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ORIGINAL PAPER

Expression level of a gibberellin 20-oxidase gene is associated with multiple agronomic and quality traits in barley

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Abstract The use of dwarfing genes has resulted in the most significant improvements in yield and adaptation in cereal crops. The allelic dwarfing gene *sdw1/denso* has been used throughout the world to develop commercial barley varieties. The *sdw1* gene has never been used successfully for malting barley, but only for a large number of feed varieties. One of the gibberellin 20-oxidase genes (*Hv20ox*$_2$) was identified as the candidate gene for *sdw1/denso*. Semi-quantitative real-time RT-PCR revealed that *Hv20ox*$_2$ was expressed at different levels in various organs of barley. Transcriptional levels were reduced in leaf blade, sheath, stem and rachis tissue in the barley variety Baudin with the *denso* gene. Subsequently, the relative expression levels of *Hv20ox*$_2$ were determined by quantitative real-time RT-PCR in a doubled haploid population and mapped as a quantitative trait. A single expression quantitative trait locus (eQTL) was identified and mapped to its structural gene region on chromosome 3H. The eQTL was co-located with QTLs for yield, height, development score, hectolitre weight and grain plumpness. The expression level of *Hv20ox*$_2$ was reduced fourfold in the *denso* mutant, but around 60-fold in the *sdw1* mutant, compared to the control variety. The reduced expression level of *Hv20ox*$_2$ enhanced grain yield by increasing the number of effective tillers, but had negative effects on grain and malting quality. The *sdw1* gene can be used only in feed barley due to its severe reduction of *Hv20ox*$_2$ expression. The gene expression marker for *Hv20ox*$_2$ can be used to distinguish different alleles of *sdw1/denso*.

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Introduction

The development of molecular markers and construction of high-density maps has enhanced our ability to associate phenotypic variation with particular genomic regions and this has provided the basis of marker-assisted selection for traits of interest in breeding programmes. Unfortunately, most of the economically important traits in crops are quantitatively inherited, and therefore understanding the molecular nature of the genotype–phenotype association is much more difficult since the genomic region containing the genes for the desired traits is very large. Furthermore, the marked regions may contain many genes, making it difficult to validate the quantitative trait markers associated with the causative genetic variant. Another way to study the association between genes and phenotype is the genetical genomics strategy (Jansen and Nap 2001). Variations in phenotype are due to differences in the expression of the underlying genes (Potokina et al. 2004). Variation of gene expression could be treated as a heritable quantitative trait similar to other phenotypic data in segregating populations (Jansen and Nap 2001; Brem et al. 2002; Gibson and Weir 2005). Recently developed technologies such as mRNA microarray chips (Schadt et al. 2003; Kirst et al.

2004; Shi et al. 2007; Druka et al. 2008; Potokina et al. 2004, 2008a, b; Lapitan et al. 2009; Bernardo et al. 2009), cDNA-AFLP (Suprunova et al. 2007) and quantitative real-time PCR (qRT-PCR) (Potokina et al. 2004) have been successfully applied to monitor gene expression in different genotypes and to identify genes contributing to traits that display quantitative variation. QTL analysis for variation in gene expression has been described as expression QTL (eQTL) (Jansen and Nap 2001; Doerge 2002; Gibson and Weir 2005). To date, eQTL analyses have been reported in yeast (Brem et al. 2002), Eucalyptus (Kirst et al. 2004), wheat (Jordan et al. 2007), barley (Potokina et al. 2004, 2006; Druka et al. 2008; Lapitan et al. 2009), Drosophila (Rifkin et al. 2003), mouse (Schadt et al. 2003; Dos et al. 2005), maize (Schadt et al. 2003; Shi et al. 2007) and humans (Schadt et al. 2003). The association of gene expression with genetic maps has resulted in the identification of gene regulatory regions and candidate genes for traits within the genome.

The *sdw1/denso* gene has been one of the most successful semi-dwarfing genes used in barley breeding programmes worldwide. Its suitable semi-dwarfing phenotype with improved lodging resistance has contributed to a higher harvest index and positively affected a number of agronomic and quality traits including yield, heading date, screenings, malt extract and test weight (Mickelson and Rasmusson 1994; Thomas et al. 1995; Bezant et al. 1997; Powell et al. 1997; Yin et al. 1999; Hellewell et al. 2000; Ellis et al. 2002; Li et al. 2006; Von Korff et al. 2006; Cuesta-Marcos et al. 2009). Although *sdw1* and *denso* are allelic, barley breeders have realised that the two alleles have different impacts on breeding programmes. The *denso* mutant has been used to develop malting barley (Rasmusson 1991; Mickelson and Rasmusson 1994; Ivandic et al. 1999; Hellewell et al. 2000; Zhang et al. 2006), while *sdw1* has been limited to feed barley varieties (Mickelson and Rasmusson 1994; Fettell et al. 1999) and breeders have not known why *sdw1* was unsuitable for malting barley. In our previous study, an *sdw1/denso* candidate gene was isolated which is an ortholog of the rice *sd1* gene encoding a gibberellin (GA) 20-oxidase enzyme (Jia et al. 2009).

