GA-20 oxidase as a candidate for the semidwarf gene *sdw1/denso* in barley

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Abstract

The barley *sdw1/denso* gene not only controls plant height but also yield and quality. The *sdw1/denso* gene was mapped to the long arm of chromosome 3H. Comparative genomic analysis revealed that the *sdw1/denso* gene was located in the syntenic region of the rice semidwarf gene *sd1* on chromosome 1. The *sd1* gene encodes a gibberellic acid (GA)-20 oxidase enzyme. The gene ortholog of rice *sd1* was isolated from barley using polymerase chain reaction. The barley and rice genes showed a similar gene structure consisting of three exons and two introns. Both genes share 88.3% genomic sequence similarity and 89% amino acid sequence identity. A single nucleotide polymorphism was identified in intron 2 between barley varieties Baudin and AC Metcalfe with Baudin known to contain the *denso* semidwarf

gene. The single nucleotide polymorphism (SNP) marker was mapped to chromosome 3H in a doubled haploid population of Baudin × AC Metcalfe with 178 DH lines. Quantitative trait locus analysis revealed that plant height cosegregated with the SNP. The *sdw1/denso* gene in barley is the most likely ortholog of the *sd1* in rice. The result will facilitate understanding of the molecular mechanism controlling semidwarf phenotype and provide a diagnostic marker for selection of semidwarf gene in barley.

Electronic supplementary material

The online version of this article (doi:10.1007/s10142-009-0120-4) contains supplementary material, which is available to authorized users.

Keywords: Semidwarf, Comparative mapping, Candidate gene, Gibberellic acid, SNP, Plant height, *sdw1/denso*

Introduction

Semidwarf varieties of rice and wheat averted a potential food shortage resulting from the rapid expansion of the world population in the 1960s and 1970s through providing lodging resistance, improved harvest index, and more efficient utilization of the environment (Milach and Federizzi 2001). The most popular high yield semidwarf variety in rice was “IR8” which was derived from the *sd1* semidwarf gene (Kush 1993). The *Rht1* and *Rht2* dwarfing genes contributed to the “Green Revolution” in wheat (Milach and Federizzi 2001).

Semidwarf genes have been extensively explored in barley breeding programs to reduce plant height and improve resistance to lodging. Such characteristics are adapted to heavy fertilizer application and dense field planting. Therefore, a higher harvest index, which is associated with high grain yield in barley, can be achieved.

There are four types of short stature in barley, including *brachytic*, *erectoid*, *uzu*, and *sdw* (Sears et al. 1981; Foster and Thompson 1987). *Brachytic* and *erectoid* genotypes have not performed well in American and Australian malting barley breeding programs, possible due to their reduced kernel size or low rate of grain filling (Mickelson and Rasmusson 1994; Fettell et al. 2001. The *uzu* varieties have been confined to China, Japan, and the Korean peninsula. Nearly 80% of the 147 Chinese barley short strawed varieties and entries carried the uzu dwarf genes (Zhang 1994, 2000). In 1930s, *uzu* varieties occupied 70% of the barley acreage in Japan and more than 30% of the acreage in Korean peninsula (Takahashi and Yamashi 1951). At present, more than half of the hull-less barley cultivated in southern part of Japan are *uzu* types (Saisho et al. 2004). Chono et al. (2003) have reported that the uzu genotype showed significantly reduced response to the brassinolide and its specific single nucleotide polymorphism (SNP) was responsible for the substitution of amino acid residue in the *HvBRI1* gene, which might be homologous to the rice *d61*, a rice homolog of the *Arabidopsis* BR-*insensitive 1* (BRI1; Yamamuro et al. 2000).

The *sdw1* (named as *sdw* previously) barley mutant stock has been used to develop several short stature cultivars for feed barley production in western USA, Canada, and Australia (Mickelson and Rasmusson 1994; Fettell et al. 2001). In contrast, the *denso* gene, which has been shown to be allelic to the *sdw1*, has gained wide acceptance in European malting barley

(Rasmusson 1991; Mickelson and Rasmusson 1994; Ivandic et al. 1999; Hellewell et al. 2000; Zhang et al. 2006). The alleles of *sdw1* and *denso* were derived from different sources. The gene *sdw1* was from a Norwegian variety "Jotun" and *denso* was from the Danish variety "Abed Denso" and the Czech mutant variety "Diamant". Both have similar agronomic traits, such as late heading, low seed weight, lower yield, and high screening (Mickelson and Rasmusson 1994; Hellewell et al. 2000). However, some varieties with *sdw1/denso* displayed increased grain yield, such as feed barley UC 828 (Gallagher et al. 1996), better grain size, and lower screening, for example, the malting barley Baudin from Western Australia.

