypes has also been reported for other cereals such as wheat (Walker-Simmons 1987) and barley (Benech-Arnold et al. 1999).

Previous research with two sorghum inbred lines displaying contrasting sprouting behavior [IS9530, resistant to pre-harvest sprouting (PHS); and RedlandB2, susceptible to PHS] has shown that, in addition to lower embryonic sensitivity to ABA, imbibed grains from RedlandB2 accumulate higher levels of active gibberellins in the embryo as compared with IS9530 (Pérez-Flores et al. 2003). In this previous work a sorghum gene encoding a GA20ox was cloned. Expression analysis during imbibition showed a higher expression of this gene in less dormant RedlandB2 grains as compared with the dormant line. Sequencing of the sorghum genome in recent years (Paterson et al. 2009) offered the possibility to study previously unknown *GA20ox* homologs and other genes of interest.

With the aid of gibberellin biosynthesis inhibitors, we first investigated at which developmental stages is gibberellin *de novo* synthesis most relevant to the expression of dormancy. Secondly, we aimed to extend the expression analysis to other sorghum genes encoding putative gibberellin metabolism enzymes in order to evaluate the existence of potential regulatory sites involved in the expression of dormancy. Transcriptional profiles of 12 sorghum putative gibberellin metabolism genes during incubation of caryopses from both lines (IS9530 and RedlandB2) were obtained and compared with the evolution of active GA_1 and GA_4 and their respective catabolites (GA_8 and GA_{34}) in the embryo. Taken together, the results presented in this work support that different gibberellin levels observed in immature, imbibed sorghum grains from two lines with contrasting dormancy result from combined regulation at the transcriptional level of both *GA20ox* (synthesis) and *GA2ox* (inactivation) genes.

Results

Effect of gibberellin synthesis inhibitors on germination at different developmental stages

The pattern of dormancy release for sorghum inbred lines RedlandB2 and IS9530 was followed through a germination

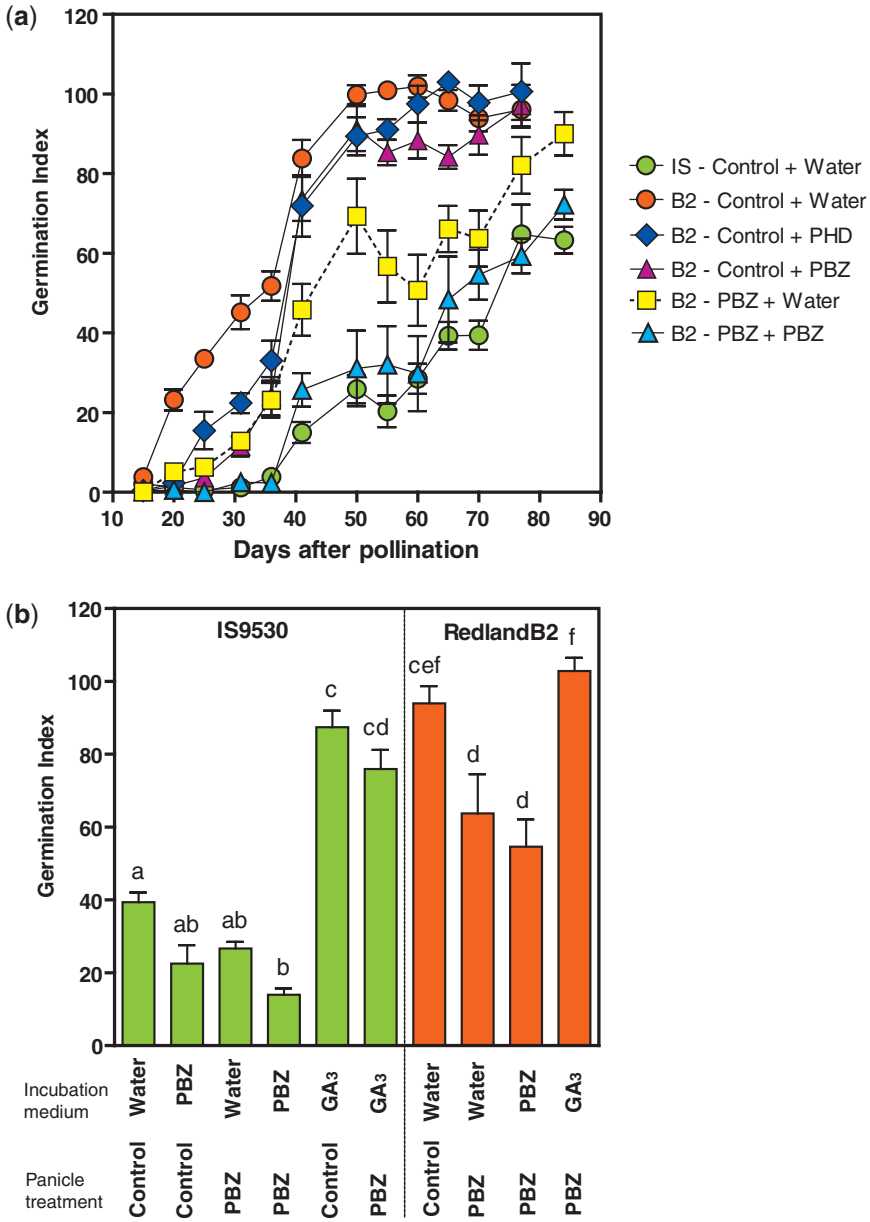

Fig. 1 (a) Dormancy release during seed development in sorghum (*S. bicolor*) and in response to gibberellin biosynthesis inhibitors. At different days after pollination (DAP), grains were harvested and incubated for 12 d to obtain a germination index (GI). The GI was followed for grains from untreated panicles (Control) of IS9530 (sprouting resistant; green circles) and RedlandB2 (sprouting susceptible; red circles). For RedlandB2, grains from control plants were also incubated in paclobutrazol (PBZ, 100 mg l^{-1}, magenta triangles) or prohexadione-Ca (PHD, 100 p.p.m., blue diamonds). The GI is also shown for RedlandB2 grains from panicles that had been sprayed with PBZ (1 g l^{-1}) during early development (at 3, 7 and 11 DAP) and incubated in water (yellow squares) or in PBZ 100 mg l^{-1} (cyan triangles). All incubations were done at 25°C. The approximate time of physiological maturity (PM) was 42 DAP for both lines. Each data point is the mean of three biological replicates (three field plots) assessed in triplicate. Bars indicate the SEM ($n = 3$). (b) GI for IS9530 (green bars) and RedlandB2 (red bars) grains harvested at 65 DAP from control or from PBZ-treated panicles. Harvested grains from both treatments were incubated either in water, PBZ100 p.p.m. or GA_3 100 μM. Bonferroni's multiple comparisons test was done with GI values for both lines at this sampling date (65 DAP), and different letters on top of bars indicate significant differences (P-value < 0.05) between treatments.

index (GI; see Materials and Methods for calculation) as shown in **Fig. 1a**. This index ranges from zero (no germination after 12 d) to 120 (all seeds germinated on day 1). As observed previously (Steinbach et al. 1995, Steinbach et al. 1997), RedlandB2 caryopses incubated in water were capable of germinating since early stages of development [i.e. 20 days after pollination (DAP)]. In contrast, IS9530 caryopses remained dormant for a longer period and were capable of germinating only after physiological maturity (PM).

The existence of a requirement for gibberellin *de novo* synthesis for germination was assessed in developing RedlandB2 caryopses by incubating grains in the presence of either paclobutrazol (PBZ; 100 mg l^{-1}; Crestar, Zeneca Agricultural) or prohexadione-Ca (PHD; 100 p.p.m.). PBZ inhibits Cyt P450-dependent monooxygenases that catalyze oxidation of *ent*-kaurene and therefore blocks gibberellin synthesis at an early step. PHD is a structural mimic for 2-oxoglutarate, a co-substrate of dioxygenases that catalyze the last steps leading to active gibberellins (Rademacher 2000). Data in **Fig. 1a** support that the requirement for gibberellin *de novo* synthesis for germination of RedlandB2 caryopses depends on the developmental stage: inhibition of germination by incubation in PBZ and PHD solutions was stronger during early development, disappeared around PM (35 DAP), and a weaker response only to PBZ was observed again between PM and harvest maturity. Dormancy release in RedlandB2 was delayed when young panicles were sprayed at 3 and 7 DAP with PBZ (1 g l^{-1}; **Fig. 1**), and germination was further inhibited when grains from PBZ-treated panicles were incubated in 100 p.p.m. PBZ solution (**Fig. 1a**). Both treatments with PBZ had an additive effect on inhibition of germination (**Fig. 1**) as compared with each treatment alone, suggesting that both gibberellins that accumulate during early development and those synthesized during incubation contribute to the germination response. Germination of grains from PBZ-treated panicles was restored by addition of GA_3 to the incubation medium (**Fig. 1b**). In an independent but similar experiment, incubation of 37 DAP grains in GA_3 (100 μM) + PBZ (100 p.p.m.) reverted the effect of PBZ alone (data not shown).