In the present study, we used quantitative real-time PCR (qRT-PCR) to test the transcripts of the candidate gene, which is differentially expressed among different alleles. Combining gene expression information, agronomic trait data and QTL analysis, we gained further insight into the regulation of the candidate gene expression and elucidated its possible effect on agronomic and quality traits. Furthermore, a gene expression marker (GEM) was developed to screen *sdw1* and *denso* alleles.

Materials and methods

Materials

The barley varieties used in the present study included AC Metcalfe, Baudin, Diamant, Jotun, UC828 and Yerong. AC Metcalfe is a medium to tall malting barley variety and was used as a control. This variety was developed at the Agriculture & Agri-Food Canada Experimental Station at Brandon, Manitoba, and is currently the best malting barley variety in Canada. Baudin is a semi-dwarfing malting barley variety, developed by the Western Australian Department of Agriculture and Food and is currently the No. 1 malting barley variety in Western Australia. UC828 is a semi-dwarfing six-row feed barley released by the University of California Agricultural Experiment Station. Yerong is a semi-dwarfing dual-purpose (feed and graze) barley variety released by the NSW Department of Agriculture. Diamant and Jotun are *denso* and *sdw1* semi-dwarfing mutants, respectively.

A doubled haploid (DH) population was developed from the cross of Baudin/AC Metcalfe with 178 DH lines. A molecular linkage map was constructed with 163 AFLP and 70 SSR markers (Cakir et al. 2011). The total length of the map was 1,306.6 cM with an average marker density of 5.6 cM per marker. This population was used to map grain yield, plant height, development score, grain plumpness and hectolitre weight. A subpopulation of 67 DH lines was selected from the above population to map the eQTL of $Hv20ox_2$. Selection of the DH lines was based on the distribution of plant height and the segregation ratio (close to 1:1 ratio) of $Hv20ox_2$ alleles (Jia et al. 2009).

Glasshouse experiments

The six barley varieties and 67 DH lines were grown in a glasshouse at the Western Australian State Agricultural Biotechnology Centre. For the quantitative real-time RT-PCR (qRT-PCR) test, stems of each line or variety were harvested 24 days after sowing. For the semi-quantitative RT-PCR test, leaf blades, leaf sheaths, stems, rachises, immature spikes and roots of parental lines were collected 15 days after heading. All samples were immediately frozen in liquid nitrogen and stored at −80°C until used.

In the test of seedling growth response to gibberellic acid (GA_3), four seeds of Baudin were planted in a 20 cm × 20 cm pot. Each pot was irrigated with a different concentration of GA_3: 0, 1, 10, 30 and 50 ppm. Each treatment had three replications and AC Metcalfe was used as a control.

Field trials and agronomic traits

The mapping population (178 DH lines) and its parents were planted in Western Australia in two consecutive

years. The trial site was located in the high rainfall agricultural zone in order to achieve the maximum growing potential for the semi-dwarfing genotypes. The DH lines and parents were planted in 1 × 5 m plots and for convenience; the same randomised design was used each year. Parental and local barley varieties were used as grid controls for spatial analysis.

Development scores were recorded using the Zadoks scale, which is a descriptive system and provides details on plant growth stages from 0 to 99 (Zadoks et al. 1974). The date of heading should be at stage 55 according to the Zadoks scale. Plant height was measured at maturity. After harvest, the barley grains were cleaned and measured for yield, plumpness and hectolitre weight.

In addition, ten plants of Baudin and AC Metcalfe were selected at random to measure the lengths of spikes, flag leaves and internodes. The first internode was the portion from the collar (base of spike) to the uppermost node. Internodes 2–4 were counted downwards from the uppermost node.

RNA isolation and cDNA synthesis

Tissue samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was isolated using the RNeasy plant Mini kit (QIAGEN) and RNase-Free DNase (QIAGEN) was applied to remove genomic DNA contamination. RNA yield and quality were determined using a NanoDrop ND-1000 spectrophotometer. First-strand cDNA was synthesised from 2.0 µg of total RNA using the Im Prom-TM Reverse Transcription System (Promega).

Semi-quantitative RT-PCR analysis

Single-strand cDNA was amplified using gene-specific primers for *Hv20ox*$_2$ to investigate transcription levels in various tissues of Baudin and AC Metcalfe. Two primers were used, sdw1exn23F-GTACTGCGGCAAGATGAAG GA and sdw1exn23R-TGTACCGTCCGTTAGACAGAG which were designed from the *Hv20ox*$_2$ sequence (Jia et al. 2009). The absence of genomic DNA contamination was further confirmed by the size of the RT-PCR products across intron 2 between exon 2 and exon 3. The reported barley *Actin* gene sequence (AY145451) was used as a control. The PCR primers were Hvactin F (TGTTCCCAGGTATCGCT GAC) and Hvactin R (ActinF1 GCCAGACTCGTCG TACTCATC). PCR cycling conditions consisted of 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final extension cycle of 72°C for 5 min.