The barley genome with 5.3 billion base pair is one of the largest diploid genomes in cereal crops and has been well mapped in the last two decades using a range of molecular markers (Ahn et al. 1993; Druka et al. 2000; Dunford et al. 1995; Gale et al. 2002; Gale and Devos 1998; Han et al. 1998; Kurata et al. 1994; Sherman et al. 1995; Smilde et al. 2001; Van Deynze et al 1995, 1998). Based on sequence comparison and existing comparative maps, many of the previously mapped barley and oat cDNAs were located to the predicted homologous locations across grass species (Sorrells 2000; Bellgard et al. 2004). Sorrells et al. (2003) aligned a large number of mapped wheat expressed sequence tags (ESTs) with rice genome sequences. The comparative mapping strategy has been employed to identify useful markers and candidate genes from rice to barley and wheat (Dunford et al. 2002; Li et al. 2004; Smilde et al. 2001). In the present study, we report the isolation of the barley semidwarf gene *sdw1/denso* based on rice–barley comparative mapping and develop a diagnostic marker for selection of the target gene.

Materials and methods

Materials

A doubled haploid population was generated from the F1 of a cross Baudin/AC Metcalfe using the technique of another culture. The population comprises of 178 DH lines. Baudin and AC Metcalfe have excellent malting quality and are major malting barley in the international markets. Baudin is a semidwarf variety bred from a cross of Stirling/Franklin and Franklin was selected from a cross of Shannon/Triumph. The *denso* semidwarf gene in Baudin was from Triumph which is the parental line for among the current malting barley varieties. A molecular linkage map was constructed with 163 AFLP and 70 SSR markers (Cakir et al. 2009). Total length of the map was 1,306.6 cM with an average marker density of 5.6 cM per marker.

Field trials

The mapping population and its parents were planted in Western Australia in two consecutive years. The trial sites were located in the high rainfall agricultural zone of Western Australia cereal growing areas for maximizing the expression of plant height. Each DH line and the parents were planted in 1 × 5 m plots. For convenience, all sites had the same randomized design. The plant height was measured by average of five individuals in each plot before maturity.

Database searches and sequence alignment

Several public databases were used for the comparison between barley, wheat, and rice:

1. Closely related the rice semidwarfing gene *sd1* (*OsGA20ox2*) sequences were identified by using sequence query against the rice database hosted by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) or the Gramene database (http://www.gramene.org/index.html).
2. Rice PAC/BAC clones containing predicted genes were positioned on the physical map and tightly linked markers identified in rice genome browser on gramene (http://www.gramene.org/index.html).
3. Syntenic regions were determined between barley and rice on the basis of common restriction fragment length polymorphisms (RFLP) markers using the comparative map on Gramene (http://www.gramene.org/index.html), barley genomic database (http://barleygenomics.wsu.edu/arnis/linkage_maps/maps-svg1.html), and Smilde et al. (2001).
4. The predicted gene structure was identified according to the rice BAC/PAC clones by using the Rice Genome Automated Annotation System (http://ricegaas.dna.affrc.go.jp) or the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).
5. The sequences analysis was using the Australian National Genomic Information Service (http://www.angis.org.au/html/index.html). The sequence alignments were made using amino acid sequences for closely related gibberellic acid (GA)-20 oxidase enzymes involved in barley and rice.

