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Seed dormancy QTL identification across a *Sorghum bicolor* segregating population

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Abstract Pre-harvest sprouting (PHS) in *Sorghum bicolor* is one of the main constrains for its production in the central region of Argentina, as grain maturation often coincides with rainy or high environmental humidity conditions. The obtention of more dormant genotypes with higher PHS resistance has always been a desirable trait for breeders but the typical quantitative nature of seed dormancy makes its manipulation difficult through classical breeding. Dissecting this quantitative variability into quantitative trait loci (QTL) is a main concern especially in cereal species. In this work, a sorghum segregating population including 190 families was genotyped with microsatellite markers and the *SbABI5* candidate gene. A genetic map encompassing 96 markers and a total length of 1331 cM was built. Seed dormancy was phenotyped in F_3 and F_4 panicles in two contrasting Argentinean environments (Castelar and Manfredi). Six seed dormancy QTL for mature grains were identified (*qGI-1*, *qGI-3*, *qGI-4*, *qGI-6*, *qGI-7* and *qGI-9*) with the aid of QTL Cartographer and QTLNetwork, three of them (*qGI-3*, *qGI-7* and *qGI-9*) being co-localised by both approaches. No epistasis was detected for the identified QTL but QTL-by-environment interaction was significant for *qGI-7* and *qGI-9*. Interestingly, seed dormancy candidate genes

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SbABI3/VP1 and *SbGA20ox3* were located within *qGI-3*, which makes them noteworthy candidate genes for this QTL.

Keywords Pre-harvest sprouting · QTL · Seed dormancy · *Sorghum bicolor* · SSRs

Introduction

Grain sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most relevant cereal worldwide in terms of production and acreage, after maize, wheat, rice and barley. Argentina is included among the first ten sorghum producers, being the fourth most important summer crop in the country, after soybean, maize and sunflower. Pre-harvest sprouting (PHS) is one of the main constrains for sorghum production in the central region of Argentina, as the grain filling period takes place during the end of summer and the beginning of autumn, when rainy or high environmental humidity conditions prevail. In general, genotypes prone to suffer PHS are those whose maturing grains are released from dormancy prematurely, sometimes even prior to physiological maturity (PM), thus germinating untimely in the mother plant if water becomes available (Rodríguez et al. 2015). PHS causes significant economic losses due to alterations in grain quality that result from both initiation of germination and fungi attack facilitation, which, in turn, can terminate seed viability. Resistance to PHS through the obtention of genotypes with a longer lasting dormancy has long been a desirable trait for breeders, though rarely evaluated or included in breeding programmes, because of the large amount of germination assays required for phenotyping. Moreover, the fact that seed dormancy is a typical complex trait makes its manipulation through classical breeding techniques difficult (Anderson et al. 1993).

Seed dormancy is governed by multiple genes and is clearly influenced by the environment to which the mother plant is exposed during seed development (Black et al. 1987; Nakamura et al. 2011; Biddulph et al. 2005; Gualano and Benech-Arnold 2009). Therefore, determining the genetic structure of dormancy in agronomically relevant species such as grain sorghum emerges as a priority, and one of the most appropriate approaches to achieve this goal is to dissect that variability into quantitative trait loci (QTL). In this sense, QTL analysis is a powerful tool to i) confirm the role of candidate genes whose participation has been suggested from physiological studies on a genetic basis and ii) advance in the discovery of new genomic regions, potentially useful for the manipulation of this trait. Also, QTL analysis should allow us to recognise which allelic variants are behind the existing intraspecific variability. In this direction, considering that not only dormancy imposition but also its expression is particularly coordinated in each species, and that environmental conditions affect this process, it is necessary to carry out species-specific genetic studies.

In the last years, many works have informed dormancy-related QTL, not only in the model species *Arabidopsis thaliana*, but also in agronomically relevant ones. Moreover, the molecular and genetic progress that has taken place during the last decade has allowed the identification of genes responsible for those detected QTL. Alonso-Blanco et al. (2003) identified the *DOG1* (Delay of Germination1) as a strong QTL for seed dormancy in *Arabidopsis*, and the high resolution mapping of this genomic region allowed the isolation of the *DOG1* gene (and its allelic variants), which is related to dormancy inception during seed maturation in this species (Bentsink et al. 2006). On the other hand, the *SDR4* QTL was identified and characterised in rice (Lin et al. 1998), and the responsible gene, *SDR4,* encodes a dormancy regulatory-specific protein, although its precise biochemical function is still unknown (Sugimoto et al. 2010). Dormancy QTL *SD1* and *SD2* were identified in barley (Han et al. 1996; Prada et al. 2004), with a *GA-20oxidase* being one of the proposed genes responsible for the last QTL. Later, Mori et al. (2005) reported a QTL located in wheat chromosome 3A for which Nakamura et al. (2011) identified

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TaMFT gene, a repressor of wheat germination, as the gene responsible for this QTL. Recently, Barrero et al. (2015) suggested *PM19-A1* and *PM19-A2* as the responsible genes for wheat major QTL located in chromosome 4AL, which had been identified across multiple populations (Mares and Mrva 2014).

Most of the research aiming to elucidate the basis of PHS resistance in sorghum has been carried out using the system composed by genotypes RedlandB2/IS9530. RedlandB2 is a sprouting-susceptible inbred line whose grains are released from dormancy well before PM; in contrast, IS9530 is a sprouting-resistant inbred line whose grains start to be released from dormancy after PM but at a slower rate than RedlandB2. Previous work with these lines showed that sprouting behaviour is well correlated to the caryopses dormancy depth and dormancy release rate during seed maturation (Steinbach et al. 1995). Other features, such as the panicle architecture, glumes or glumellae presence and seed tannin content, which could actually affect the relationship between dormancy and PHS susceptibility, have been shown not to be involved in IS9530/RedlandB2 PHS behaviour (Steinbach et al. 1995). Premature release from dormancy in RedlandB2 grains has been ascribed to a reduced sensitivity of their embryos to the germination inhibitory action of abscisic acid (ABA) and an abnormal accumulation of active gibberellins (GAs) (Steinbach et al. 1995, 1997; Rodríguez et al. 2009, 2012). Both processes (faulty ABA sensitivity and GA inactivation) have been found to be differentially regulated in this line at the transcriptional level of genes involved in ABA signalling and GA catabolism (Rodríguez et al. 2009, 2012). QTL searches for PHS resistance in immature sorghum grains were attempted previously by Lijavetzky et al. (2000) using a RedlandB2 x IS9530 segregating population. However, the population size used in that study and the impossibility to physically locate QTL flanking markers to move towards fine mapping make new QTL analysis necessary for sorghum seed dormancy. Also, phenotyping for sprouting tolerance in sorghum should consider the temporal pattern of dormancy release during grain development and maturation stages since different mechanisms might be operating, as reported by Rodríguez et al. (2012). In this context, phenotyping for QTL analysis related to the expression of dormancy in immature and mature grains (before and after PM) would be of great benefit, as the role of candidate genes could be confirmed, and new genes controlling different dormancy mechanisms both before and after PM could be discovered.