Identification of sorghum genes encoding putative gibberellin metabolism enzymes

Genes encoding gibberellin metabolism enzymes have been described in several species. Those reported for *Arabidopsis thaliana* (Hedden and Phillips, 2000) and *Oryza sativa* (Sakamoto et al. 2004) were used as query sequences to search for putative orthologs in the sorghum genome database. Thirteen loci were identified in the sorghum genome that encode putative enzymes involved in gibberellin metabolism, and are listed in **Table 1**. Phylogenetic relationships for the predicted protein sequences found in sorghum and their putative orthologs from maize, rice and Arabidopsis are shown in **Fig. 2**.

A single sequence with highest identity was found in sorghum (**Table 1**) for each gene encoding putative orthologs for both terpene cyclases [*ent*-copalyl diphosphate synthase, (CPS) and *ent*-kaurene synthase (KSB)] and monooxygenases [*ent*-kaurene oxidase (EKO) and *ent*-kaurenoic acid oxidase (EKAH)] involved in the conversion of geranyl-geranyl pyrophosphate to GA_{12}. Sorghum predicted proteins SbCPS, SbKSB, SbEKO and SbEKAH were most closely related to the corresponding orthologs from maize (identities were 80, 75, 93 and 81%, respectively), and identity decreased for rice and Arabidopsis (**Fig. 2a, b**). Although the predicted SbKSB shared 67% identity with rice OsKS2, both sorghum and maize KSB hypothetical proteins (**Fig. 2a**) lacked the SAYDTAWVA conserved domain that is present in all *Oryza* and Arabidopsis terpene cylases.

Three sorghum genes were identified that encode putative GA20oxs and are referred to as *SbGA20ox1*, *SbGA20ox2* and *SbGA20ox3*, respectively. Among these genes, *SbGA20ox1* had been partially cloned and reported by Pérez-Flores et al. (2003).

Table 1 Plant GDB identification codes for selected sorghum sequences, and PCR primers used in this work

Gene name	PlantGDB ID code	Fw primer	Rev primer
SbCPS	Sb01g021990	CCACCCACGCCGTATCA	GGGCGATGCTAAGATCAATGA
SbKSB	Sb06g028210	CGGTGATCAATGAGCCACTAAAG	CCTCCCTCTCCCACACAACA
SbEKO	Sb10g022520	TGGACAAATCTTGGAGGCACTA	AACGCGGTGTTCCATGAGA
SbEKAH	Sb10g000920	TGCGGCTCGTCAACATCTC	GCAGCTTACCGTTCACGAAGA
SbGA20ox 1	Sb01g000580	CGCTTCGCGTCCAAGCT	GCCCTGGTCGTCGGAGTAG
SbGA20ox 2	Sb09g020760	AGACGCTCTCCTTCGGCC	TCGCGTTGCAGTAGTTCTGATAC
SbGA20ox 3	Sb03g041900	GACTACTTCACCGGCACCCTC	CCAGGCTCAGCTCCAGCA
SbGA3ox1	Sb03g004020	GAGGCGGAGCGGAGGAT	GCACCGAGGGTACCAGTTGA
SbGA3ox2	Sb09g005400	GCTTCTGCGACGTGATGGA	CCGAGCGCCATGAAGAATAG
SbGA2ox1	Sb09g004520	CGGCACAGGGTAATTGCAA	GGCGCAGCGAAGTAGATTG
SbGA2ox2	Sb03g013450	GTCCGCCTTCTTCATCAACG	GAAGTAGATGGTGGACAGCCG
SbGA2ox3	Sb03g035000	CACGAGGAGTACACAAGCACACA	GGGCAAGCACGCTGAGAA
SbGA2ox4	Sb09g006490	TTGATGTGTTGTAAACTGTGTCATGA	CTTCACCTCAACATTTAGCCACC
SbActin	Sb01g010030	GCCGTCCTCTCTCTGTATGC	CGAGCTTCTCCTTCATGT
SbGID1	Sb09g020080	AGGCCACCGTGGGTTTCT	CTCCTCCATCACCTCGTGGTA

Sorghum genes were named after their putative orthologs in reference species.

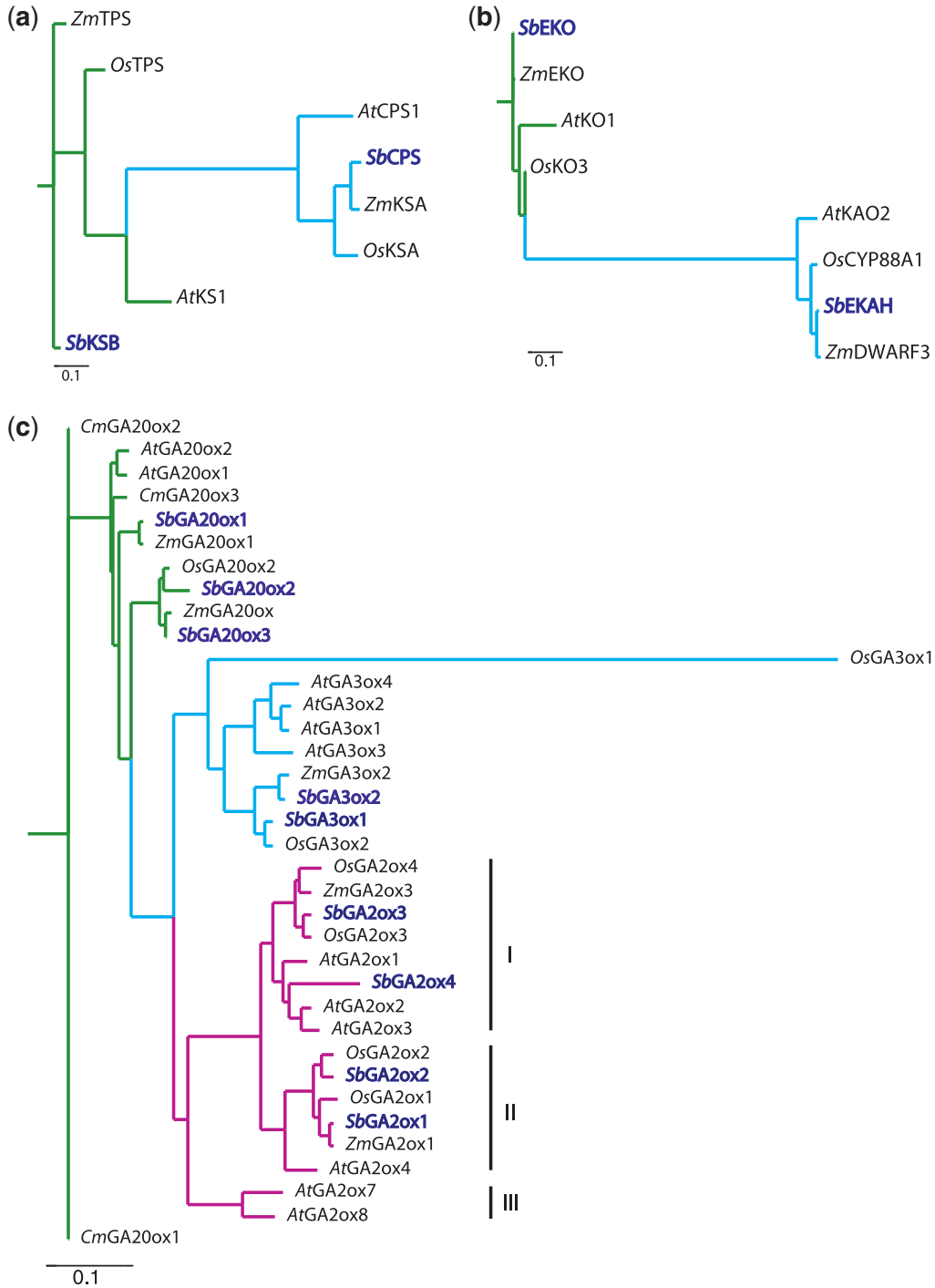

Fig. 2 Phylogenetic relationships for gibberellin metabolism enzymes known in several species and putative orthologs in sorghum identified in this work. Phylogenetic trees are shown for: (a) gibberellin metabolic diterpene cyclases (CPS/KSA and TPS/KSB); (b) gibberellin metabolic Cyt P450-dependent monooxygenases (KO/EKO and KAO/EKAH); (c) gibberellin metabolic 2-ODDs (soluble di-oxygenases) GA20ox, GA3ox and GA2ox. At, Arabidopsis; Cm, pumpkin; Os, rice; Sb, grain sorghum; and Zm, maize. Scale bars indicate the number of changes per position for a unit branch length. Identification codes for reference sequences from other species are listed in **Supplementary Table S1**. Protein alignments are shown in **Supplementary Fig. S4**.