Isolation of the target gene using PCR from barley genomic DNA

Some polymerase chain reaction (PCR) primer sequences were designed based on the sequence of rice dwarf gene *sd1* and its conserved regions with wheat and maize EST available from the GenBank Nucleotide sequence database. The sequence number of rice dwarf gene-*sd1* was AF465255 named as *Oryza sativa* cultivar Nipponbare gibberellin-20 oxidase gene. The conserved regions were with *Sorghum bicolour* (AF249881.1), *Triticum aestivum* mRNA sequence (Y14007.1), and *Zea mays* (PCO130576). The proposed sequences isolated from barley were amplified with the following primers: forward primer (Ex1F1), 5′-ACGGGTTCTTCCAGGTGTC, and reverse primer (Gsp4), 5′-GTGGATATATTACCCCATTGGCTCGTAG, were used to partially amplify exon 1; forward primer (sdwn2), GAGTACTGCGGGAAGATGAA, and reverse primer (sdwex2R), CATGAAGGTGTCGCCGATGT, were used for exon 2; forward primer (Jex3F), CTAACGGACGGTACAAGAGC, and reserve primer (Jex3R2), CAGGTGAAGTCCGGGTAGTG, were used to partially amplify exon 3; forward primer (sdwn1), CGGACTACGAGCCAATGG, and reverse primer sdwex2R, were used to amplify intron 1 and exon 2; two pairs of primers were used to amplified intron 2, one primer pair is forward primer (JIN2F), GTC ATC AAC ATC GGC GAC ACC TTC ATG, and reverse primer (In2R), GTA CGT ACA TGG CTT GGC ATC, another primer pair is forward primer (In2F), GAT GCC AAG CCA TGT ACG TAC, and reverse primer (JIN2R), AGG CAG CTC TTG TAC CGT CCG TTA GA.

PCR reaction was comprised of 1.5 mM MgCl2, 1× PCR buffer II, 200 μM dNTP, 1.2 U *AmpliTaq* DNA polymerase, 0.4 μM of each primer, and 100 ng of template DNA, in a final volume of 25 μl with sterile distilled water. PCR cycling conditions consisted of an initial

denaturation step of 94°C for 4 min, followed by 30–35 cycles of 94°C for 1 min, 55°C for 45 s, 72°C for 1 min, and a final extension cycle at 72°C for 5 min. PCR products were cloned into a pGEM-T Easy Vector (Promega), and at least two independent clones from each PCR product were sequenced by using an automated sequencing system (ABI 377, Applied Biosystems).

SSCP analysis

Single-strand conformation polymorphism (SSCP) was considered as a simple and efficient technique for the detection of single base substitutions. The SSCP method followed previously described procedures (Savov et al. 1992; Zietkiewicz et al. 1997; Martins-lopes et al. 2001) and was optimized with a 12% polyacrylamide gel (acrylamide/bisacrylamide ratio of 37.5:1) in 0.5× Tris–borate–ethylenediaminetetraacetic acid and run at room temperature for 22–32 h.

Data analysis

MapManager (Manly 1993) was employed to construct the linkage map by combining the genotypic data. Quantitative trait locus (QTL) analyses were performed using the software package QTLNetwork (Yang et al. 2005). In this software, the QTL effects are estimated by the Monte Carlo Markov Chain method/mixed linear model approach. Permutation tests (Doerge and Churchill 1996) were carried out using 1,000 iterations at 1-cM intervals. A minimum separation of 10 cM (“filtration window”) was used to define individual adjacent QTLs. The QTL Network calculates a P value for significance and in the present study a threshold of $P < 0.05$ was used to declare a significant QTL.

JMP software was used for statistical analyses (SAS Institute). Association of the marker with plant height was calculated using stepwise regression analysis by the "Fit-model" function of JMP software (SAS Institute). A probability of $P < 0.01$ was used to claim association between the markers and traits.

Results

Comparative mapping of the *sdw1/denso* region of barley with rice

The barley semidwarf gene *sdw1/denso* was previously mapped to Bin 13 of chromosome 3H by using of phenotype as a probe (Barua et al. 1993; Laurie et al. 1993; Hellewell et al. 2000; http://barleygenomics.wsu.edu/arnis/linkage_maps/maps-svg1.html). This bin contains eight RFLP markers including the rice probe R1545. Initial BLASTN search using the marker sequences demonstrated that this barley bin is syntenic to rice chromosome 1 where the rice semidwarf *sd1* gene has been mapped previously (Sasaki et al. 2002; Spielmeyer et al. 2002, 2004). The synteny of rice chromosome 1 and barley chromosome 3H has been reported and 26 common markers covering more than 95% of the genetic length of both chromosomes (Smilde et al. 2001). A set of six RFLP conserved markers (ABG499, C191, R1545, ABC161, C742, R1014) were mapped in *sdw1/denso* region and showed synteny with the rice *sd1* gene region. Both *sdw1/denso* and *sd1* were close to the marker R1545 (Supplementary Fig. 1). The colinearity of the molecular markers indicated that the *sdw1/denso* in barley is most likely ortholog of *sd1* in rice. The *sd1* gene (*Os20ox2*) encodes an oxidase enzyme (GA20ox-2) involved in the biosynthesis of gibberellin (Sasaki et al. 2002; Spielmeyer et al. 2002).