The recent progress that has taken place in grain sorghum has generated valuable genetic resources that can be used for the identification of molecular markers and for the detection of genes governing different agronomic relevant traits. In this context, a fundamental event of recent years was the concretion of sorghum line BTx623 genome sequencing (Paterson et al. 2009), which immediately led to the generation of a physical map for this species. Among the five most relevant cereals worldwide, the sorghum genome was the smallest (750 Mb), after rice. On the other hand, during the last decades, different types of molecular markers have been developed, including RFLPs, AFLPs, RADPs, SSRs, DArTs and SNPs, which have been successfully used in the development of genetic maps for grain sorghum (Bhattramakki et al. 2000; Haussmann et al. 2002; Menz et al. 2004; Mace et al. 2009; Ramu et al. 2009; Satish et al. 2009). The number of microsatellites or SSRs (simple sequence repeats) identified in sorghum has particularly increased in the last decade (Yonemaru et al. 2009). The multiallelic nature, its codominant transmission, simple PCR detection, frequency of appearance, genome coverage and small DNA amounts required for its detection make SSRs very valuable in QTL mapping studies (Powell et al. 1996).

This work aimed to identify seed dormancy QTL in mature and immature sorghum grains, using a segregant mapping population generated from the crossing of two inbred lines, IS9530 (highly dormant, PHS resistant) and RedlandB2 (weakly dormant, PHS susceptible). Phenotyping was conducted under two contrasting Argentinean environments, Castelar (Buenos Aires Province) and Manfredi (Córdoba Province). Our results allowed the detection of six QTL for seed dormancy expression in mature grains. In silico and synteny analysis of the detected QTL was also carried out. Altogether, the results presented in this work should be a step towards the elucidation of the genetic bases governing sorghum seed dormancy complementing previous and future physiological work. The detected genomic regions need to be explored in detail in order to identify the responsible genes and dig into their function and the regulatory mechanisms in which they are involved.

Materials and methods

Plant material and field experiments

IS9530 (high dormancy, PHS resistant) and RedlandB2 (low dormancy, PHS susceptible) inbred lines were crossed to produce an F_1 population, which was selfed to generate an F_2 generation. A complete F_2 panicle (1800 seeds) was sown during 2008–2009 in a randomised complete design at the INTA Biotechnology Institute experimental field (Castelar, Buenos Aires, 34 °C 36′S) together with the parental lines. The anthesis date was recorded for every plant; 190 plants with the same flowering date were selected to perform the genotyping, phenotyping and selfing to produce F_3 families. F_3 generation was grown during 2009–2010 at the INTA Biotechnology Laboratory experimental field (Manfredi, Córdoba, 31 °C 49′S). A randomised complete block design (RCBD) was used, with 3 blocks including 15 plants for each F_3 family (190 families). Both crops were fertilised and irrigated, and pests and weeds were controlled following standard procedures for the region.

Seed dormancy measurement (phenotyping)

Seed dormancy was indirectly measured as germination absence in incubated seeds under standardised humidity and temperature conditions. Fifty seeds were incubated in petri dishes with distilled water at 25 °C. Germinated seeds (i.e., radicles emerged through the seminal covers) were recorded daily over 12 consecutive days, and a germination index (GI) was calculated according to Steinbach et al. (1995). This index reflects not only the final number of germinated seeds but also the rate of germination. Phenotyping was performed for both the selected 190 F_3 panicles and their derived 186 F_4 panicles, as four families failed to establish in the field. For F_3 phenotyping, grain sampling and germination assays were conducted at 34 and 45 days after pollination (DAP), before and after PM, respectively. Occurrence of PM was estimated to be around 36–40 DAP and was visualised as the appearance of a black layer in the grain base (i.e. chalaze). Although both samples differed only in 10 days, physiological changes occurring in this time window are important, as not only grain humidity diminishes considerably but also endosperm programmed cell death takes place. Three samples of 50 seeds were incubated as described before for GI measurement. At the same time, GI was also calculated for the parental lines (IS9530 and RedlandB2) at 34 and 45 DAP. During phenotyping of F_4 panicles, between 5 and 8 plants per family within each block and with the same flowering date were sampled at 45 DAP. Seeds were pooled together, and three replicates of 50 seeds were incubated for 12 days; an average GI score was calculated for each family within a block and considered as one biological repetition. Therefore, three GI values (one from each block) were used as biological repetitions in the QTL analyses.

Phenotypic data were organised in three data sets for further analysis:

- *GI1* Germination index of F_3 seeds harvested at 34 DAP from F_2 plants, grown in Castelar (Buenos Aires), during the 2008–2009 campaign. Biological replicates are not available since each GI value was obtained from an individual plant as the mean of three pseudo-replicates (3 petri dishes with 50 grains each).
- *GI2* Germination index of F_3 seeds harvested at 45 DAP from F_2 plants, grown in Castelar (Buenos Aires), during the 2008–2009 campaign. Biological replicates are not available since each GI value was obtained from an individual plant as the mean of three pseudo-replicates.
- *GI3* Germination index of F_4 seeds harvested at 45 DPA from F_3 plants, grown in Manfredi (Córdoba), during the 2009–2010 campaign. The GI value is the mean of three biological replicates, resulting from the randomised complete block design.

Broad sense (h^2_B) and narrow sense (h^2_N) heritability for GI2 and GI3 were estimated with QTLNetwork, from variance components V_A (additive variance), V_G (genetic variance) and V_E (environmental variance) (Yang et al. 2008).

Allelic variant identification in candidate genes for seed dormancy

With the aim of including functional markers in the genetic map, size polymorphisms in candidate genes that had been previously associated with contrasting dormancy in IS9530 and RedlandB2 lines were analysed. In silico searches of candidate genes *ABI1*,

ABI2, *ABI3*, *ABI4*, *ABI5*, *GA2oxidase1 GA2oxidase3* and *PkABA1* were performed in the PlantGDB *S. bicolor* database. These genes had shown differential expression between IS9530 and RedlandB2 lines during grain incubation. Introns and 3′ and 5′ UTR regions as well as some exons from these candidate genes were amplified by PCR for both genotypes (IS9530 and RedlandB2). To identify size polymorphic regions, PCR products were sequenced for both genotypes and aligned using MultAlin software (Corpet 1988). Whenever a polymorphism was detected, primers were designed to amplify that region and used as a functional marker to be incorporated in the genetic map. Candidate gene IDs are included in Table S1.