The sequence LPWKET, which is considered to be involved in the binding of the gibberellin substrates and is conserved in all OsGA20ox proteins (e.g. at residues 128–133 of OsGA20ox1; Sakamoto et al. 2004) is present in the three sorghum genes encoding putative GA20oxs. Other genes encoding 2-oxoglutarate-dependent dioxygenases with an overall high identity to GA20ox were also found in the sorghum genome, but a detailed analysis of their sequence revealed that

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the LPWKET domain necessary for GA20ox activity is not conserved and they were therefore excluded from the expression analysis. Identity among sorghum GA20oxs ranged from 45% (for SbGA20ox1 and SbGA20ox2, which were the least similar) to 60.1% (for SbGA20ox2 and SbGA20ox3). Each sorghum GA20ox shared higher identity with a different GA20ox from other monocots (rice or maize), but still grouped separately from those belonging to Arabidopsis and pumpkin (**Fig. 2c**).

Two genes encoding putative GA3oxs were identified for sorghum, and named *SbGA3ox1* and *SbGA3ox2*. Both sorghum homologs shared 53.8% identity, and each one was more closely related to another GA3ox from maize or rice (>78% identity between SbGA3ox1 and OsGA3ox2, and 76% between SbGA3ox2 and ZmGA3ox2).

Four genes encoding putative GA2oxs involved in gibberellin inactivation were identified and named *SbGA2ox1*, *SbGA2ox2*, *SbGA2ox3* and *SbGA2ox4*. These predicted sorghum proteins shared a stronger identity with GA2oxs from other species (including dicots) than among themselves (**Fig. 2c**), and appear to reflect the existence of homologs with different functions across plant species, as mentioned previously in Sakamoto et al. (2004). Identity among sorghum GA2oxs within the same group ranged from 55.1% for SbGA2ox1 and SbGA2ox 2 (group II, in **Fig. 2c**) to 32% for SbGA2ox3 and SbGA2ox4 (group I). In contrast, SbGA2ox3 was most similar to OsGA2ox3 (82.7%), SbGA2ox2 was similar to OsGA2ox2 (68.9%) and SbGA2ox1 was similar to ZmGA2ox1 and OsGA2ox1 (81.7 and 64.3%, respectively). Among putative sorghum GA2oxs, SbGA2ox4 was the most different from the other sorghum homologs, and the level of identity was similar to that observed with other GA2oxs from other species (e.g. 31.8% with AtGA2ox1).

A sorghum sequence encoding a putative ortholog for the gibberellin receptor gene *GID1* (*GIBBERELLIN INSENSITIVE DWARF1*; BN001196) was identified by *in silico* search and reported by Voegele et al. (2011). This sequence shares a high identity (82.9%) with its rice ortholog.

Transcripts from all these genes (except *SbGA2ox2*) were detected in our quantitative PCR assays with cDNA from sorghum embryos, although expression levels varied greatly among them (as reflected by relative expression values shown in **Fig. 4**).

Evolution of GA_1 and GA_4 content in immature, imbibed sorghum grains

The embryonic content of GA_1 and GA_4 (both active gibberellins) was measured in immature (30 DAP) grains after different incubation times during phase II of imbibition (i.e. before embryo growth begins) (**Fig. 3**; numerical data in **Supplementary Table S2**).

Active GA_4 levels (**Fig. 3**) were similar in both lines during the first 3 d but, after a 4 d incubation period, GA_4 reached a significantly higher value in less dormant RedlandB2 than in IS9530 (255.8 vs. 99.3 ng g^{-1} DW; two-tailed *P*-value = 0.0227). This 2.5-fold increase anticipated the completion of germination of the first grains observed on day 6. In parallel, GA_1 levels were slightly higher in RedlandB2 throughout the 4 d period evaluated (**Fig. 3**, inset), but differences were not significant at any time point.

An earlier (i.e. after 12 and 24 h) increase in GA_4 content had been reported previously for 40 DAP grains from these two lines (Pérez-Flores et al. 2003). This was probably related to the lower dormancy level evidenced by a faster germination response (germinated grains were first observed at 24 h) in 40 DAP grains as compared with more dormant 30 DAP grains used in this work.

Expression of gibberellin metabolism genes and *SbGID1* (gibberellin receptor) in immature (30 DAP) grains

Based on previous results (Pérez-Flores et al. 2003), we assessed the possibility that higher GA_4 levels observed in RedlandB2 might involve differential regulation at the transcriptional level of other gibberellin metabolism genes in addition to a *GA20ox* gene. In this previous work, higher GA_4 levels were related to an increased expression of a *GA20ox* gene in the embryo of less dormant, imbibed RedlandB2 grains. It is relevant to note that PCR determinations reported in Pérez-Flores et al. (2003) were carried out with primers designed on a sequence encoding a GA20ox from rice. We recently compared the sequence of these original primers with all three sorghum GA20oxs (*SbGA20ox1*– *SbGA20ox3*). As both *SbGA20ox1* and *SbGA20ox3* have similar binding sites for these primers and also give origin to amplicons of a similar size, it is highly

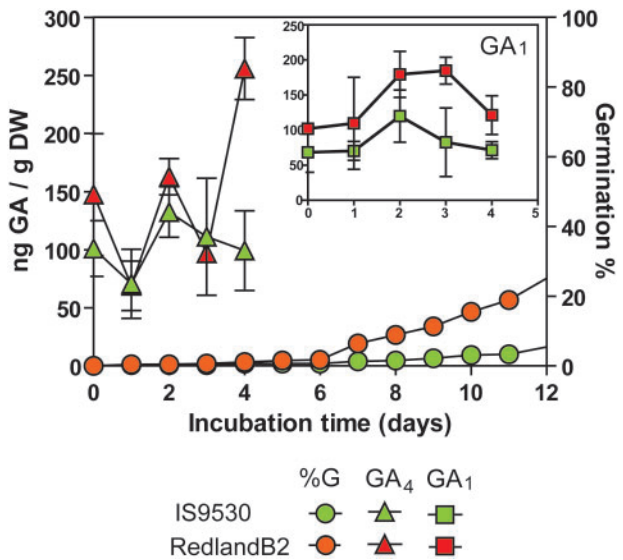

Fig. 3 Evolution of germination percentage (circles) and embryonic content of GA_4 (triangles) and GA_1 (inset, squares) as a function of incubation time for sorghum caryopses from RedlandB2 (sprouting susceptible; red symbols) and IS9530 (sprouting resistant; green symbols). Numerical gibberellin values are shown in **Supplementary Table S2**. Caryopses were harvested at 30 DAP and incubated at 20°C. Each data point is the mean of three biological replicates.

probable that transcripts from these two genes were not distinguished during PCR assays reported by Pérez-Flores et al. (2003).

We hypothesized that the expression of gibberellin biosynthetic genes would be enhanced and/or gibberellin inactivation genes would be repressed in RedlandB2 grains as compared with IS9530. The results confirmed the existence of different expression patterns between both lines for genes involved in several steps of gibberellin metabolism (**Fig. 4c, d**). A relatively early increase of transcript levels was observed for most genes encoding putative gibberellin synthesis enzymes (**Fig. 4c**) in more dormant IS9530: expression of *SbEKO*, *SbEKAH*, *SbGA20ox2*, *SbGA20ox3* and *SbGA3ox1* increased after 2 or 3 d and reached higher values in embryos from dormant IS9530 grains as compared with less dormant RedlandB2, and decreased afterwards by day 4. Nevertheless, induction of gibberellin biosynthesis genes during the first 3 d in IS9530 dormant grains was accompanied by a strong promotion of gibberellin inactivation genes, namely *SbGA2ox1* and *SbGA2ox3* (**Fig. 4d**). Both *SbGA2ox1* and *SbGA2ox3* were expressed at relatively high levels as compared with the rest of the genes. Expression of *SbGA2ox4* in both lines was, on average, about 100 times lower as compared with *SbGA2ox1* and *SbGA2ox3*, and we were not able to detect *SbGA2ox2* transcripts in any of our samples. These results suggest that temporary induction of gibberellin synthesis genes observed in dormant IS9530 grains, which may lead to a higher synthesis activity, is probably counterbalanced by a higher gibberellin inactivation rate as deduced by strong expression of *SbGA2ox1* and *SbGA2ox3* (**Fig. 4c, d**).