Isolation and analysis of the candidate gene family

Due to the barley–rice synteny and the conservation of the gene family, the semidwarf gene *sdw1/denso* from barley might have similar gene structure to the rice semidwarf gene *sd1*. The full sequence of *sd1* (AF465255) isolated from *O. sativa* cultivar Nipponbare is 6,590 bp, which includes three exons and two introns (Fig. 1a). A fragment of 2,413 bp of *Hv20ox2* was isolated from barley by using the primers based on the conserved domain of the *Os20ox2* genes in rice (Fig. 1b). By comparing with the *Os20ox2* gene, the barley gene *Hv20ox2* shared the same structure as the rice *Os20ox2* with three exons and two introns. The isolated barley gene fragment contains the entire intron 1, intron 2, exon 2, and parts of exon 1 and exon 3. The *Hv20ox2* cDNA sequence obtained in this study and its deduced amino acid sequence shared 88.3% and 89% identity with the rice *sd1 Os20ox2* gene, respectively. The *Hv20ox2* gene sequence was also compared with two other GA20-oxidases genes published in barley. Its amino acid shared 57% with GA20-oxidase-1 (*Hv20ox1*, accession no. AY551428.1) mapped on chromosome 5H and 54% with GA20-oxidase-3 (*Hv20ox3*, accession no. AY551429.1) mapped on chromosome 3H. Moreover, the genes of *Hv20ox1* and *Hv20ox3* are different from *sd1* due to the significant differences from *sd1* in the amino acid sequences (Spielmeyer et al. 2004). The amino acid similarity of these two genes compared with the *sd1* was 50% and 47%, respectively. The *Hv20ox2* intron 1 and intron 2 sequences were blasted against NCBI database but no matching sequence was found. This indicates that the *Hv20ox2* gene sequence has not been reported so far. Thus, *Hv20ox2* is a new GA20-oxidase gene different from the other GA20-oxidase genes previously published in barley and is the more likely ortholog gene of the rice semidwarf gene *sd1*.

Mapping the candidate gene in a doubled haploid barley population

DNA sequences of 2,413 bp were compared between a medium tall barley AC Metcalfe and a semidwarf barley Baudin (known to contain the *sdw1/denso* gene). It seems that the *Hv20ox2* gene is highly conserved in barley. The exons of the two varieties showed identical nucleotide sequence (data not shown). An A/G substitution was identified in the intron 2 (Fig 1c). The single nucleotide polymorphism was used to genotype 178 DH lines in the Baudin × AC Metcalfe DH population (Fig. 1d). Combined with previously published data in the same population (Cakir et al. 2009), linkage analysis showed that the SNP was mapped to the long arm of chromosome 3H (Fig 2a). The *Hv20ox2* locus mapped 9.6 cM proximal to Bmag0013 and 26.3 cM distal to xp11m48A93 in this population. In the barley consensus map, Bmag0013 mapped on chromosome 3H and is flanked by the markers MWG2190 and WG110 (http://wheat.pw.usda.gov/cgi-bin). Both markers were mapped in the bin 13 of chromosome 3H where the *sdw1/denso* gene has been assigned to by phenotype (Barua et al. 1993; Laurie et al. 1993; Hellewell et al. 2000). The results of genetic mapping provide the evidence to support the colinearity between the *sd1* and *sdw1/denso* regions.

QTL analysis of the plant height in barley

We conducted QTL analysis to establish whether there is association between *Hv20ox2* locus and plant height in the Baudin × AC Metcalfe DH population. The plant height varied from 25 to 75 cm in the DH population (Supplementary Fig. 2). The distribution of plant height indicated that there was one major gene controlling plant height, although the evidence exists suggesting some other minor genes. QTLs for plant height were identified on chromosomes 1H, 2H, 3H, and 7H (C Li, unpublished). A major QTL for plant height was mapped to the interval between Bmag0013 and xp11m48A93 on chromosome 3H (Fig. 2b). This single

QTL accounts for 59% of the phenotypic variation for plant height in two seasons and *Hv20ox2* was colocalized with the peak of the QTL (Fig. 2b). This observation again supports the hypothesis that *Hv20ox2* in barley is the most likely ortholog of *sd1* in rice.