Cloning vector

The RT-PCR product was purified using a QIA quick PCR purification kit (QIAGEN) and cloned into a pGEM®-T Easy Vector (Promega). The pGEM®-T plasmids were then transformed into JM109 competent cells (Promega) and potential recombinants were selected using the blue/white colony screening method. Plasmid DNA was purified from fresh *E. coli* cells. The presence of pGEM®-T plasmids containing DNA inserts was confirmed by restriction digest screening and sequencing.

Quantitative real-time PCR

Primers for qRT-PCR were the same as the RT-PCR described above. The qRT-PCR was performed with Rotor Gene RG3000 using SYBR Green to monitor dsDNAs synthesis. The reaction contained 12.5 µl of SYBR Green PCR Master Mix (Applied Biosystems), 2.5 µl of each gene-specific primer (10 nm), 5 µl of pre-synthesised cDNA (20× dilution) and 2.5 µl injection water. All qPCR reactions were carried out in triplicate. The standard curve for the *Hv20ox*$_2$ gene was generated using a tenfold dilution series of the plasmid containing *Hv20ox*$_2$ inserts. A sample containing cDNA from AC Metcalfe stem tissue was included in all real-time PCR runs for calibration purposes, and was used to compare results across the plates. In order to account for the differences in target RNA in each sample, *Hv20ox*$_2$ gene quantities were normalised to the barley *Actin* as an internal housekeeping gene. The amplification thermal cycling conditions were as follows: 95°C at 10 min, followed by 45 amplification cycles of 10 s at 95°C and 15 s at 60°C and 20 s at 72°C. After quantitative real-time amplification, the relative amount of target cDNA in the experimental samples was calculated from the linear regression of the standard curve. The relative quantity of each sample was determined by the ratio of target DNA to the endogenous reference.

Data analysis

The linkage map of the Baudin × AC Metcalfe DH population was based on Cakir et al. (2011) and Jia et al. (2009). Quantitative trait loci (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. The QTL Network calculates a P value for significance and a threshold of $P < 0.05$ was used to

declare a significant QTL. A minimum separation of 10 cM ('filtration window') was used to define individual adjacent QTLs. Association of the alleles with gene expression content was calculated using a stepwise regression analysis by the "Fit model" function of JMP software (SAS Institute). Significant mean differences between Baudin and AC Metcalfe for spike length, flag leaf length and internode length were calculated by means of a *t* test. Correlation and spatial analyses were carried out using linear regression and fit model functions in SPSS 10.0 (SAS Institute).

Results

Expression profiles of *Hv20ox*$_2$ and its effect on phenotype

Semi-quantitative RT-PCR analysis was conducted to investigate the tissue expression of *Hv20ox*$_2$ in barley. The results showed that *Hv20ox*$_2$ was expressed at different levels in all tissues tested; the *Hv20ox*$_2$ transcript was highly accumulated in leaf blades, stems, rachises and immature spikes whilst root tissue showed the lowest expression level of *Hv20ox*$_2$ (Fig. 1). The semi-dwarfing barley variety Baudin had lower expression levels of the gene than the medium-tall variety AC Metcalfe in leaf blade, leaf sheath, stem and rachis tissue.

Plant height is the result of culm length and spike length, and the culm length consists of culm internodes. Therefore, the lengths of spikes, flag leaves and internodes were compared between Baudin and AC Metcalfe at maturity (Fig. 2). As expected, Baudin had significantly shorter flag leaves. There were no differences in spike length between Baudin and AC Metcalfe. Thus, the height difference between the two varieties was the result of variation in internode length. There were relatively small differences between the two varieties in the length of the top two internodes and much larger differences between the bottom two internodes. It appears that the effect of the semi-dwarfing gene is reduced in the youngest internodes (Fig. 2).

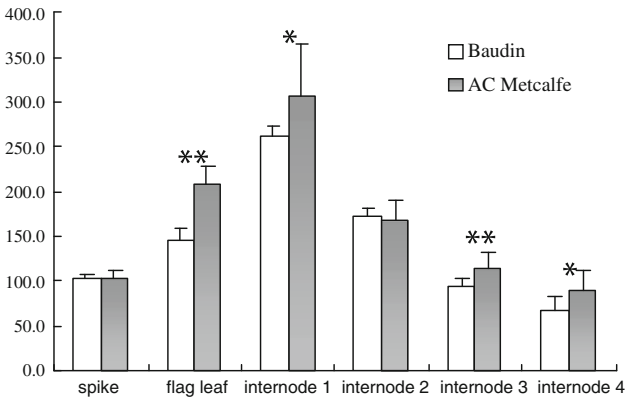

Fig. 2 Length (mm) of spikes, flag leaves and internodes in Baudin and AC Metcalfe. *White bars* Baudin and *grey bars* AC Metcalfe. *Bars* SE. *, ** Significant at the 5 and 1% levels, respectively

eQTL mapping

Low expression of *Hv20ox*$_2$ was observed in Baudin based on the semi-quantitative RT-PCR results. Subsequently, the relative expression level of *Hv20ox*$_2$ was estimated for Baudin, AC Metcalfe and 67 DH lines by quantitative real-time PCR. The variation in *Hv20ox*$_2$ transcript abundance in the DH population was normally distributed and suitable for eQTL analysis (Fig. 3a). Following analysis, a single QTL was detected on the long arm of chromosome 3H (Fig. 3g and Table 1). The eQTL mapped to the same region as the *Hv20ox*$_2$ gene and explained 37% of phenotypic variation (Table 1).