A different pattern was observed for RedlandB2: transcript levels of several gibberellin biosynthesis genes remained almost unchanged (*SbEKO* and *SbGA20ox1*) or increased smoothly (*SbGA20ox3*) between days 1 and 3, and were induced by day 4 (**Fig. 4c**). At this time point (i.e. 1 d prior to completion of germination of the first grains, **Fig. 4a**), expression values for both *SbGA20ox1* and *SbGA20ox3* had increased in RedlandB2 and were higher than those observed in the more dormant IS9530 (that had been decreasing since day 2 or 3; **Fig. 4c**). In parallel, transcript levels of the inactivation genes *SbGA2ox1* and *SbGA2ox3* either increased slightly or remained unchanged in RedlandB2 as compared with previous days, and most of the time values were below those observed for IS9530 (**Fig. 4d**). The 6-fold increase observed for *SbGA20ox3* and *SbGA20ox1* between days 1 and 4 in RedlandB2 (**Fig. 4c**) suggests that the rise of GA_4 levels in this line in a similar experiment (**Fig. 3**) might result from an increased synthesis activity in combination with reduced inactivation rates. Expression profiles for *SbGA3ox1* and *SbGA3ox2* did not relate to this possible increase in gibberellin synthesis, but might also not be limiting the gibberellin biosynthetic rate. This possibility is in accordance with Huang et al. (1998) who reported higher active gibberellin levels in Arabidopsis transgenic plants overexpressing *AtGA20ox*.

Expression profiles for most of these genes were also obtained for grains from the same crop but harvested at 42 DAP (**Supplementary Fig. S2**). Among all gibberellin biosynthetic genes, expression of *GA2ox* genes corresponded best with changes in dormancy occurring between 30 and 42 DAP (**Supplementary Fig. S2a**): the observed induction of *SbGA2ox1* and *SbGA2ox3* in 30 DAP IS9530 grains was absent at 42 DAP (**Supplementary Fig. S2c**) and expression of *SbGA2ox3* was further reduced in RedlandB2 as compared with 30 DAP. Hormonal data at 42 DAP are lacking, but gibberellin *de novo* synthesis was still contributing to promote germination in RedlandB2 (GI was reduced from 69 in water to 39 in the presence of 100 p.p.m. PBZ).

Expression of a sorghum gene encoding the putative gibberellin receptor GID1 was also analyzed in this experiment (**Fig. 4b**), as Arabidopsis homologs *AtGID1a* and *AtGID1c* have been observed to be necessary for germination. Transcript levels of *SbGID1* increased sharply during the first 2 d in IS9530 grains and decreased by day 4 to the same level as in RedlandB2.

Evolution of GA_8 and GA_{34} (catabolites) as related to active gibberellin levels and gene expression profiles in imbibed, immature grains

Inactivation of active C_{19}-gibberellins is catalyzed by GA2oxs. Transcript levels of two sorghum genes encoding putative GA2oxs, *SbGA2ox1* and *SbGA2ox3*, were induced in IS9530 grains after imbibition (**Fig. 4d**), with maximum expression values observed between days 2 and 4 (at day 2, *SbGA2ox1* transcript levels were >4-fold higher than those observed in RedlandB2). A higher expression of *SbGA2ox3* in IS9530 vs. RedlandB2 was also observed in two other independent experiments in different years (data not shown). These results suggested that the inactivation rate might be increased in dormant IS9530 grains, resulting in lower gibberellin levels as compared with RedlandB2. This possibility was assessed by quantifying both GA_4 and GA_1 and their respective catabolites, GA_{34} and GA_8 (**Fig. 5a**). Temporal changes in GA_4 levels were accompanied by opposing changes in GA_{34}, and this was observed in both lines. The observed increase in GA_4 content (from 96 ng g^{-1} DW on day 3 to 255 ng g^{-1} DW on day 4) in RedlandB2 was accompanied by a proportional decrease in GA_{34} (from 207 to 64 ng g^{-1} DW; **Fig. 5a**, numerical data are shown in **Supplementary Table S2**). Indeed, GA_{34} content was negatively correlated with GA_4 values observed in grains that had been imbibed between 1 and 4 d (**Fig. 5b**). This negative correlation was significant in both lines, with a P-value <0.02, and supports the notion that GA_4 levels during incubation are regulated, at least in part, by conversion into GA_{34}.

Effect of exogenously applied GA_{3+4} on transcript levels of gibberellin metabolism genes and *SbGID1*

Negative feedback regulation at the transcriptional level for several gibberellin biosynthesis genes has been reported in

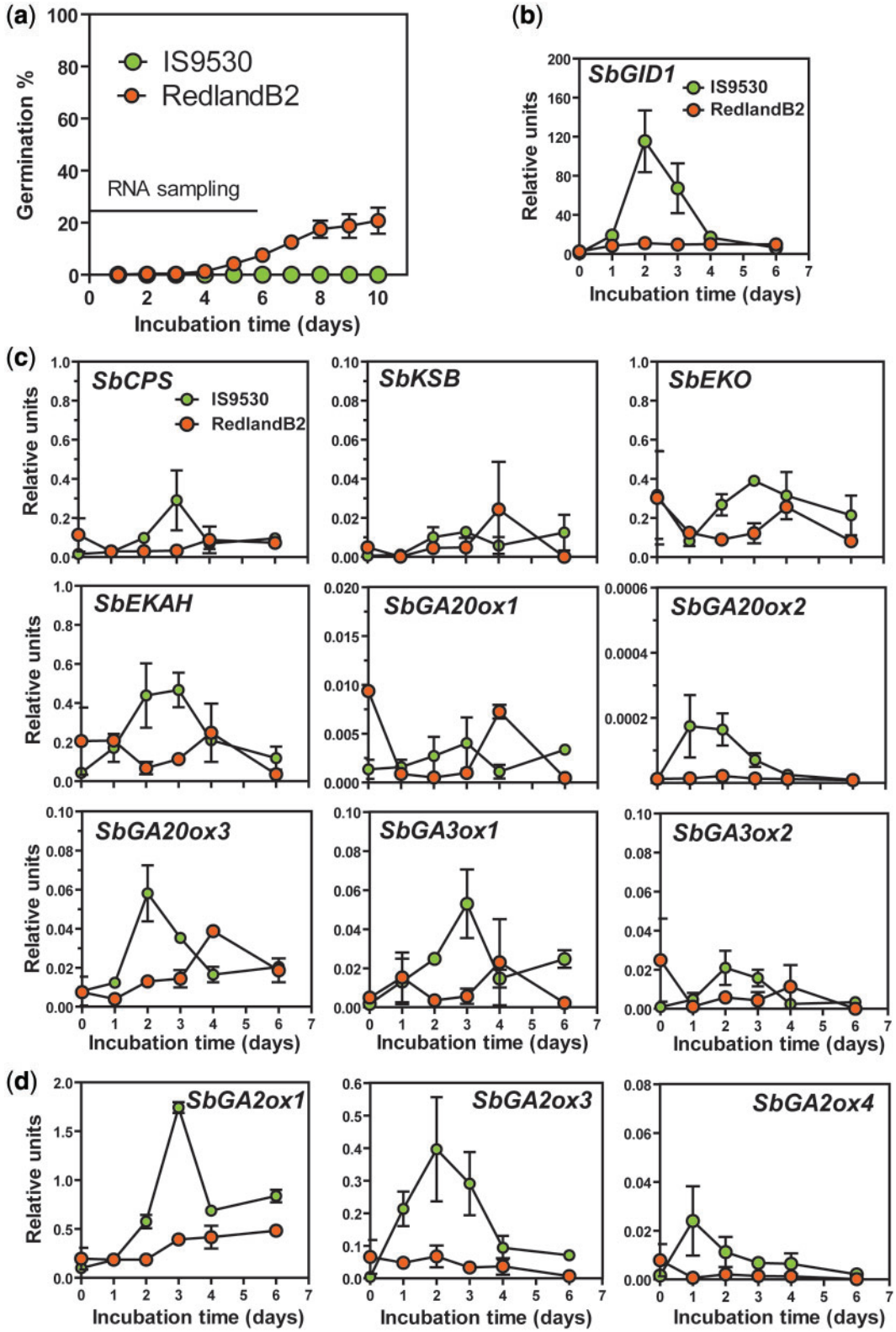

Fig. 4 Embryonic transcript levels for gibberellin metabolism genes and *SbGID1* (gibberellin receptor) during incubation of immature (30 DAP) sorghum grains from IS9530 (green circles) and RedlandB2 (red circles). The germination response is shown in (a). Transcript levels (relative to *SbActin*) are shown for *SbGID1* (b), putative gibberellin biosynthesis genes (c) and putative gibberellin inactivation genes (d). Incubation was done at 20°C. Each data point is the average of two biological replicates (two field plots). Bars indicate the SEM.