Marker analysis on segregant population (genotyping)

Genotyping was carried out in F_2 plants. A leaf from each of the F_2 plants that had been phenotyped and a leaf from the parental lines were collected, lyophilised and ground with a TissueLyser (Quiagen). Genomic DNA was extracted with CTAB according to Watson and Thompson (1986), and working dilutions (20 ng μl^{-1}) were prepared. SSR primer pair sequences were obtained from public sorghum databases (Bhattramakki et al. 2000; Kong et al. 2000; Schloss et al. 2002; Wang et al. 2012; Li et al. 2009; Brown et al. 1996; Upadhyaya et al. 2012) and synthesised in an external facility (Alfa DNA, Canada). Highly polymorphic SSRs were preferred to increase the chance of their being polymorphic between the parental lines used in this work. Pairs of primers were ordered for these markers, with one of them being tagged with a fluorophore (FAM, HEX or NED). Presence of polymorphism between RedlandB2 and IS9530 parental lines was first checked for approximately 250 SSR, from which 132 markers were polymorphic. Informative markers were amplified by PCR in the whole F_2 population, resulting in 106 SSRs with appropriate amplification. Each PCR reaction contained 1X buffer, 1.5 mM Mg_2Cl, 0.2 mM dNTPs, 0.425 U Taq Platinum, 0.25 µM primers and 20 ng template DNA. PCR reaction conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 39 cycles of 94 °C (denaturation) for 30 s, 50 °C (annealing) for 30 s and 72 °C (extension) for 45 s. A final extension step of 10 min at 72 °C was added after the last cycle. PCR products were subjected to capillary electrophoresis in an ABI 3130 Genetic Analyzer (Applied Biosynthesis), and the results were analysed using GeneMapper 4.0.

Linkage map construction

A goodness-of-fit test at a 1:2:1 ratio for each SSR was performed by means of chi-square analysis and markers showing distorted segregation were excluded. Linkage analysis was performed using JoinMap 3.0 software (Van Ooijen and Voorrips 2001). CentiMorgan (cM) genetic distances between adjacent loci were estimated with the Kosambi mapping function (Kosambi 1943). A minimum LOD score of 4 and a maximum recombination frequency of 50 % were used. Those markers that were not assigned to any of the linkage groups were excluded from the genetic map. Physical position of markers and marker order were corroborated through comparison against the *S. bicolor* physical map (2.1 version), with the aid of the Gbrowse tool (Paterson et al. 2009).

QTL analysis

Three statistical methodologies were used to locate putative QTL and estimate their effects: (1) composite interval mapping or CIM (Zeng 1993, 1994) to identify the main effect QTL, (2) multiple trait mapping or MTM (Jiang and Zeng 1995) to identify the main QTL and to evaluate QTL-by-environment interaction (QTLxE) interactions and (3) mixed-model-based composite interval mapping or MCIM (Yang et al. 2008) to identify the main QTL, epistatic interactions between different QTL and QTLxE as well. CIM and MTM were performed in Windows version of QTL Cartographer 2.5 (Wang et al. 2006) and MCIM was implemented through QTLNetwork 2.0 (Yang et al. 2008).

For CIM analysis, model 6 from the Zmapqtl procedure was employed. Automatic forward stepwise and backward elimination regression methods were used for cofactor selection. Cofactors within 5 cM on either side of the QTL position were not included in the Zmapqtl model. The likelihood of a QTL and its corresponding effect were estimated every 0.5 cM. The critical LOD statistic value was determined for each data set with permutation tests (1000 repetitions) and 0.05 significance level (Churchill and Doerge 1994).

Percentages of phenotypic variance explained by the QTL and gene effects (dominant and additive) were determined for each significant QTL using CIM results.

Considering that PHS phenotyping had been carried out in both seeds from F_3 panicles and F_4 panicles at 45 DAP, an MTM analysis was performed in order to reduce the residual variance that results from measuring the same trait in two environments and to estimate the QTLxE interaction. For MTM analysis, GI2 and GI3 phenotypic data were used. The LOD threshold value for the QTL position (a and d effects) and QTLxE interaction was determined through permutation tests (1000 repetitions) and a 0.05 significance level (Churchill and Doerge 1994). In order to determine whether the detected QTL exhibited QTLxE interaction, the LOD statistic value for QTLxE in the QTL's most probable position (maximum LOD value) was compared to the QTLxE LOD threshold value. Significant QTLxE interaction was considered only in case the LOD value exceeded that threshold. CIM and MTM detected the QTL position, which was also expressed using a 95 % confidence interval (CI), i.e., a 1-LOD interval, according to Van Ooijen (1992). QTL were named with a *q* letter, followed by GI (germination index) and the corresponding chromosome number.

QTLNetwork 2.0 was used to analyse the main additive (*A*) and dominance (*D*) effects, epistatic effects and QTL-by-environment interaction across all tested environments (Yang et al. 2008). Threshold F values for an experiment-wise significance level of 0.05 were determined by performing 1000 permutations. Tests to detect QTL were conducted at 1-cM intervals with a window size of 10 cM (Yang et al. 2008). A Monte Carlo Markov Chain approach was used to estimate the main and epistatic QTL effects.

In silico and synteny analysis of detected QTL

In silico analysis of the sequences encompassed within the QTL intervals was performed with *S. bicolor* Gbrowse (Paterson et al. 2009). QTL flanking SSR markers were positioned in the physical sorghum map, and the genomic regions included in the intervals were carefully scanned registering ID (identification code), PFAM (protein family) codes and GO (gene ontology) terms for every gene identified. GO terms were grouped into GO classes according to the Plant_GOslim classification using CateGOrizer (Hu et al. 2008). Sorghum gibberellin metabolism genes (*SbCPS*, *SbKSB*, *SbEKO*, *SbEKAH*, *SbGA20ox1*, *SbGA20ox2*, *SbGA20ox3*, *SbGA3ox1*, *SbGA3ox2*, *GA2ox1*, *SbGA2ox2*, *SbGA2ox3*) and abscisic acid signaling genes (*SbABI1*, *SbABI2*, *SbABI3/VP1*, *SbABI4*, *SbABI5* y *SbPKABA*) were physically located within the sorghum genome with the aim of determining whether some of them were included within the detected QTL.

A synteny analysis between sorghum-detected QTL and wheat QTL or PHS-related genes was performed. Sorghum *qGI-3* location was directly carried out using the available syntenic analysis for wheat chromosome 3B (Glover et al., 2015), which is syntenic to sorghum chromosome 3. As no detailed synteny analysis between wheat and sorghum has been done for the remaining chromosomes, the location of sorghum *qGI-1*, *4*, *6,7* and *9* in rice chromosomes was assessed using the Gramene tool for synteny analysis, and then the regions identified in rice were compared to wheat main PHS QTL as described by Cabral et al. (2014).

Results

Phenotypic evaluation

F_2 and F_3 crops were conducted during 2008–2009 in Castelar (Buenos Aires Province) and during 2009–2010 in Manfredi (Córdoba Province), respectively. Along with carrying out field trials in two different locations and years, alternative sowing dates were also chosen (i.e. 20 October and 10 December for Castelar and Manfredi, respectively), leading to contrasting environmental conditions explored by F_2 and F_3 crops (Fig. 1). In particular, the grain filling period from anthesis until grain sampling date was exposed to a higher mean temperature in Castelar (24.5 °C) than in Manfredi (21.6 °C). Mean daily radiation for this same period was also higher in Castelar (24.4 MJ m^{-2}) than in Manfredi (18.5 MJ m^{-2}). Water and nutrients were not limiting in either of the locations as crops were irrigated and fertilised whenever necessary. Soil type was also different between locations, as Castelar had a typical Argiabol, poorly drained soil, with a strong textural Bt horizon, and Manfredi soil was a deep, well-drained entic Haplustol.