Association of the plant height with SNP allele

Based on the polymorphism of the SNP (Fig. 1d), the Baudin × AC Metcalfe DH population can be divided into two groups. One group has the allele of "Baudin" and the other group has the allele of "AC Metcalfe". The segregation ratio of "Baudin"-type and "AC Metcalfe"-type was close to 1:1 ratio. In Baudin group, the plant height ranged from 25 to 55 cm with an average value of 40 cm. The average plant height was 60 cm in "AC Metcalfe" group, ranging from 45 to 75 cm (Supplementary Fig. 3). It seems that the lines with the denso allele of *Hv20ox2* gene reduces nearly 20 cm plant height compared with the tall counterparts. Hellewell et al. (2000) reported that the *sdw1* allele reduced height by 10 to 20 cm. Therefore, the denso allele of *Hv20ox2* has similar effect on reducing plant height as the *sdw1*.

Discussion

Fundamental research has revealed that most of the dwarfing genes were involved in the GA biosynthetic and signal transduction pathway in cereals. Some of them have been isolated successfully. In rice, six of seven GA metabolic enzymes (CPS, KS, KO, KAO, GA20ox, and GA3ox) were identified in 18 GA-deficient mutants, which gave phenotypic expressions ranging from semidwarf to severe dwarf (Sakamoto et al. 2004). The wheat *Rht1* semidwarf gene appears to participate in gibberellin signaling and it is ortholog gene of the maize d8 and

the *Arabidopsis* gene AtGAI (Peng et al. 1999). Analysis of barley *grd5* mutants displaying gibberellin-responsive dwarf genotypes showed that *HvKAO1* gene corresponds to the *grd5* locus and produces a functional enzyme in vitro (Helliwell et al. 2001). Similarly, a potential candidate open reading frame for the GA-insensitive dwarfing gene *sdw3* of barley was identified in rice, based on the *sdw3* region on barley 2HS with rice chromosome 7 L (Gottwald et al. 2004). Comparative analysis revealed that these homolog genes are found at syntenic chromosomal locations with high levels of conservation among different plant species (Spielmeyer et al. 2004). Furthermore, by utilizing each barley gene encoding GA biosynthetic and catabolic enzymes, except *Hv20ox3* and *Hv3ox1*, it was possible to identify closely related syntenic regions of rice (Spielmeyer et al. 2004). These indicated that known genes in model plants can serve as a cloning vehicle for synteny-based gene isolation in the large genome species like barley. In the present study, we used this strategy to target isolating the barley semidwarf gene *sdw1/denso*. Comparative mapping revealed that the *sdw1/denso* region in barley is syntenic to the *sd1* gene of chromosome 1 in rice and the gene (*Hv20ox2*) isolated from barley showed conserved gene structure and a high degree of sequence similarity with the rice *sd1* gene (Fig 1). Further mapping results demonstrated that *Hv20ox2* was located in the syntenic region of barley chromosome 3H and cosegregated with a major QTL controlling plant height in barley (Fig. 2). It is well known that the *sdw1/denso* mutants reduced plant height and were sensitive to gibberellic acid (Franckowiak and Pecio 1992). These are also the characteristics of the rice *sd1* mutant. Thus, *sdw1/denso* in barley is the most likely ortholog gene of the *sd1* in rice which carries the mutation in the gene (*Os20ox2*) encoding an oxidase enzyme (*GA20ox-2*) involved in the biosynthesis of gibberellin (Sasaki et al. 2002; Spielmeyer et al. 2002).

Although the gene function of GA_{20}-oxidase in barley is uncertain, their ortholog genes in rice have been studied extensively. The *OsGA20ox2* (*sd1*) gene controls the step from GA_{53} to $GA_{44,}$ resulting in the levels of GA_{44}, GA_{19}, GA_{20}, GA_1, GA_{29}, and GA_8 in *sd1-1* (an allele of *sd1*) being lower than wild type, whereas the amount of GA_{53} in *sd1-1* was slightly higher (Spielmeyer et al. 2002; Sakamoto et al. 2004). Therefore, the level of GA_1 is reduced resulting in dwarf phenotype. Based on the work in rice, the sd1 orthologs gene *Hv20ox2* (barley) is predicted to control the step from GA_{53} to GA_{44}.