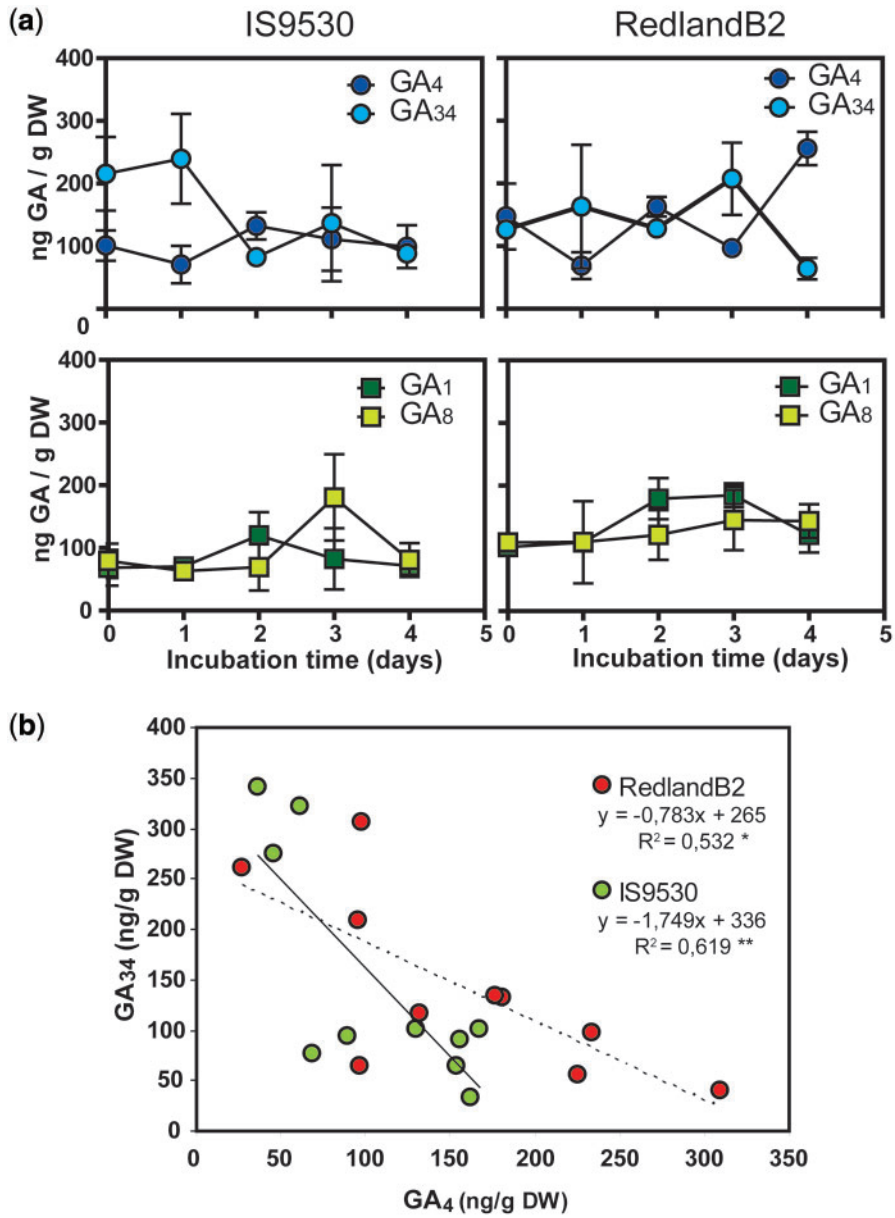

Fig. 5 Embryonic levels of active gibberellins (GA_4 and GA_1) and their respective catabolites (GA_{34} and GA_8) in imbibed sorghum grains (30 DAP) from IS9530 and RedlandB2. (a) Evolution of GA_4 and GA_{34} (upper panel) and GA_1 and GA_8 (lower panel) during grain incubation at 20°C. Germinated grains were first observed in RedlandB2 on day 6. Each data point is the mean of three replicates of 120 embryos, and bars indicate the SEM. (b) Linear regression between GA_4 and GA_{34} embryonic content in IS9530 (green circles) and RedlandB2 (red circles) grains imbibed between 1 and 4 d. Each data point is a biological replicate. *P*-values of regression coefficients (two-tailed test) were 0.0167 for RedlandB2 and 0.0069 for IS9530.

different species (Hedden and Phillips 2000, Pimenta Lange and Lange 2006, Dai et al. 2007). We tested whether the existence of a similar mechanism in sorghum accounted for the higher expression of *SbGA20ox* and *SbGA3ox* genes observed in IS9530 dormant grains as compared with RedlandB2. Incubation of dormant IS9530 grains (30 DAP) in a 100 μM GA_{3+4} solution enhanced germination; however, the expression of *SbGA20ox1*, *SbGA20ox3*, *SbGA3ox1* and *SbGA3ox2* was not clearly reduced (and in some cases it was even enhanced, such as *SbGA20ox1* after 2 d) as compared with the control in water (**Fig. 6**). A reduced expression according to a negative regulatory mechanism was observed only for *SbGA20ox2* on days 1 and 2. So far, with the exception of *SbGA20ox2*, our data do not support the existence of a negative feedback regulatory mechanism controlled by active gibberellin levels as clearly as has been reported for other gibberellin biosynthetic genes in other species.

The existence of a feed-forward regulatory mechanism of *AtGA2ox* genes involved in C_{19}-gibberellin 2-oxidation has been reported to occur in seeds (Ogawa et al. 2003) and

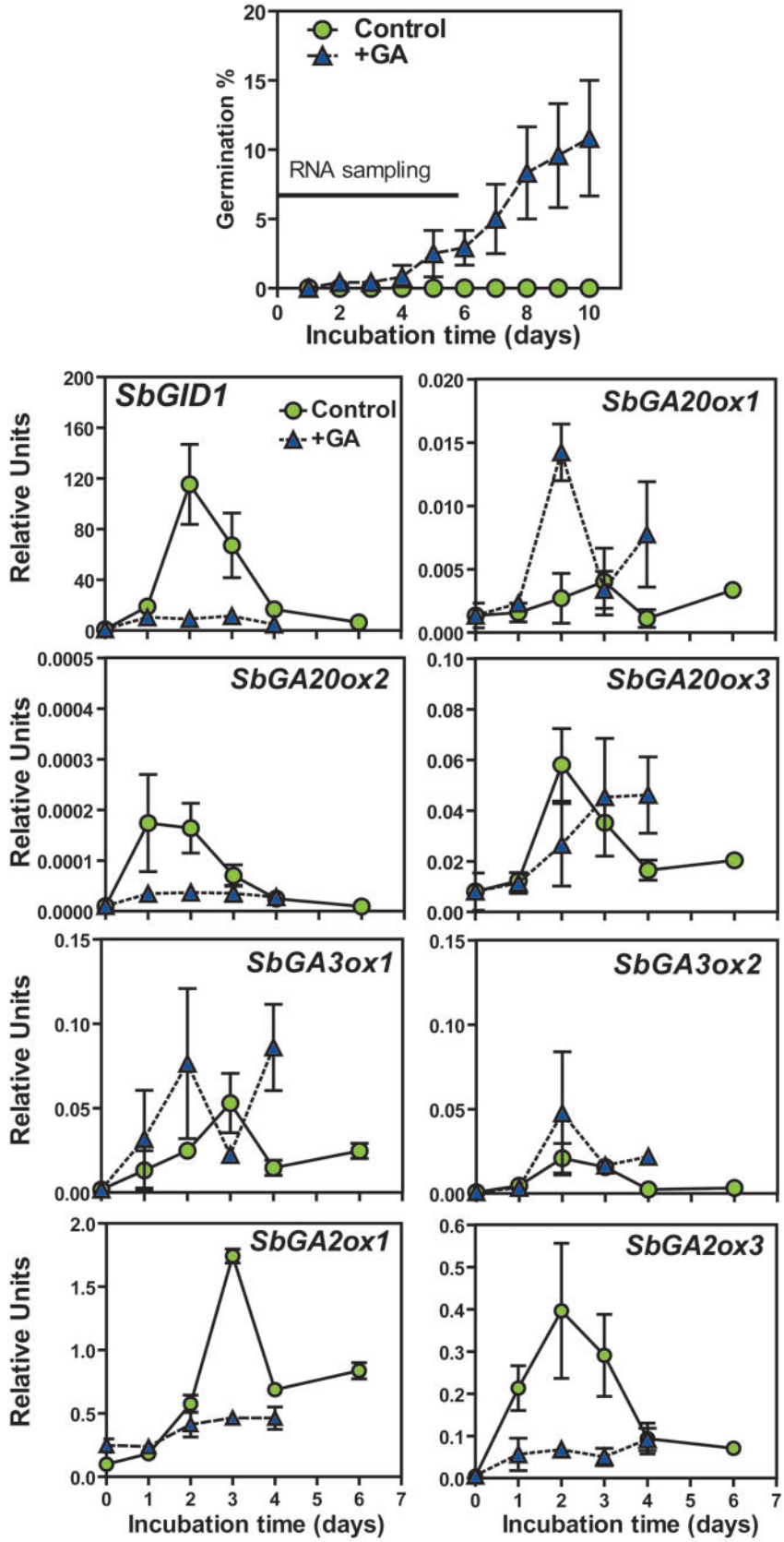

Fig. 6 Effect of applied GAs on embryonic expression of sorghum genes encoding putative 2-ODDs (soluble di-oxygenases) and *SbGID1*. IS9530 immature grains (30 DAP) were incubated at 20°C either in water (circles) or a 100 μM GA_{3+4} solution (triangles). GA_{3+4} solution contained 96% GA_3 and 4% GA_{4+7}. Evolution of the germination percentage is shown in the upper panel. Relative units are gene expression values relative to *SbActin*. Each data point is the average of two biological repetitions (seeds sampled from two field plots). Bars indicate the SEM.

seedlings (Rieu et al. 2008). In sorghum immature (30 DAP) grains, the opposite was observed for both *SbGA2ox1* and *SbGA2ox3* (**Fig. 6**). Treatment with 100 μM GA_{3+4} interfered with the induction observed in IS9530 grains during the first 3 d of incubation in water (**Fig. 6**), and the resulting patterns resembled those observed for *SbGA2ox1* and *SbGA2ox3* in RedlandB2 incubated in water (**Fig. 4d**).