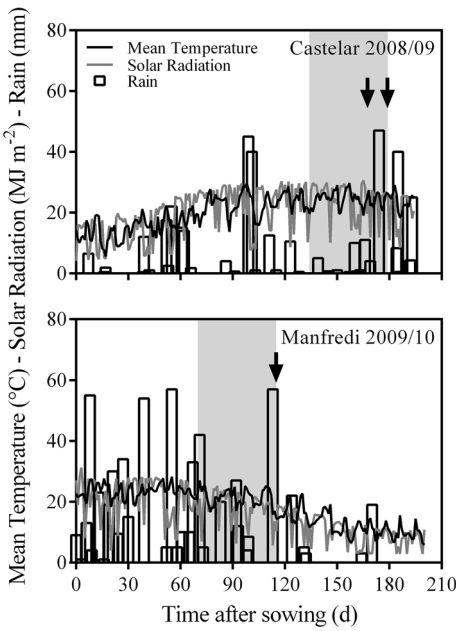

Fig. 1 Climatic factors in each evaluation environment. Details of the mean daily temperature evolution (°C), mean daily incident solar radiation (MJ m^{-2}) and daily rain (mm) for the whole cycle during 2008/2009 (Castelar) and 2009/2010 (Manfredi) crops. *Grey bars* indicate the period comprised between anthesis and seed sampling, which is pointed to with arrows (34 DAP and 45 DAP in Castelar and 45 DAP in Manfredi)

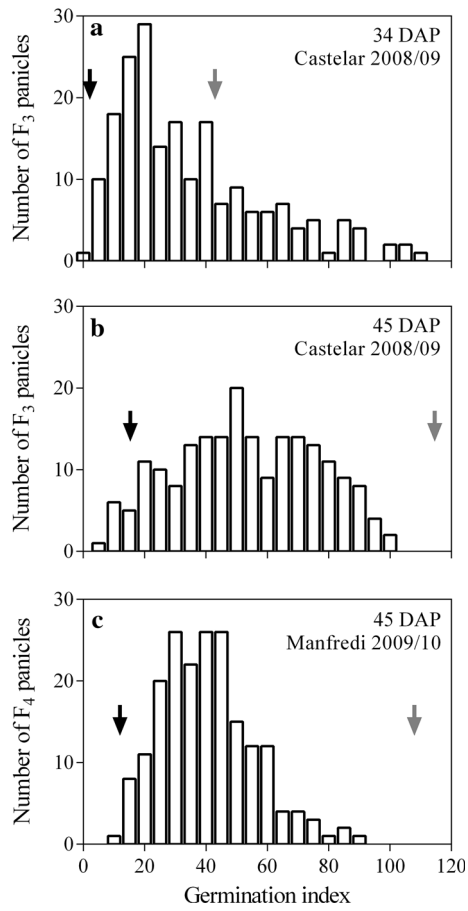

Fig. 2 Germination index (GI) frequency distribution for the mapping population. **a** GI for F_3 seeds sampled at 34 days after pollination (DAP) in Castelar (Buenos Aires). **b** GI for F_3 seeds sampled 45 DAP in Castelar (Buenos Aires). **c** GI for F_4 seeds sampled at 45 DAP in Manfredi (Córdoba). *Black* and *grey arrows* denote the GI value for parental lines IS9530 and RedlandB2, respectively, measured in those same environments

Phenotypic evaluation of the segregating population was carried out through GI measurement, as described in the Materials and Methods section. Both parental lines (IS9530 and RedlandB2) showed contrasting GIs in all sampling events (34 DAP and 45 DAP in Castelar and 45 DAP in Manfredi). As expected, IS9530 exhibited high GI values in agreement with being a PHS-resistant line, and RedlandB2 showed low GI values in accordance with a PHS-susceptible genotype. A large variation among the 186 F_3 or F_4 panicles was detected for GI as expected for a quantitative trait, with values ranging from 0 to 110 (maximum GI value is 120). GI2 values showed a rather normal distribution, and the GI3 frequency distribution was slightly skewed towards lower values (Fig. 2). The GI1 frequency distribution was quite skewed towards lower values, according to the early sampling moment chosen (i.e. 34 DAP), when it is expected that an important number of plants are highly dormant. Positive transgressive segregation was detected for the GI1 data set, where some plants exhibited higher GI values than the RedlandB2 parental line and among GI2 where a few individuals showed lower GI values than the IS9530 parental line.

Broad sense (h^2_B) and narrow sense (h^2_N) heritability for GI2 and GI3 was estimated through QTLNetwork. Values of $h^2_B = 0.28$ and $h^2_N = 0.20$ were obtained for GI2, while for GI3 heritabilities of $h^2_B = 0.19$ and $h^2_N = 0.19$ were estimated. These values suggest that 28 and 19 % of the phenotypic variation is explained by the genotypic component of variance for GI2 and GI3, respectively, with an important environmental variation for these traits.

Allelic variant identification within dormancy candidate genes

With the aim of generating functional markers (i.e. markers located within sorghum seed dormancy candidate gene sequences), introns and UTR regions together with some exons of *ABI1, ABI2, ABI3/VP1, ABI4, ABI5, GA2oxidase1 GA2oxidase3* and *PkABA* genes were PCR amplified and sequenced for both parental lines (IS9530 and RedlandB2). Sequence alignment for those amplicons revealed an InDel in the *SbABI5* gene, which consisted of the presence of CAGCAG bases in the RedlandB2 *SbABI5* first exon (+40 bp from ATG) and its absence in IS9530 (Fig. S1). The detected InDel was used to develop a functional marker named "ABI5", which was evaluated along with the other SSRs in the mapping population. For the remaining candidate genes' examined sequences, no allelic variation between RedlandB2 and IS9530 was found.

Molecular marker analysis and linkage map construction

Approximately 250 SSRs were evaluated in IS9530 and RedlandB2 parental lines. One hundred thirty-two (132) SSRs were polymorphic between parental lines and 106 were appropriately amplified in the whole mapping population. Markers *Xgap365*, *Xcup05*, *Xtxp42*, *Xtxp45*, *Xtxp162* and *Xtxp221* exhibited distorted segregation and so they were excluded from linkage analysis. Genetic distance between markers was estimated using JoinMap 3.0 (Van Ooijen and Voorrips 2001) and the physical position of all the markers along *S. bicolor* chromosomes was verified. The obtained genetic map comprised 96 markers and had a total length of 1331 cM with an average spacing between markers of 15.47 cM (Fig. 3). Markers *Xtxp8*, *Xtxp273*, *Xcup42c* and *Xtxp79* were not assigned to any of the linkage groups and were excluded from the genetic map.