The semidwarf gene *uzu* is also located on barley chromosome 3H, but is not allelic to the *sdw1/denso*. This semidwarf gene is encoded by *HvBRI1* and it was shown that a single nucleotide substitution in the gene correlated with the semidwarf phenotype (Chono et al. 2003). Similarly, in the present study, a fragment of 2,413 bp of *Hv20ox2* was isolated from two barley varieties and the comparison of DNA sequences from two barley varieties Baudin (semidwarf) and AC Metcalfe (medium) revealed only a single nucleotide substitution (Fig. 1). Mapping of this SNP showed that the gene did colocate with *sdw1/denso*.

The *sdw1/denso* is one of the most important genes in barley breeding and has already contributed to the improvement of many new barley varieties, due to its suitable semidwarf phenotype and stiff straw for high yielding environments. Besides the magnitude of the effect of this gene on reducing plant height, the alleles at the *sdw1/denso* locus also have significant different effects on a number of agronomic traits. Given all of the association between *sdw1/denso* alleles and agronomic traits (Barua et al. 1993; Bezant et al. 1997; Coventry et al. 2003; Foster and Thompson 1987; Hellewell et al. 2000; Laurie et al. 1993; Mickelson and Rasmusson 1994; Nedel et al. 1993; Powell et al. 1997; Thomas et al. 1991, 1994; Yin et al.

1999), it is not surprising that there are still so many barley varieties carrying the gene. In the present study, we isolated the gene sequence and identified the polymorphism for the *sdw1/denso* gene. This result will provide a tool to select this gene in the breeding programs and to understand the association of *sdw1/denso* alleles with a range of traits.

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Fig. 1 Gene structure of the gene encoding GA20-oxidase, a rice gene *Os20ox2*, b barley gene *Hv20ox2*, c single nucleotide polymorphism and the primers for amplification of the SNP in the *boxes*, d segregation of the SNP in Baudin × AC Metcalfe DH population

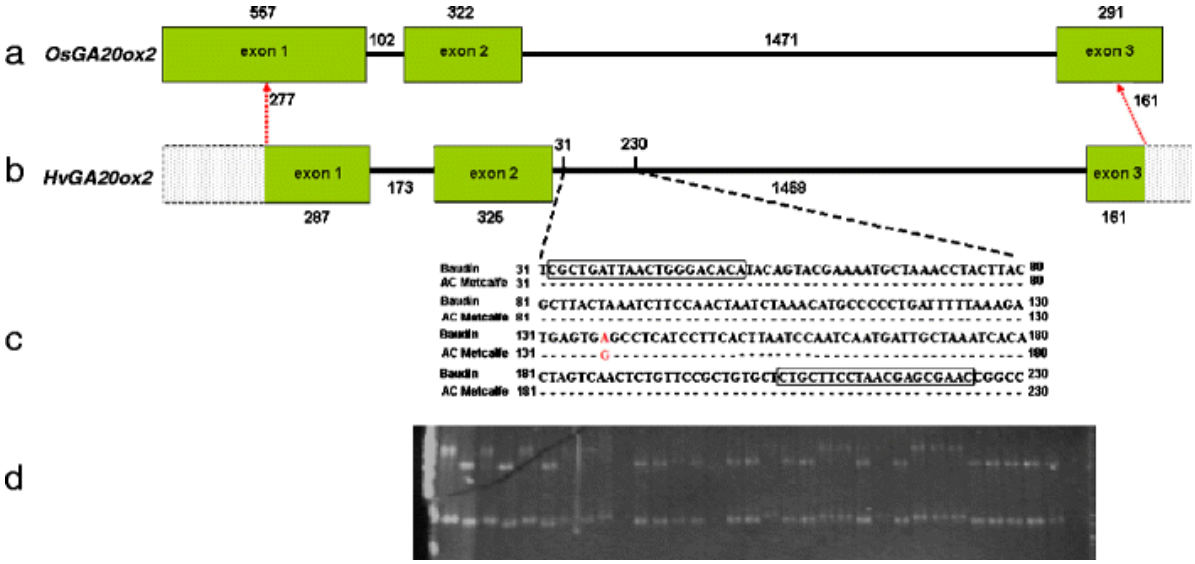

Fig. 2 Chromosome location of the *Hv20ox2* (a) and QTL controlling plant height (b) on chromosome 3H in the Baudin × AC Metcalfe DH population based on the single nucleotide polymorphism. The GA20-oxidase gene *Hv20ox2* (a) cosegregated with the QTL peak for plant height (b)