Both Arabidopsis homologs *AtGID1a* and *AtGID1c* are transcriptionally repressed in seeds incubated with GA_{4+7} (Voegele et al. 2011). A negative regulation of sorghum *GID1* by gibberellins was also tested in this experiment (**Fig. 6**). In accordance with this possibility, *SbGID1* was inhibited by 100 μM GA_{3+4} in IS9530, and values were close to those observed in RedlandB2 incubated in water (**Fig. 4b**).

Discussion

We investigated the transcriptional regulation of putative gibberellin metabolism genes during the expression of dormancy in developing sorghum grains by comparing two inbred lines with contrasting sprouting behavior (IS9530, sprouting resistant; and RedlandB2, sprouting susceptible). A contribution to germination by gibberellin *de novo* synthesis was evidenced most clearly for RedlandB2 before physiological maturity (i.e. before 40 DAP), as suggested by experiments with gibberellin biosynthesis inhibitors added to the incubation medium (**Fig. 1**). Therefore, we focused our experiments for both hormone and gene expression analysis on immature grains (30 DAP). This moment is also of interest because it is at approximately between 20 and 40 DAP that RedlandB2 grains begin to lose dormancy precociously, accompanied by a marked decrease in embryo sensitivity to ABA (Rodríguez et al. 2009).

Transcriptional data obtained in sprouting-resistant IS9530 grains support the existence of an early activity of gibberellin synthesis which is promptly suppressed by an increased deactivation rate, maintaining low gibberellin levels in this line. A higher deactivation rate was suggested by a strong promotion of gibberellin inactivation genes (*SbGA2ox1* and *SbGA2ox3*) at the same time as we observed an increased expression of two gibberellin synthesis genes (*SbGA20ox1* and *SbGA20ox3*; **Fig. 4**) during early incubation of dormant IS9530 grains, which did not lead to an increase in active gibberellin levels (**Fig. 3**). Nevertheless, overall GA_{34} levels were not found to be higher in IS9530 as compared with RedlandB2 during this incubation period (**Fig. 5**). This might be explained by the fact that GA_{34} is the first expected product after C_{19}-gibberellin 2-oxidation of GA_4 substrate, and GA_{34} is further converted by GA2ox into the GA_{34} catabolite through a second oxidative step (Hedden and Phillips 2000). In contrast, a different coordination of gibberellin synthesis and deactivation processes seems to operate in immature grains of RedlandB2. Expression of the gibberellin biosynthetic genes *SbGA20ox1* and *SbGA20ox3* was observed to increase towards later stages of imbibition phase II, while gibberellin inactivation genes *SbGA2ox1* and *SbGA2ox3* remained low and stable throughout incubation. This pattern is in accordance with the increase in GA_4 content observed in this line after 4 d of incubation (2 d before radicle growth was initiated; **Fig. 3**).

Although, as mentioned before, we did not find overall higher levels of GA_{34} in dormant IS9530 as compared with RedlandB2, GA_{34} values corresponded to simultaneous and opposing changes in GA_4 throughout incubation for both genotypes (**Fig. 5**). Indeed, the increase in GA_4 content observed on day 4 in RedlandB2 grains was coupled to a similar drop in GA_{34}. The existence of a negative correlation between GA_4 and GA_{34} indicates that the inactivation process is actively regulating GA_4 levels, and that the gibberellin catabolic rate is not a direct consequence of gibberellin levels as has been described for mature, non-dormant Arabidopsis seeds (Ogawa et al. 2003). Transcriptional and hormone analysis by Ogawa et al. (2003) showed that increased synthesis of active gibberellins, rather than reduced deactivation activity, is what leads to the increase in GA_4 levels during wild-type seed germination. These authors observed that all *AtGA2ox* transcripts remained at low levels before radicle emergence, suggesting that altered *AtGA2ox* transcript abundance is not the principal mechanism for the increase in GA_4 levels. Also, the curve for GA_{34} nearly paralleled that for GA_4, suggesting that the formation of GA_{34} is dependent on the amount of GA_4. Indeed, in another work, transcript abundance of four *AtGA2ox* genes increased in response to applied GA_3 in plants (Rieu et al. 2008), further demonstrating the existence of a feed-forward regulatory mechanism in Arabidopsis. A similar regulatory mechanism seems to operate in rice seedlings: *OsGA2ox3* expression was enhanced in seedlings overexpressing *YAB1* (which is normally enhanced by active gibberellins, and represses *OsGA3ox*), supporting the existence of a feed-forward regulatory mechanism involved in regulation of *OsGA2ox3* (Dai et al. 2007). In contrast to these observations with Arabidopsis and rice, incubation of dormant IS9530 grains in 100 μM GA_{3+4} prevented the induction of *SbGA2ox1* and *SbGA2ox3* otherwise observed during the first 3 d of incubation in water (**Fig. 6**).

Malting barley has also served as a monocot model for research in dormancy, usually by comparing freshly harvested (dormant, while stored at −20°C) and after-ripened (non-dormant) mature grains. Expression of dormancy in barley involves the capacity to maintain high embryonic ABA levels in the imbibed grain (Benech-Arnold et al. 2006) and this is related to the repression of an ABA inactivation gene, *HvABA8′OH* (Millar et al. 2006). In contrast, gibberellin metabolism has been suggested not to play a role in the expression of contrasting levels of dormancy: gibberellin-deficient barley mutants germinate normally (Chandler and Robertson 1999), and Jacobsen et al. (2002) observed that GA_1 content increased after germination but not before. Additionally, transcriptional analysis of gibberellin metabolism genes in dormant and after-ripened barley grains does not support a role for gibberellin metabolism in dormancy release (Barrero et al. 2009).

Nevertheless, all these works compare two 'extreme' states, and not intermediate states during after-ripening and as embryo sensitivity to ABA decreases, as has been reported by Benech-Arnold et al. (1999). In this work, treatments with PBZ increased dormancy of a sprouting-susceptible barley cultivar, suggesting a role for gibberellins in counterbalancing the effect of ABA.

Expression results with *GA20ox1* and *GA20ox3* obtained in this work are in accordance with results reported previously by our group (Pérez-Flores et al. 2003). In both cases, expression of a *GA20ox* gene during incubation was observed to increase in RedlandB2 grains together with an increase in GA_4. Nevertheless, comparisons between expression profiles in both studies should consider that PCR primers used in the previous work were not able to distinguish between transcripts from both *SbGA20ox1* and *SbGA20ox3* (only the first of these was partially cloned and sequenced; *SbGA20ox3* remained unknown until recently). In the present work, three sorghum homologs encoding GA20oxs were identified and analyzed separately by quantitative PCR. Different timing in gene expression and hormone levels can also be expected to occur, as grains used in both studies belonged to different developmental stages (30 and 40 DAP) and exhibited different degrees of dormancy. As field experiments also imply variability in environmental conditions that might affect the processes under study, to have a more robust scenario, crop cultivation and incubation experiments were repeated with immature grains (30–35 DAP; before PM) in three different years. Gene expression for several genes and also changes in active GA_4 content showed similar tendencies to those presented here (data are shown only for 1 year).

It is known that some GA20oxs have a catalytic function. Radi et al. (2006) demonstrated that two pumpkin GA20oxs (encoded by *CmGA20ox1* and *CmGA20ox2*) can convert gibberellin precursors GA_{12} and GA_{53} to non-active GA_{25} and GA_{17}, which are subsequently converted into inactive catabolites. Positive conversion of GA_{12} and GA_{53} into GA_{20} leading to active gibberellins in pumpkin seeds is carried out by a third GA20ox, CmGA20ox3. Alignment of sorghum and pumpkin GA20oxs shows that sorghum sequences are closely related to CmGA20ox3 and other known GA20oxs (**Fig. 2c**), and clearly separated from CmGA20ox1 and CmGA20ox2. This supports a functional similarity between sorghum GA20oxs reported here and GA20oxs whose activity leads to active gibberellin products.