Seed dormancy quantitative trait analysis

Composite interval mapping

To identify main effect QTL, CIM analysis was conducted. Table 1 summarises the results obtained for CIM analysis in immature and mature seeds in two contrasting environments (Manfredi and Casterlar). LOD threshold values were estimated through permutation tests for each data set, obtaining the following values: GI1: 7.39, GI2: 3.68 and GI3: 3.54. No significant QTL was obtained for immature seeds (IG1) but two significant QTL were identified for mature grains in Castelar (IG2). The first QTL was located in chromosome 3 and was named *qGI-3*. It was positioned at 129.5 cM (LOD = 3.77, Fig. S2), linked to the *Xtxp38* marker and exhibited a significant additive effect of 9.02 (i.e. it increases GI value in 9.02 units) and a dominance effect of −1.32. This QTL explained 8.13 % of the total phenotypic variation for GI2. An additional QTL was also found for GI2 in chromosome 7, named *qGI-7,*, located at 75.01 cM (LOD = 5.16, Fig.S2), linked to the *Dsenhsbm7* marker. The phenotypic variation (R^2) explained by *qGI-7* was 17.41 %, and it exhibited an additive effect of −14.17, indicating that this QTL diminishes the GI value in 14.17 units, with a dominance effect of 4.36.

QTL analysis performed for GI3 data (GI for mature grains in Manfredi) led to the identification of one QTL in chromosome 9, named *qGI-9,* located in the vicinity of *Xtxp107*, at 83.01 cM (LOD = 8.90, Fig. S3). This QTL explained 17.95 % of the total phenotypic variation observed, with an additive effect of −8.73 (i.e. this QTL decreases GI in 8.73 units) and a dominance effect of 0.47.

Multiple trait mapping

A multiple trait mapping analysis was also performed, considering GI2 and GI3 as replicates of the same trait (mature grains of 45 DAP sampled at Manfredi and Castelar). LOD threshold value for additive and dominance effects obtained by a permutation test was 4.85 for the "joint trait" (GI2 and GI3). Four significant dormancy QTL were identified located in chromosomes 4, 6, 7 and 9, named *qGI-4*, *qGI-6*, *qGI-7* and *qGI-9*, respectively (Table 1). *qGI-4* was linked to the *Xtxp29* marker and was located at 158.51 cM (LOD = 4.99), while *qGI-6* was in the vicinity of sam44008 and positioned at 94.51 cM (LOD = 6.83). *qGI-7* was linked to marker *Dsenhsbm7* and located at 76.01 cM (LOD = 5.80), whereas *qGI-9* was associated with the *Xtxp107* microsatellite and placed at 84.51 cM (LOD = 8.74, Fig. S4). Additive and dominance effects were not estimated for the "joint trait". In order to test the QTLxE interaction for the

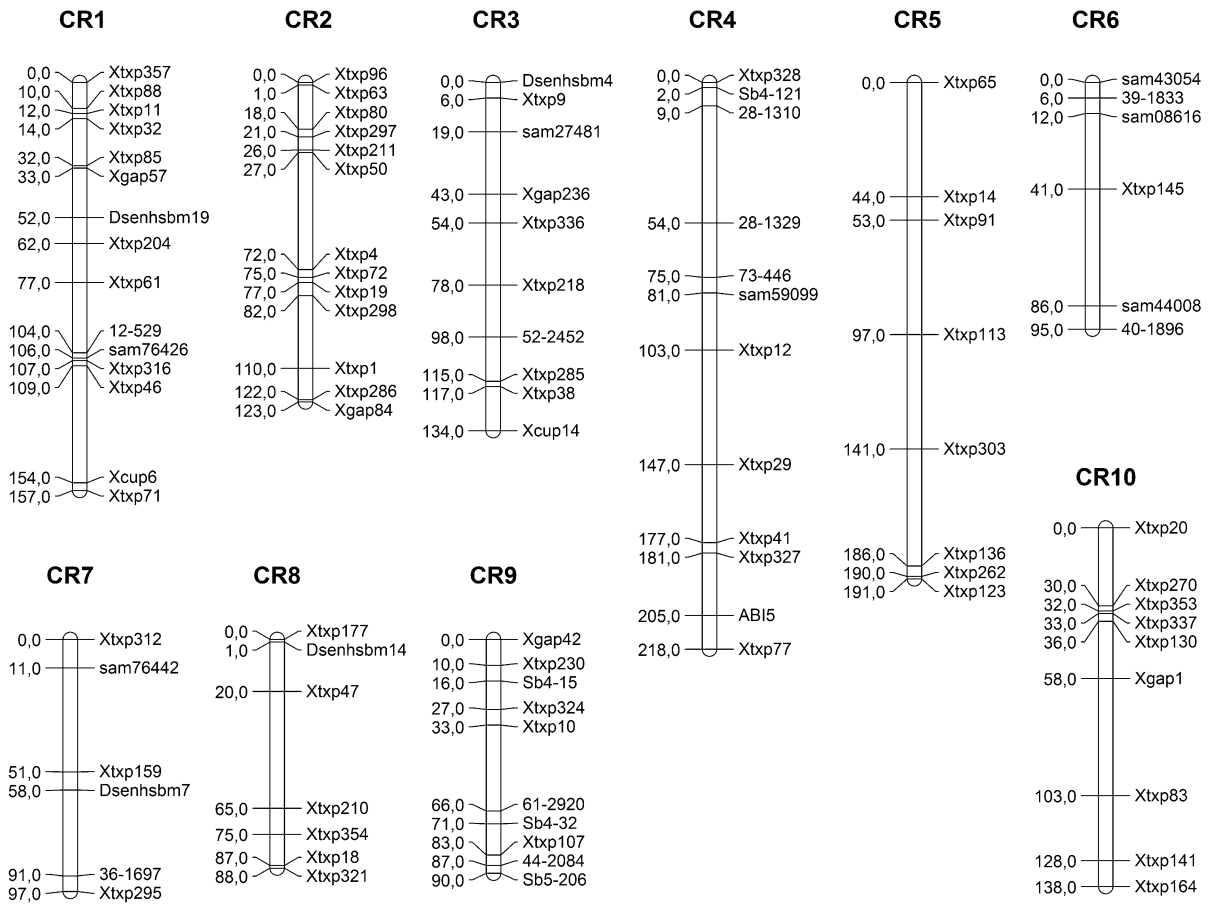

Fig. 3 Genetic linkage map for the F_2 grain sorghum population, derived from IS9530 and RedlandB2 inbred lines, built with 96 SSRs segregation analysis. Marker names are shown on the *right side* of each chromosome and genetic distances between markers (in cM) are indicated on the *left side*

detected QTL, the LOD value for QTLxE interaction in the QTL's most probable position was compared to a threshold value previously estimated by the permutation test (LOD = 4.75). The LOD value for the four detected QTL was below the critical LOD threshold, indicating that none of the QLTs had significant QTLxE interactions.