QTLs for agronomic traits

To investigate whether there was any association between the eQTL for *Hv20ox*$_2$ and agronomic traits, we measured plant height, yield, development scores, hectolitre weight and plumpness in the Baudin × AC Metcalfe population with 178 DH lines and 234 molecular markers. All traits demonstrated a continuous distribution of phenotypic values (Fig. 3b–f). Using the QTL network software to combine data from both years, QTLs for all five traits were identified on the long arm of chromosome 3H and co-located with the *Hv20ox*$_2$ eQTL (Fig. 3g–l) and the

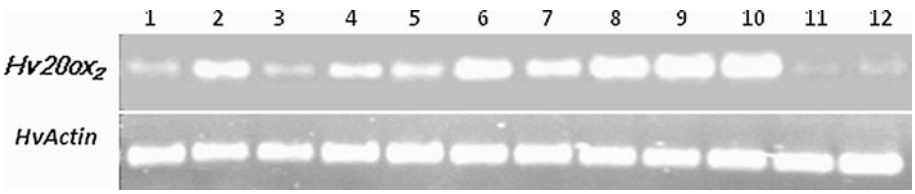

Fig. 1 Semi-quantitative RT-PCR results of *Hv20ox*$_2$. Amplicons in various tissues of Baudin and AC Metcalfe harvested 24 days after planting, were detected. *Lanes 1*, *3*, *5*, *7*, *9* and *11* were amplified from cDNAs of leaf blade, leaf sheath, stem, rachis, unopened flower and root tissue in Baudin. *Lanes 2*, *4*, *6*, *8*, *10*, and *12* were amplified from cDNAs of leaf blade, leaf sheath, stem, rachis, unopened flower and root tissue in AC Metcalfe. *HvActin* was used as a control