Several authors have reported the existence of a negative feedback regulation at the transcriptional level for several gibberellin synthesis genes (Hedden and Phillips 2000, Pimenta Lange and Lange 2006, Dai et al. 2007). We tested whether the existence of a similar mechanism in sorghum accounted for the higher expression of *SbGA20ox* and *SbGA3ox* genes observed in IS9530 dormant grains as compared with RedlandB2. Incubation of dormant IS9530 grains (30 DAP) in a 100 μM GA_{3+4} solution enhanced germination but, apart from *SbGA20ox2*, expression of *SbGA20ox1*, *SbGA20ox3*, *SbGA3ox1* and *SbGA3ox2* was not clearly reduced as compared with the control in water (**Fig. 6**). In contrast, transient increases in transcript abundance in response to gibberellin treatment were observed for some of these genes. So far, our data do not support the existence of a negative feedback regulatory mechanism controlled by active gibberellin levels as clearly as has been reported in other species. In addition, endogenous GA_1 and GA_4 levels were not different between both lines during the first 3 d of incubation, and therefore cannot be related to the higher expression of *GA20ox1*, *GA20ox2* and *GA3ox* found in IS9530 as compared with RedlandB2 within this period of time.

Gibberellins are recognized by a soluble receptor, GID1/DWARF1 (Ueguchi-Tanaka et al. 2005). Three homologs for *GID1* exist in Arabidopsis, and two of them (*AtGID1a* and *AtGID1c*) are required for germination (Voegele et al. 2011). Expression of *GID1* appears to be repressed in imbibed seeds committed to germination, as observed in Arabidopsis seeds incubated with GA_{4+7} (Voegele et al. 2011) and in barley after-ripened grains imbibed for 18 h (Barrero et al. 2009). In our experiments, expression of *SbGID1* agrees with these observations and further supports a negative regulation of this gene by active gibberellins and/or a low dormancy state (in which gibberellin levels may be low, but a higher gibberellin/ABA signaling balance results from low sensitivity to ABA).

Despite the fact that we observed that exogenously applied gibberellins reduced the expression of *SbGA2ox1* and *SbGA2ox3* in IS9530 grains, endogenous gibberellin levels are not likely to be the reason for higher expression of these genes in dormant IS9530 grains. Among other possible factors (different from endogenous gibberellin levels) regulating the expression of *SbGA2ox1* and *SbGA2ox3* in dormant IS9530 grains, ABA signaling could be involved. At the developmental stage addressed in this work, embryos of dormant IS9530 grains display a strong sensitivity to ABA. In contrast, in less dormant RedlandB2, ABA sensitivity (and expression of ABA signaling components) is reduced (Rodríguez et al. 2009). *In silico* analysis of a 557 bp sequence upstream of the transcription initiation point of *SbGA2ox3* led to the identification of several *cis*-regulatory elements related to ABA and gibberellin signaling (**Supplementary Fig. S3**). A gibberellin down-regulatory element was found at position −196 upstream of the TATA box. This, together with the presence of several ABA-related elements (RY repeat, CE and ABRE) within the promoter region close to the TATA box, suggested that the expression of *SbGA2ox3* might be enhanced by ABA and repressed by gibberellin-dependent factors. In a recent work, we reported the expression profiles of a set of sorghum putative ABA signaling candidate genes including *SbABI5*, *SbABI4*, *SbPKABA1* and *SbVP1/ABI3*. All of them were induced in the embryo during incubation of IS9530 dormant grains and peaked around days 2 and 3 in a similar experiment to that shown here, and this pattern was also followed by sorghum ABI5 protein (Rodríguez et al. 2009). These patterns coincide with the

observed profiles of *GA2ox* genes analyzed in this work, and suggest a possible regulation of *GA2ox* genes by ABA signaling transcription factors. Future work is needed to explore this possibility.

Whether a direct interaction between ABA signaling and gibberellin metabolic pathways occurs or not in sorghum grains, it is clear by now that these two hormones act antagonistically both in the inception of dormancy during early development and in the expression of dormancy. White et al. (2000) worked with developing maize caryopses and proposed that a gibberellin signal early in embryogenesis establishes a competence for vivipary which is counterbalanced by an ABA signal. Maize kernels are viviparous if the ABA signal is reduced or abolished (as in the *vp-5* mutant, which is ABA deficient, or with the application of an ABA biosynthesis inhibitor) and normal dormancy is restored in the *vp-5/d1* double mutant (impaired in both ABA and gibberellin synthesis); these seeds also survive desiccation and germinate. Although sorghum differs from maize in the presence of a more prolonged dormancy (and has no vivipary, even when treated with an ABA biosynthesis inhibitor), both species are closely related and can be expected to share similar mechanisms regarding the control of the maturation vs. germination pathways. In sorghum, the lack of response to gibberellin biosynthesis inhibitors observed for non-dormant RedlandB2 strongly suggests that, similar to maize, gibberellin *de novo* synthesis is no longer required for germination when sensitivity to ABA becomes sufficiently low, and pre-existing gibberellins may be enough or even unnecessary to trigger germination. A similar pattern can be observed for IS9530, although both response to PBZ in the incubation medium (**Supplementary Fig. S1**) and embryo loss of sensitivity to ABA (Rodríguez et al. 2009) are displaced towards later developmental stages.

The promotion of dormancy by treatments with PBZ and PHD in sorghum grains might result from additional changes in other hormones, such as an increase in ABA levels (due to inhibition of ABA 8′-hydroxylase by PBZ) or a decrease in ethylene synthesis [due to inhibition of ACC (1-aminocyclopropane-1-carboxylate) oxidase by PHD; Rademacher 2000]. Nevertheless, quantification of embryonic ABA in developing sorghum grains showed that ABA levels were not affected in grains from panicles that had been sprayed with PBZ during early development (Steinbach et al. 1997). In contrast, gibberellin levels in 37 DAP RedlandB2 grains were severely reduced by a similar treatment with PBZ (Benech-Arnold et al. 2003). Increased dormancy in grains coming from PBZ-sprayed panicles was completely reverted by incubation in a 100 μM GA_3 solution (**Fig. 1b**). In addition to gibberellins, ethylene also antagonizes ABA inhibition of germination and promotes endosperm cap rupture necessary for germination of Arabidopsis and *Lepidium sativum* (garden cress) seeds (Linkies et al. 2009). Previous experiments in which germination of 30 DAP grains of IS9530 and RedlandB2 was not promoted by incubation in 0.1 mM ACC suggested that ethylene is not involved in the expression of dormancy at this stage (unpublished results), allowing us to discard effects of PHD through possible changes on ethylene levels. In addition to its effect on GA20ox activity, PHD blocks gibberellin inactivation by GA2oxs, as all these enzymes use 2-oxoglutarate as co-substrate (Rademacher et al. 2000). This may account for the smaller effect of PHD in our system, where both gibberellin synthesis and inactivation pathways have a regulatory role. Although side effects for PBZ and for PHD cannot be ruled out (even in the case where the germination response is restored to control levels by addition of gibberellins), evidence in sorghum grains supports that the main observed effects on germination are related to the inhibition of gibberellin biosynthesis.

The tissues surrounding the embryo have an active role in the hormonal control of embryo growth. For example, ABA degradation upon imbibition is related to increased activity of ABA 8′-hydroxylases in the endosperm cap of Arabidopsis seeds (Okamoto et al. 2006) and in the coleorhiza of barley non-dormant grains (Barrero et al. 2009). Germination of Brassicaceae species such as *L. sativum* and Arabidopsis involves both endosperm cap weakening and increased growth potential that lead to radicle protrusion through the ruptured endosperm. In a model proposed by Linkies et al. (2009), endosperm cap weakening in *L. sativum* seeds is initiated by gibberellins from the embryo and further promoted by ethylene, in parallel with degradation of ABA in the endosperm cap. Oracz et al. (2011) carried out a detailed analysis of gibberellin metabolism in this species: the authors showed that gibberellin metabolism is active in both the radicle and endosperm cap, but the main active gibberellin species produced, GA_4 and GA_6, and the expression of gibberellin metabolism genes differed between both organs. In germinating barley grains the coleorhiza cells separate and elongate accompanying early radicle growth, and this organ is finally disrupted as the radicle continues to expand (Barrero et al. 2009). Tissue-specific transcriptome analysis by these authors showed that the expression of ABA metabolism and signaling genes in the coleorhiza correlates with the germination response of dormant and non-dormant (after-ripened) barley grains (Barrero et al. 2009). Our work in sorghum has not considered tissue-specific studies so far, but complex radicle–coleorhiza cross-talk is expected to exist. As all measurements were done with whole embryos (including the coleorhiza), we cannot discriminate possible differences in organ-specific regulation of hormone and transcript levels presented here.