Mixed-model-based composite interval mapping

In order to compare the QTL identified through QTL Cartographer and to identify epistatic interactions between different QTL, QTL analysis was also performed with QTLNetwork. In the first place, a one-dimensional analysis was achieved for GI1, GI2 and GI3. No QTL was detected for the GI1 trait, but 3 QTL were identified for GI2 in chromosomes 1 (*qGI-1*), 3 (*qGI-3*) and 7 (*qGI-7*), while for GI3, one QTL in chromosome 9 was observed (*qGI-9*) (Table 2). Estimated additive values were −6.39, 8.70, −18.06 and -9.06 for *qGI-1*, *qGI-3, qGI-7* and *qGI-9*, respectively, while dominance effects were estimated in −14.14, −0.65, 9.51 and 0 for these same QTL. h^2_N (V_A/V_P) values estimated by QTLNetwork ranged between 0.0154 (*qGI-1*) and 0.1894 (*qGI-9*). A two-dimensional analysis was also performed with QTLNetwork for GI2 and GI3 (GI for mature grains in Castelar and Manfredi), which allows the identification of epistatic interactions. No epistatic interaction between QTL was detected but an additive by environmental interaction was found for both *qGI-7* and *qGI-9* (Fig. 4), indicating that QTL effects would not be constant through the Castelar and Manfredi environments.

Table 1 Main effect QTL for seed dormancy traits identified by QTLCartographer CIM and MTM analysis

Trait	Chr.	QTL	Flanking markers	LOD	Additive effect	Dominance effect	R^2 (%)	Peak position (cM)	95 % Confidence support interval (cM)	Positive allele	Environment
CIM											
GI2	3	*qGI-3*	*Xtxp38–Xcup14*	3.77	9.02	−1.32	8.13	129.5	117–133.5	RedlandB2	Cast.
GI2	7	*qGI-7*	*Dsenhsbm7–36-1697*	5.16	−14.17	4.36	17.41	75.01	63.6–87.0	IS9530	Cast.
GI3	9	*qGI-9*	*Xtxp107–44-2084*	8.9	−8.73	0.47	17.95	83.01	76.5–89.5	IS9530	Manf.
MTM											
GI2–GI3	4	*qGI-4*	*Xtxp29–Xtxp41*	4.99	–	–	–	158.51	153.1–169.8	IS9530	Mean
GI2–GI3	6	*qGI-6*	*sam44008–40-1896*	6.83	–	–	–	94.51	93.6*	IS9530	Mean
GI2–GI3	7	*qGI-7*	*Dsenhsbm7–36-1697*	5.8	–	–	–	76.01	65.8–89.7	IS9530	Mean
GI2–GI3	9	*qGI-9*	*Xtxp107–44-2084*	8.74	–	–	–	84.51	75.9–86.8	IS9530	Mean

GI2 germination index for 45 DAP grains phenotyped at Castelar; *GI3* germination index for 45 DAP grains phenotyped at Manfredi. *Chr.* chromosome, *QTL* QTL name, *Flanking markers* QTL flanking markers, *LOD* maximum QTL LOD value, R^2 *(%)* % of phenotypic variance explained by the QTL. Environment: *Cast.* Castelar; *Manf.* Manfredi; *Mean* joint data from Castelar and Manfredi

* *qGI-6* upper limit of the 95 % confidence support interval could not be estimated as the QTL was located at the edge of the chromosome

Co-localised QTL identified through QTL Cartographer and QTLNetwork

QTL analysis carried out in this work for GI1, GI2 and GI3 traits detected six genomic regions related to seed dormancy. Among these QTL, *qGI-1* was only identified by QTLNetwork, while *qGI-3*, *qGI-7* and *qGI-9* had already been observed with QTL Cartographer. On the other hand, *qGI-4* and *qGI-6* were only identified by QTL Cartographer through MTM, leading to the final result of 3 QTL being co-localised by both softwares. The estimated additive effect values were similar for almost all QTL. For almost all QTL, the positive allele came from the high dormant parent IS9530, except for *qGI-3*, which came from RedlandB2.

QTL in silico and synteny analysis

All detected QTL were successfully anchored on the *S. bicolor* genome assembly v2.1 (Paterson et al. 2009) through the QTL flanking SSR physical position. Physical size of these intervals ranged from 500.021 to 5.658.391 pb and genetic size varied from 4 to 33 cM (Table 3). Intervals spanning the genomic region comprised by the QTL were used to identify all the genes included within the six QTL. The number of genes within these intervals ranged from 75 (*qGI-9*) to 547 (*qGI-3*) and a relatively constant relationship between interval size and the number of genes included within those regions was found (i.e. genes/kb ranged from 0.08 and 0.15). However, gene density was variable between intervals when considering genetic size (i.e. genes/cM), which ranged from 3.633 (*qGI-4*) and 32.176 (*qGI-3*) (Table 3). A total amount of 1655 genes were included considering the 6 QTL intervals, from which 1187 genes (71.72 %) had at least 1 PFAM domain annotation and they were included in 1 of the 584 different annotations registered (Table S2). Some PFAM codes were interesting for seed dormancy expression, such as the protein kinase domain, cytochrome P450, bZIP transcription factor, B3 DNA binding domain, AP2 domain, 2OG-Fe(II) oxygenase superfamily and methyltransferase domain; 53.17 % of the genes had at least one GO term assigned and they were organised into 72 GO classes (Table S3), which showed that for every QTL a number of genes with a wide variety of predicted functions, including nuclear, cytoplasmic and membrane proteins, were identified. An important fact to

Table 2 Main effect QTL for the seed dormancy trait identified by QTLNetwork (MCIM method)

Trait	Chr.	QTL	Flanking markers	*F*	Additive effect	Dominance effect	Peak position (cM)	Support interval	Positive allele	Environment
1D										
GI2	1	*qGI-1*	*Xtxp32–Xtxp85*	8.9	−6.39	−14.14	26	19.5–32.5	IS9530	Cast.
GI2	3	*qGI-3*	*Xtxp38–Xcup14*	8.66	8.7	−0.65	132.5	121.5–133.5	RedlandB2	Cast.
GI2	7	*qGI-7*	*Dsenhsbm7–36-1697*	14.69	−18.06	9.51	75	66–85.5	IS9530	Cast.
GI3	9	*qGI-9*	*Xtxp107–44-2084*	21.46	−9.06	0.00	93	84.5–97	IS9530	Manf.
2D										
GI2–GI3	1	*qGI-1*	*Xtxp32–Xtxp85*	7.68	−2.87	−9.69	26	21–32.5	IS9530	Mean
GI2–GI3	3	*qGI-3*	*Xtxp38–Xcup14*	5.91	5.59	2.64	132	123–133.5	RedlandB2	Mean
GI2–GI3	7	*qGI-7*	*Dsenhsbm7–36-1697*	9.46	−12.05	5.99	74	65.5–82.5	IS9530	Mean
GI2–GI3	9	*qGI-9*	*Xtxp107–44-2084*	5.45	−5.61	0.07	94.5	80.5–99.5	IS9530	Mean

GI2 germination index for 45 DAP grains phenotyped at Castelar, *GI3* germination index for 45 DAP grains phenotyped at Manfredi, *Chr.* chromosome, *QTL* QTL name. *Flanking markers* QTL flanking markers, *F* maximum QTL *F* value. Environment: *Cast.* Castelar; *Manf.* Manfredi; *Mean* joint data from Castelar and Manfredi

mention is that *qGI-3* included genes *SbABI3/VP1* (Sobic.003G398200) and *SbGA20ox3* (Sobic.003G379500), two of the already characterised candidates that have been described as having a role during seed dormancy expression. On the other hand, the five remaining QTL did not include known seed dormancy expression candidate genes.