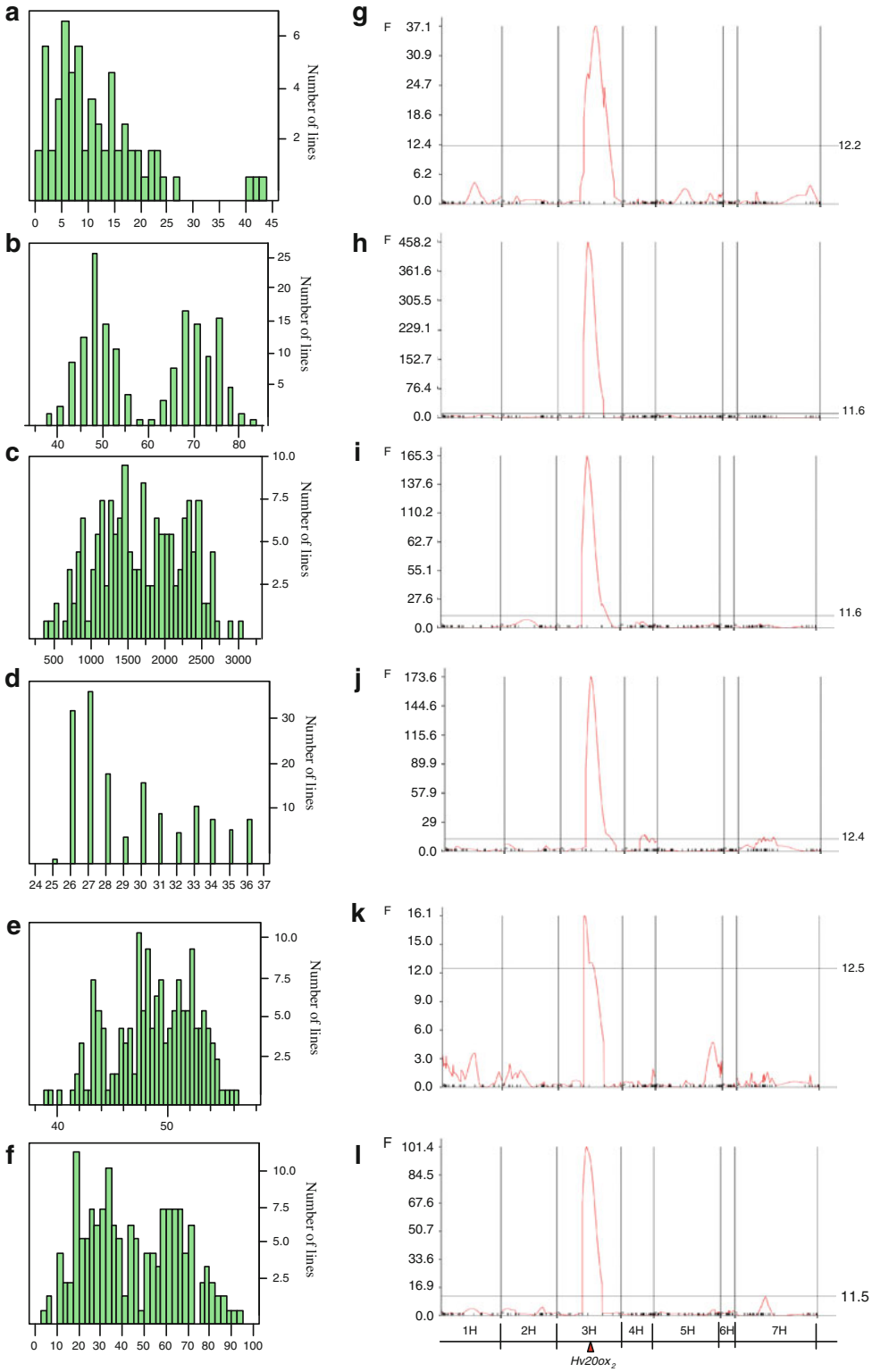

Fig. 3 Frequency distribution (**a**–**f**) and whole-genome quantitative trait locus (QTL) scans (**g**–**l**) for *Hv20ox*$_2$ gene expression level and agronomic traits in the Baudin × AC Metcalfe doubled haploid population. **a** and **g** for *Hv20ox*$_2$ expression level; **b** and **h** for plant height; **c** and **i** for yield; **d** and **j** for development score; **e** and **k** for hectolitre weight; **f** and **l** for plumpness; *1H–7H* barley chromosomes. The genetic location of the *Hv20ox*$_2$ gene is located in the *lower panel*

structural gene of *Hv20ox*$_2$ (Jia et al. 2009). The marker intervals, the explained phenotypic variation and the additive effects for each trait are listed in Table 1. The single QTL explained between 9 and 72% of phenotypic variation. As expected, the QTL had the largest effect on plant height and explained 72% of the phenotypic variation. The QTL also had a surprisingly large effect on grain yield and accounted for 49% of the variation for this trait. All QTLs in this region had additive effects and the trait increasing allele was derived from AC Metcalfe, except for

Table 1 Marker Interval, explained genetic variation (r^2) and additive effect of the QTL associated with *Hv20ox*$_2$ polymorphism for plant height, yield, development score, grain hectolitre weight, grain plumpness and the relative expression level of *Hv20ox*$_2$

Traits	Intervals	r^2 of QTL	Additive effect	Positive parent	r^2
Express of *Hv20ox*$_2$	GA20OX–Xp11m48A93	0.37	−6.8	ACM	1
Height	Bmag0013–GA20OX	0.72	−10.8	ACM	0.45**
Yield	GA20OX–Xp11m48A93	0.49	408.6	Baudin	−0.385**
Development score	GA20OX–Xp11m48A93	0.50	−2.3	ACM	0.332**
Hectolitre weight	Bmag0013–GA20OX	0.09	−1.2	ACM	0.111
Plumpness	Bmag0013–GA20OX	0.37	−13.5	ACM	0.200

r^2 a regression coefficients between *Hv20ox*$_2$ expression patterns and each agronomic trait

** Significant at 1% levels

grain yield, which was derived from the semi-dwarfing variety Baudin.

Association of the gene expression with the *Hv20ox*$_2$ allele

Based on an SNP polymorphism in *Hv20ox*$_2$, the Baudin × AC Metcalfe DH population can be divided into two subpopulations; lines carrying the Baudin allele and lines with the AC Metcalfe allele (Jia et al. 2009). The relative expression level of *Hv20ox*$_2$ ranged from 1.10E−04 to 1.78E−03 with an average value of 6.88E−04 in 31 Baudin allele lines. The average relative expression level was 1.62E−03 in 36 AC Metcalfe allele lines, ranging from 4.82E−04 to 4.33E−03 (Fig. 4). Comparison of the *Hv20ox*$_2$ expression levels between the two subgroups revealed that the AC Metcalfe allele subpopulation had 2.4 times higher expression of *Hv20ox*$_2$ than the Baudin allele subpopulation.