To conclude, alignments show that gibberellin metabolism enzymes are highly conserved in their structure and probably in their function. Nevertheless, interspecific variability exists regarding regulation of these genes and/or their protein products. It is important to identify the regulatory steps operating in different species of agronomic importance in order to develop breeding strategies leading to improvement in their performance or quality. Our work presents new evidence on the genes involved in the regulation of gibberellin levels during the expression of grain dormancy (i.e. when processes leading to germination are activated or repressed) and within the context of

natural variability for dormancy and PHS behavior. To our knowledge, this is the first time that transcriptional regulation of gibberellin metabolism has been addressed in immature seeds, and in relation to dormancy and sprouting behavior. Moreover, the results presented here improve our knowledge on potential regulatory sites that contribute to intraspecific variability in dormancy and pre-harvest sprouting in sorghum.

Materials and Methods

Plant material

Two sorghum [*S. bicolor* (L.) moench] inbred lines displaying contrasting patterns of dormancy release, IS9530 (sprouting resistant, highly dormant until well after PM) and RedlandB2 (sprouting susceptible, low dormancy even prior to PM), were sown in the experimental field of the Facultad de Agronomía de la Universidad de Buenos Aires, Argentina, during summer of 2003/2004, 2006/2007 and 2009/2010. Three plots 24 m^2 each were sown for each genotype in a completely randomized design. Anthesis date (anthers exposed in the middle third of the panicle) was recorded for each plant. For each experiment, seeds from the middle third of the panicle were harvested from 8–10 plants with the same anthesis date and within the same field plot, and pooled together. Two field plots were used as biological repetitions for each genotype in gene expression analysis. Experiments began on the same day as the seeds were harvested. For hormone analysis, three separate groups of plants were pooled into three biological repetitions. To obtain samples of embryos for RNA and hormone analysis, sufficient Petri dishes (9 cm diameter, plus 6 ml of distilled water and two discs of filter paper) containing 50 seeds each were incubated in chambers with constant temperature of 20°C. Incubation chambers had no artificial light, but seeds incubated in transparent plastic Petri dishes were repeatedly exposed to ambient light during scoring events. Sampling for RNA and hormone extraction was carried out as follows: after different incubation periods, embryos (embryonic axis plus scutellum) were dissected carefully with a scalpel from the surrounding tissues (endosperm, testa and pericarp), flash-frozen in liquid N_2 and stored at −80°C until processed. Germinated grains (visible radicle) were not sampled; therefore, only embryos from grains that had not completed germination (and, thus, were still on phase II of imbibition) were dissected and stored.

Incubation experiments were done with immature grains harvested at 30 and at 42 DAP. Whole grains from IS9530 were also incubated in 100 μM GA_{3+4} solutions. GA_{3+4} solution (100 μM) contained: 96% GA_3 and 4% GA_{4+7} (all gibberellins from Sigma). Incubation experiments in water for RNA extraction were repeated in three different years with similar results. Simultaneously with each experiment for RNA and hormone extraction, grains were also incubated (three replicates of 50 seeds on two layers of filter paper plus 6 ml of distilled water) under the same experimental conditions. Germination (radicle protrusion) was scored daily and expressed as a percentage of total grains.

Treatments with PBZ (Crestar, Zeneca Agricultural) were done as follows: during the 2003/2004 campaign, within each plot, several panicles of both lines were sprayed repeatedly at 3, 7 and 11 DAP with PBZ solution (1 g l^{-1}), and control panicles were sprayed with water. For germination assays, a lower concentration was used (100 mg l^{-1}).

To follow dormancy release during seed development, a germination index (GI; Steinbach et al. 1995) was calculated as follows:

$$GI = [12 \times (n_i = 1) + 11 \times (n_i = 2) + 10 \times (n_i = 3) + \ldots + 1 \times (n_i = 12)]/(N/10)$$

where n_i is the number of seeds germinated on day 'i' of incubation, and N is the number of incubated seeds. Each GI value was obtained from a 12 d assay in which germination was scored daily. Values for GI can range from zero (no germinated seeds after 12 d) to 120 (all seeds germinated on the first day). Incubation temperature for these experiments was 25°C.

In silico search for sorghum sequences encoding putative orthologs for gibberellin metabolism enzymes and phylogenetic analysis

Identification of sorghum sequences with high identity to the reference genes was done by searching the PlantGDB *S. bicolor* database (sorghumGDB, http://www.plantgdb.org/). The identification codes for the selected sorghum sequences are shown in **Supplementary Table S1** and the alignments between the predicted sorghum and reference proteins are shown in **Supplementary Fig. S4**. Alignments of protein sequences were performed using the CLUSTALW program (Thompson et al. 1994) with default parameters. Phylogenetic analyses were done using PhyML Best AIC Tree package in order to find the best AIC-protein model over all available PhyML (Guindon and Gacuel 2003). The resulting phylogenetic trees were edited with ETE software (Huerta-Cepas et al. 2010). All analyses were performed using Phylemon2 suite, http://phylemon.bioinfo.cipf.es/. (Tarraga 2008, Sánchez et al. 2011).

RNA extraction and real-time quantitative PCR

Between 30 and 45 embryos (70–100 mg) per sample were ground to powder with a mortar and pestle in liquid N_2, and added to 600 μl of the RA1 extraction buffer included in the Nucleospin RNA plant mini-kit (Macherey-Nagel) for total RNA extraction. PVP-40 (Sigma) was added to the RA1 buffer to a final concentration of 1% (wv). After 5 min centrifugation, clear supernatant was used in the extraction protocol as described in the kit's manual. RNA quality was assessed in a 1% agarose gel stained with ethidium bromide. After reverse transcription (Promega reactives and dT15 primer), cDNA was diluted 1:5 in water, and used as template for quantitative real-time PCR, in an ABI7500 (Applied Biosystems) with Roche SYBR-green

master mix. PCR standard conditions for ABI7500 were used. Each sample consisted of 150–200 seeds pooled together from plants in a single field plot, and incubated for a certain period of time (two biological repetitions—field plots—were done for each genotype). Two replicates for each PCR were pipetted within the same 96-well PCR plate. The 'quantitative method' as described in the ABI7500 user's manual was used, with standard curves for each amplicon. After a first PCR trial, the size and unicity of the product was checked in an agarose gel, and the excised amplicon was purified (Nucleospin Extract kit, Macherey-Nagel) and used to create a set of serial dilutions (1 : 10 each step) covering a wide range of Ct values (usually ranging from 16 to 32–33). The relative number of copies obtained for each cDNA species was normalized against *SbActin* transcript values. *SbActin* levels (data not shown) did not change significantly during incubation of whole grains or in response to hormone treatments in imbibed caryopses (Rodríguez et al. 2009). Data points on each graph are the mean of two biological repetitions (two field plots), and bars in all graphs indicate the SE. To make comparisons among different genes possible, an additional quantitative PCR run was made using a few samples as template but tested simultaneously with all primer pairs (including *SbActin*) in the same plate. Differences in Ct values for the different genes in the same sample were used to compare their expression level.

Primers for quantitative real-time PCR were designed with the Primer Express software (Applied Biosystems), and primer sequences for each gene are listed in **Table 1**.

Gibberellin extraction, purification and quantitation

Gibberellin analysis was carried out with IS9530 and RedlandB2 grains (30 DAP) from plants sown in 2009 and harvested in 2010. Intact caryopses were incubated in distilled water for various times. After each incubation period, three samples (biological triplicates) of 150 embryos were excised from grains of each genotype and frozen in liquid N_2. Samples were freeze-dried, ground to powder with a mortar and pestle and weighed (~300–400 mg per sample). Gibberellin extraction was performed with 5 ml of methanol : water (80 : 20) at 4°C. After centrifugation (15 min, maximum speed), the buffer was collected and the pellet was then re-extracted with 2 ml of fresh buffer for an additional 4 h. A 50 ng aliquot of each of deuterated GA_1, GA_4, GA_8 and GA_{34} (L. Mander, Australian National University, Canberra, Australia) was added as internal standards. After methanol evaporation in a speed-vac, the volume of the remaining aqueous fraction was adjusted with water to 15 ml, and the pH was lowered to 2.5 with diluted HCl. The 15 ml aqueous extract was then partitioned three times against 5 ml of ethyl acetate (water saturated). After solvent evaporation, samples were taken to the University of Río Cuarto (Córdoba, Argentina) for hormone analysis. The ethyl acetate fraction was resuspended in 50 µl of MeOH (100%), placed in specific vials and 10 µl of each sample was injected and GA_1, GA_4, GA_8 and GA_{34} determined by liquid chromatography with electrospray ionization (LC) (Waters) coupled to a tandem mass spectrometer (MS/MS) (Micromass) monitored with the Masslink Software 4.1. All gibberellin data are expressed as ng of gibberellin g^{-1} DW.

Supplementary data

Supplementary data are available at PCP online.

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