On the other hand, we addressed the possibility that QTL detected in the present work coincide with other known QTL or PHS-related genes in wheat. Syntenic analysis between sorghum and wheat has only been done in detail for wheat chromosome 3B (Glover et al. 2015), which is syntenic to sorghum chromosome 3. Although sorghum *qGI-3* lies close to wheat QTL 3B, they do not overlap and appear to result from different genes. No detailed synteny analysis between wheat and sorghum has been done for the remaining chromosomes. Nevertheless, considering that a high level of genome synteny exists among wheat, Brachypodium and rice (with the genome of these last two species being available), Cabral et al. (2014) identified candidate genes, regions and markers for pre-harvest sprouting resistance in wheat. These authors provided genomic coordinates for wheat QTL in both Brachypodium and rice genomes. Sorghum QTL (*qGI-1*, *4*, *6*, *7* and *9*) were located in rice chromosomes, and the identified regions in rice were compared to wheat main PHS QTL as described by Cabral et al. (2014). No coincidence was found as sorghum genomic regions containing *qGI-1*, *4*, *6*, *7* and *9* are syntenic to genomic regions in rice chromosomes 10, 2, 4, 8 and 5, respectively. Syntenic regions containing wheat QTL 3B and 4A are found in rice chromosomes 1 and 3 respectively, and both wheat QTL 7B.1 and 7D.2 are syntenic to regions in rice chromosome 6.

Discussion

Seed dormancy is a typical quantitative trait, resulting from the combined action of numerous genes and an important environmental effect modulating its imposition and expression. Although intraspecific variability for seed dormancy has been observed for many species, including cereals, breeding for a timely dormancy release has proven to be difficult to accomplish. The possibility of manipulating crops' dormancy level and therefore PHS response relays in the comprehension of seed dormancy genetic bases.

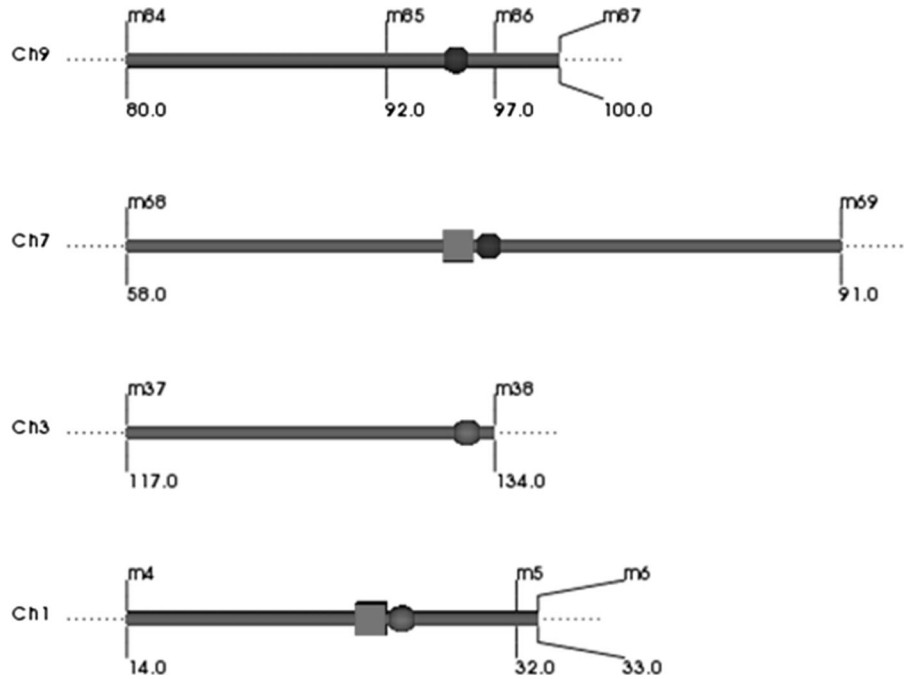

Fig. 4 Representative figure showing genetic effects and interactions between QTL, detected by QTLNetwork. Marker names are as follows: m84: *Sb4-32*; m85: *Xtxp107*; m86: *44-2084*; m87: *Sb5-206*; m68: *Dsenhsbm7*; m69: *36-1697*; m37: *Xtxp38*; m38: *Xcup14*; m4: *Xtxp32* and m5: *Xtxp85*. Genetic distances in cM are indicated below each chromosome. *Light grey circles* QTL with only additive effect; *grey squares* QTL with only dominance effect; *dark grey circles* QTL with both additive and additive × environment interaction

In this sense, QTL analysis appears to be a robust strategy for deepening the fundamentals of dormancy mechanisms through new gene discovery, genetic confirmation of the role of candidate genes and detection of the allelic gene variants responsible for the phenotypic variation. In addition, the introgression of seed dormancy QTL into commercial varieties could be a shortcut to reduce PHS in grain sorghum. In this work, we performed a QTL analysis for seed dormancy traits in immature (34 DAP) and mature grains (45 DAP) from a RedlandB2 x IS9530 segregating population, evaluating two contrasting environments (Manfredi and Castelar), with the aid of three methodological approaches (CIM, MTM and MCIM).

Phenotypic evaluation of dormancy character in grain sorghum corroborated the typical continuous distribution of this quantitative trait in a segregating population like the one used in this work. Wide sense estimated heritability for sorghum seed dormancy of mature grains in Manfredi and Castelar showed moderate values, which were somehow lower than those obtained for other species (Silady et al. 2011; Kronholm et al. 2012; Gu et al. 2010; Prada et al. 2004). This implies that a modest proportion of the phenotypic variability shown by the character is explained by the genotypic variability (i.e. between 19 and 23.5 %) with a large amount of this genetic variance being due to additive variance, as the h^2_N and h^2_B values were very similar. A large proportion of sorghum seed dormancy variability in the field conditions explored in this work ascribed to environmental variance could lead to the idea that GI values could not be good predictors of plant breeding values. However, recurrent selection, including progeny testing, could still help improve this character through traditional breeding, despite its moderate heritability.