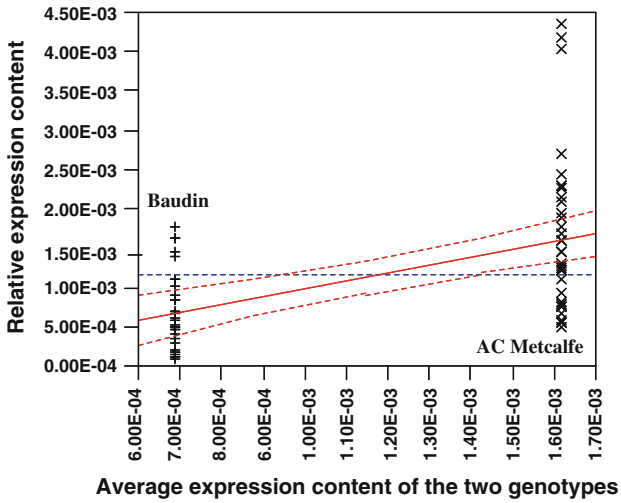

Fig. 4 Association of *Hv20ox*$_2$ alleles with expression levels in 37 Baudin × AC Metcalfe doubled haploid lines. The *lines* with Baudin genotype are marked with *plus symbol* and that with AC Metcalfe genotype marked with *multi symbol*

Association of *Hv20ox*$_2$ expression with agronomic traits

In order to identify if this gene is relevant to agronomic and quality traits, the relationship between *Hv20ox*$_2$ transcript levels and the variation in agronomic traits was tested in the Baudin × AC Metcalfe DH population using correlation analysis. There was a significant correlation between *Hv20ox*$_2$ transcript levels and plant height, yield and development scores ($P < 0.01$) which explained 45, 38.5 and 33.2% of the phenotypic variation, respectively (Table 1). Hectolitre weight and plumpness were positively correlated with the gene expression levels but not statistically significant (Table 1). Thus, high expression levels of *Hv20ox*$_2$ increased plant height and improved grain plumpness and hectolitre weight but reduced grain yield and delayed maturity.

Expression levels of *Hv20ox*$_2$ in different barley varieties

In order to test if the expression levels of *Hv20ox*$_2$ were associated with specific semi-dwarfing alleles, we measured the relative expression level of *Hv20ox*$_2$ in six barley varieties: AC Metcalfe, Baudin, Diamant, Jotun, UC828 and Yerong (Fig. 5). Three different levels of expression

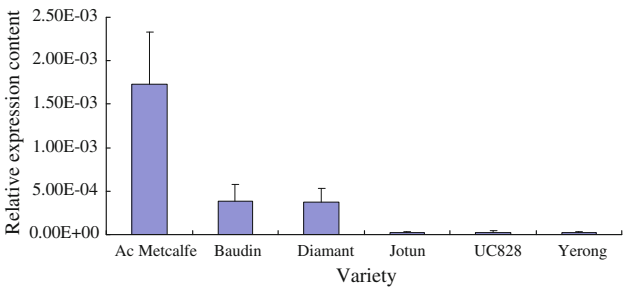

Fig. 5 Relative expression levels of *Hv20ox*$_2$ in the *sdw1* and *denso* mutants. AC Metcalfe as control; Baudin and Diamant with the *denso* gene and Jotun, UC828 and Yerong with the *sdw1* gene

Fig. 6 Effect of GA_3 on the growth of Baudin. Baudin was treated with different concentrations of GA_3. *From left to right* AC Metcalfe as control, Baudin treated with 0, 10, 30 and 50 ppm GA_3. *Bar* 2 cm

were observed; AC Metcalfe, a medium-tall malting barley variety from Canada, showed the highest expression level. Diamant, the *denso* mutant, and Baudin (a semi-dwarfing malting barley variety from Australia) had four times lower expression than AC Metcalfe. The expression level in Jotun, the *sdw1* mutant, UC828 (a semi-dwarfing feed barley variety from USA) and Yerong (a semi-dwarfing grazing and feed variety from Australia) was sixty times lower than AC Metcalfe. The expression level in semi-dwarfing malting barley Baudin was about 15 times higher than that in the semi-dwarfing feed barley varieties UC828 and Yerong.

Diamant, generated from X-ray mutagenesis in the Czech Republic, contains the *denso* semi-dwarfing gene. Diamant was one of the parents of the European malting barley variety Triumph, which has been widely used as a parent around the world to develop malting barley varieties. Baudin was selected from a cross between Stirling and Franklin and Triumph was one of the parents of Franklin. Thus, the *denso* semi-dwarfing gene in Baudin can be traced back to Diamant. Jotun is a Norwegian mutant variety for the *sdw1* semi-dwarfing gene. Although there are long pedigrees for UC828 from USA and Yerong from Australia, the semi-dwarfing gene in both varieties can be traced back to Jotun (Read and Macdonald 1991; Gallagher et al. 1996). Thus, there is allele-specific expression of *Hv20ox*$_2$ for *denso* and *sdw1*. The expression level of *Hv20ox*$_2$ in *sdw1* is almost 15 times lower than in *denso* and 60 times less than in AC Metcalfe. The expression pattern of *Hv20ox*$_2$ can be used as a GEM to distinguish different alleles of *sdw1/denso*.

Response of Baudin to exogenous gibberellin

To confirm that Baudin has the *denso* semi-dwarfing gene, we treated Baudin with 0, 10, 30 and 50 ppm GA_3. The height of Baudin was normal at 10 ppm GA_3, but increased at higher concentrations of GA_3 (30 and 50 ppm) compared with AC Metcalfe, which was unaffected by gibberellin application (Fig. 6). This is consistent with an earlier study where both barley *sdw1/denso* and rice *sd1* mutants were sensitive to GA_3 (Franckowiak and Pecio 1992). The expression levels of *Hv20ox*$_2$, however, remained unchanged by GA_3 treatments (data not shown).

Discussion

Hv20ox$_2$ as the candidate gene controlling the *sdw1/denso* semi-dwarfing phenotype

Most of the dwarfing genes in cereals are involved in the GA biosynthetic and signal transduction pathways. In rice, six of seven GA metabolic enzymes (CPS, KS, KO, KAO, GA20ox and GA3ox) have been identified in 18 GA-deficient mutants, which resulted in phenotypic expressions ranging from semi-dwarfing to severe dwarfing (Sakamoto et al. 2004). The semi-dwarfing *sd1* mutants were widely used in the development of new rice varieties, which resulted in the Green Revolution for rice yield. It is now clear that the *sd1* mutants result from a loss of function, or reduced function of the GA 20-oxidase gene which encodes an oxidase enzyme (*GA20ox-2*) involved in the biosynthesis of gibberellins (Ashikari et al. 2002; Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). In barley, the *sdw1/denso* semi-dwarfing gene has been successfully used to develop many modern barley varieties around the world. The *sdw1/denso* mutants are sensitive to GA_3 (Franckowiak and Pecio 1992) which was also demonstrated in the present study (Fig. 6). This is similar to the rice *sd1* mutants which also respond to exogenous GA_3 (Ashikari et al. 2002; Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002, 2004; Sakamoto et al. 2004). Our previous study showed that the *sdw1/denso* region in barley is syntenic to the *sd1* gene region on rice chromosome 1. The *Hv20ox2* gene isolated from barley showed conserved gene structure and a high degree of sequence similarity with the rice *sd1* gene. Further mapping results demonstrated that *Hv20ox2* was located in the syntenic region of

barley chromosome 3H and co-segregated with a major QTL controlling plant height in barley (Jia et al. 2009). The present study showed that the expression level of *Hv20ox2* was closely associated with plant height (Figs. 3, 4) and this association of gene expression with phenotypic variation provides direct evidence to support the underlying genes as the functional candidates (Jansen and Nap 2001; Potokina et al. 2004). Furthermore, the expression patterns of $Hv20ox_2$ and its strong expression in leaf blade and stem tissue were similar to the rice *sd1* mutants (Sasaki et al. 2002). Our study also demonstrated that the expression pattern of $Hv20ox_2$ could be used as a GEM to distinguish different alleles of *sdw1/denso*. This result is similar to the gene expression pattern of the different alleles of the rice *sd1* mutants (Ashikari et al. 2002; Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). Thus, we conclude that $Hv20ox_2$ is the functional gene controlling the barley *sdw1/denso* semi-dwarfing mutants.

Expression of $Hv20ox_2$ was *cis*-regulated

Regulation of gene expression is classified into two categories depending on the physical position of a structural gene related to its eQTL position. If an eQTL is located close to the structural gene position, it is considered to be *cis*-regulated but if an eQTL is located elsewhere in the genome, it is considered to act in *trans*. *Cis*-acting eQTLs have been reported to control gene expression in several species and up to one-third of eQTLs are regulated in *cis* (Gibson and Weir 2005). Based on wheat–rice synteny, 42% of 283 consensus sequences were assigned to the physical locations of structural genes and were predicted as *cis*-acting eQTLs through the Affymetrix GeneChip® Wheat Genome Array (Jordan et al. 2007). A similar study in budding yeast revealed that around 36% of genes showed segregation in expression levels via *cis*-acting regulation (Brem et al. 2002). In the present study, a single eQTL was mapped to chromosome 3H with the eQTL peak coinciding with the structural gene, suggesting that the expression of $Hv20ox_2$ is *cis*-regulated. In barley, Potokina et al. (2006) reported that the observed differences in *Cxp1* expression were the result of *cis* regulation in the Steptoe × Morex population, which resulted in the identification of barley serine carboxypeptidase 1 (*Cxp1*) as the key gene controlling malting quality. Using the same population, expression of *Rpg1*, one of the barley stem rust resistance genes, corresponded to its structural location (Druka et al. 2008).

In most studies, eQTLs accounted for approximately 25–50% of the transcriptional variation (Gibson and Weir 2005). In barley, an eQTL explained between 13 and 22% of the variation in *Cxp1* expression (Potokina et al. 2006). In the present study, the eQTL explained 37% of the variation in $Hv20ox_2$ expression, which is consistent with other eQTL analyses.

The effect of $Hv20ox_2$ on agronomic traits

Expression QTLs provide new opportunities to link genes and phenotypic traits and recently, eQTLs for specific genes were found to be coincident with QTLs for complex traits in a number of plant species. Thus, eQTL genes are good targets for candidate genes and/or molecular markers.