The QTL analysis achieved in this work did not allow the detection of QTL able to explain the phenotypic variability observed in immature grains (34 DAP). Indeed, phenotypic data in these sampling date were quite far from a normal distribution and GI1 data normalisation was not considered as an alternative, as the results obtained in that case would lose reliability and could not be compared with those coming from the mature grain data mapping analysis. The inability to find genomic regions related to dormancy expression in immature grains does not mean that they do not exist, but that probably many small effect loci are involved in the trait expression in seeds at this level of development and a larger population with a wider marker coverage could

Table 3 Main effect QTL for seed dormancy trait

QTL	Flanking markers	Physical location	Physical size (pb)	Genetic size (cM)	Genes	Gene/Kb	Gene/cM
qGI-1	*Xtxp32–Xtxp85*	Chr01:52962744–Chr01:55721536	2,758,792	18	278	0.10	15.44
qGI-3	*Xtxp38–Xcup14*	Chr03:68.132.731–Chr03:72423918	4,291,187	17	547	0.13	32.18
qGI-4	*Xtxp29–Xtxp41*	Chr04:57546281–Chr04:58537697	991,416	30	109	0.11	3.63
qGI-6	*sam44008–40-1896*	Chr07:54128269–Chr07:59786660	1,513,803	9	210	0.14	23.33
qGI-7	*36-1697–Xtxp295*	Chr06:59065206–Chr06:60579009	1,332,531	6	137	0.10	22.83
qGI-9	*Xtxp107–44-2084*	Chr09:57746020–Chr09:58246041	500,021	4	75	0.15	18.75

QTL name, flanking markers, physical location, physical size (in pb), genetic size (in cM), number of genes and gene densities (in gene/Kb and gene/cM) are indicated

probably lead to the identification of QTL at this developmental stage.

QTL analysis for dormancy in mature grains (45 DAP) resulted in the detection of 6 QTL with an impact on seed dormancy expression. Taken together, the results of all the mapping methodologies used in this work, *qGI-3*, *qGI-7* and *qGI-9*, were identified by both QTL Cartographer and QTLNetwork, suggesting that they could have an important role in seed dormancy expression. Altogether, these QTL would explain more than 25 % of the trait phenotypic variation in Castelar (*qGI-3* and *qGI-7*) and almost 18 % in Manfredi (*qGI-9*). On the other hand, phenotypic variation explained by *qGI-1*, *qGI-4* and *qGI-6* could not be estimated because of methodological limitations, but they were identified when both environments were evaluated together with no QTLxE interaction (QTL Cartographer) or AxE interaction (QTLNetwork), which suggests that they could have a stable effect through contrasting environments like Castelar and Manfredi. In an opposite direction, QTLNetwork detected AxE interaction for *qGI-7* and *qGI-9*, leading to the notion that their effects could be environment specific (i.e. *qGI-7* in Castelar and *qGI-9* in Manfredi), a fact that should be considered and tested specifically in future works. The finding of some environment-specific and some environment-stable QTL for seed dormancy is common in other published works (Miura et al. 2002; Hori et al. 2007), reflecting the complexity and large environmental sensitivity that seed dormancy expression exhibits.

According to the grain sorghum cytogenetic and physical map information available (Kim et al. 2005), the identified QTL are all located on euchromatin regions, except for *qGI-4* and *qGI-7*, which are located in heterochromatin zones, suggesting that these genomic regions might have a low recombination rate, in agreement with sorghum "shrinked" heterochromatin behaviour (Mace et al. 2009). This could lead to a major challenge when trying to perform fine mapping on this genomic region or to introgress *qGI-4* or *qGI-7* in future breeding programmes.

Along with obtaining a mapping population in a brief period of time, one of the main advantages of performing the genotyping on F_2 and phenotyping on F_3 and F_4 generations was that loci genic effects (i.e. additive and dominance values) could be estimated for the identified QTL (except for *qGI-4* and *qGI-6*). All the QTL showed a significant negative additive effect, except for *qGI-3*, which exhibited a positive additive value. *qGI-1*, *qGI-7* and *qGI-9* negative additive values indicate that IS9530 alleles diminish GI values (i.e. increases seed dormancy), which is a valuable characteristic for further breeding strategies targeted to reduce PHS. Estimated dominance values were quite low for *qGI-3* and *qGI-9*, evidencing a clear additive effect for these QTL, while *qGI-1* and *qGI-7* showed dominance values that should not be underestimated as selection will not be efficient in the earliest generations. In agreement with our findings, seed dormancy in other species such as Arabidopsis, barley and rice has also been characterised as the result of the combined action of genes with additive effects (Hori et al. 2007; Imtiaz et al. 2008; Bentsink et al. 2010) and other genetic analyses suggest that some dormancy genes may also have dominant effects (Buraas and Skinnes 1984; Bhatt et al. 1983). On the other hand, the phenotypic variability explained by our QTL is in accordance with the expected values for a

trait of quantitative nature, where it is likely that the combined action of many genes with a small or moderate effect governs trait expression. In this sense, many studies that identified seed dormancy QTL informed R^2 values of similar magnitude to those found in this work (Cai and Morishima 2002; Alonso-Blanco et al. 2003; Wan et al. 2005).

In silico analysis of the six QTL revealed a large number of genes included in those genomic regions, with a broad diversity in their predicted functions. Although some of the predicted gene functions detected could be interesting for seed dormancy expression, it is quite difficult to make assumptions about the possible genes that could be responsible for those QTL because of the large genomic regions comprised and the number of genes included within them. In this scenario, new mapping studies are needed to narrow these QTL and carry out future fine mapping analysis to identify individual genes. Of particular interest was the co-localisation of genes *SbABI3/VP1* and *SbGA20ox3* within *qGI-3*, taking into account that ABA sensitivity and GA metabolism are the most relevant physiological components to explain differences in seed dormancy behaviour for the parental lines used in this work (Steinbach et al. 1995; Gualano et al. 2007; Rodríguez et al. 2009, 2012). On the other hand, the detected QTL do not colocalise with known wheat PHS QTL and seem to result from different genes.

In summary, this work provides insight into the genetic architecture of sorghum seed dormancy. A major strength of this work is the fact that it was carried out in a segregating population derived from the cross between two inbred lines (RedlandB2 and IS9530) whose sprouting behaviour has been studied extensively from a physiological and molecular standpoint. This implies that the information gathered here can be used beyond the most obvious immediate use in marker-assisted breeding programmes. The validation across different environments of the identified QTL with permanent populations and a larger set of markers is needed. In this direction, recombinant inbred lines (RILs) derived from the population used in this work are under development and the possibility of using single nucleotide polymorphism (SNP) markers for future genotyping is being considered. High-resolution mapping of the genomic regions encompassing the detected QTL, using near isogenic lines, could finally lead to the identification of the QTL responsible genes and their underlying allelic variants. In this sense, this work provided the first step towards finding new candidate genes for seed dormancy that could serve as targets for transgenic alternatives tending to reduce PHS. At the same time, the results provided interesting genomic regions that could be considered together in future sorghum breeding programmes through marker-assisted recurrent selection (MARS).

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