In Eucalyptus, a number of genes related to lignin biosynthesis were found to regulate stem growth, lignin content and composition (Kirst et al. 2004). In barley, a serine carboxypeptidase 1 (*Cxp1*) eQTL co-located with a pQTL for diastatic power, an important trait for malting quality. Linkage disequilibrium analysis also provided evidence that the level of diastatic power in the barley seed is influenced by the level of *Cxp1* expression (Potokina et al. 2006). In the wheat population RL4452 × AC Domain, clusters of eQTLs were co-located with QTLs for phenotypic traits (Jordan et al. 2007). In their reports, eQTLs between WMC420 and WMC650 corresponded to intervals that were coincident with QTLs for grain protein and yield on chromosome 4A. Furthermore, there were 28 QTLs between GWM494 and WMC219 that were coincident with 1,000-grain weight, maturity and several flour and dough quality traits related to viscosity. Similarly, candidate genes for cell-wall digestibility were validated by gene expression analysis in a Flint × Flint maize recombinant inbred line population (Shi et al. 2007). In barley, a number of QTLs known to affect agronomic traits such as yield, yield components, seed characters, phenology, plant architecture and morphology have been mapped to Bin 13 of barley chromosome 3H where the *sdw1/denso* gene is located (Mickelson and Rasmusson 1994; Thomas et al. 1995; Bezant et al. 1997; Powell et al. 1997; Yin et al. 1999; Hellewell et al. 2000; Ellis et al. 2002; Li et al. 2006; Von Korff et al. 2006; Cuesta-Marcos et al. 2009). In the present study, QTLs for plant height, yield, development score, plumpness and hectolitre weight co-located with the $Hv20ox_2$ eQTL on the long arm of chromosome 3H. We speculate that the multiple QTLs for agronomic traits identified in this study are due to pleiotropic effects of $Hv20ox_2$.

Yield is the most important agronomic trait for barley breeding and is generally controlled by multiple genes with small effects. It is well known that the *sdw1/denso* gene region is one of the yield hot-spots and that the semi-dwarfing phenotype has been associated with yield and yield related components in more than eight mapping populations (Thomas et al. 1995; Bezant et al. 1997; Powell et al. 1997; Yin et al. 1999; Ellis et al. 2002; Li et al. 2006; Von Korff et al. 2006; Cuesta-Marcos et al.

2009). The *sdw1/denso* gene is also related to low seed weight and high screenings (Powell et al. 1985; Thomas et al. 1991). Our results are consistent with these previous studies, which saw the *sdw1/denso* semi-dwarfing gene increase grain yield but reduce grain size, hectolitre weight and grain plumpness. As the head length (an indicator for grain numbers per ear) was similar between the semi-dwarfing and tall genotypes (Fig. 2), the semi-dwarfing gene increased grain yield through increasing the number of effective tillers. This conclusion is also supported by the previous studies (Powell et al. 1985; Coventry et al. 2003). In the present study, the QTL for grain yield co-located with the eQTL of $Hv20ox_2$, but was negatively correlated (Fig. 4; Table 1), indicating that high yield is associated with lower expression levels of $Hv20ox_2$. We postulate that reduced expression of $Hv20ox_2$ in the semi-dwarfing mutants results in lower GA in the apical meristem, which inhibits apical growth, internode length, plant height and promotes the development of more tillers.

Differences in expression level of $Hv20ox_2$ in *sdw1* and *denso*

Both *sdw1* and *denso* semi-dwarfing genes have been successfully used to develop barley varieties. It is still unclear why there has never been a malting barley variety developed from the *sdw1* semi-dwarfing gene. There have been a lot of feed barley varieties with *sdw1* and many malting barley varieties have been developed using the *denso* semi-dwarfing gene. Bioactive gibberellins are not only essential regulators of barley growth and development, but also affect of α- and β-amylases, and limit dextrinase in the endosperm of barley (Groat and Briggs 1969; Jacobsen et al. 1970; Yadav et al. 2000; Evans et al. 2009). Since these enzymes are essential for malting and brewing processes in barley, Yadav et al. (2000) suggested that exogenous application of GA_3 at 15 ppm could be useful for enhancing the quality of barley malt. In the present study, both *denso* and *sdw1* have a lowered mRNA expression level of $Hv20ox_2$, presumably a consequence of reduced function of its encoding enzyme, which might be involved in the biosynthesis of gibberellins, and consequently low gibberellin content. However, the reduction in expression levels of $Hv20ox_2$ is four times lower in the *denso* mutant and 60 times lower in the *sdw1* mutant, compared with AC Metcalfe. The *denso* mutant is still widely acceptable as malting barley, which may be because it only has a modest reduction in gibberellin content. The *sdw1* mutant is doomed to be used as feed barley due to its serious shortage of gibberellins. A GEM for $Hv20ox_2$ will assist in the differentiation between *sdw1* and *denso* alleles in barley germplasm and may provide an efficient tool for screening new mutants.

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