Molecular characterization and functional analysis of barley semi-dwarf mutant Riso no. 9265

Qiaojun Jia¹,², Chengdao Li³, Yi Shang², Jinghuan Zhu², Wei Hua², Junmei Wang², Jianming Yang²* and Guoping Zhang¹*

Abstract

Background: sdw1/denso is one of the most important and useful semi-dwarf genes in barley breeding. At least four sdw1/denso alleles have been reported and HvGA20ox₂ is considered as the candidate gene. Up to date, results of studies have not univocally proven the genetic relationship between sdw1/denso and HvGA20ox₂.

Results: In the present study, a complete deletion of Morex_contig_40861 including both HvGA20ox₂ and Mloc_56463 genes was identified at the sdw1 locus from a semi-dwarf mutant Riso no. 9265. Expression of the genes encoding gibberellin biosynthesis (HvGA20ox₁ and HvGA3ox₂) were increased in the mutant compared to the wild type Bomi, while the expression of GA catabolic gene HvGA2ox₃ was decreased. Over-expression of HvGA20ox₂ could rescue the semi-dwarf phenotype and increase GAs concentration.

Conclusions: We confirmed that a GA biosynthetic enzyme HvGA20ox₂, acted as GA 20-oxidase, is the functional gene for the sdw1/denso semi-dwarfism. Lose of HvGA20ox₂ is partially compensated by HvGA20ox₁ and further feedback is regulated by gibberellin. We also deduced that the sdw1/denso allele itself affects later heading owing to its reduced endogenous GAs concentration.

Keywords: Barley, Deletion, Gene compensation, Gibberellin, Semi-dwarf

Background

Plant height is one of the most important agronomic traits in cereal crops that not only determines plant architecture, but also is closely associated with grain yield. A reduction in plant height usually leads to strong straw and high resistance to lodging, as happened in semi-dwarf rice and wheat cultivars. However, reduced plant height means lowering of canopy, which favors the epidemic spread of fungal diseases, resulting in an undesired increase of fungicide use, and a reduction of yield potential mainly due to smaller grain weight [1]. For optimizing the balance of plant height and yield, breeders have successfully utilized the semi-dwarf genes in cereal crop breeding since 1960s. The development and wide cultivation of semi-dwarf wheat and rice varieties led to a dramatic increase of cereal production worldwide [2], which is labeled as a ‘green revolution’.

The Green Revolution genes have been identified and isolated in the semi-dwarf varieties by reversed genetics. Among them, sd1 in rice is one of the most famous genes and has been widely used to produce semi-dwarf phenotype in both japonica and indica rice. It was reported that sd1 encoded one of the GA 20-oxidases and was involved in the last steps of gibberellin biosynthesis [3–6]. Semi-dwarf genes have also been extensively explored in barley breeding programs and more than 30 types of them have been described [7]. One of the most successfully used semi-dwarf genes in modern barley breeding is sdw1/denso, which was postulated as homologous to ga5 in Arabidopsis and sd1 in rice [8, 9]. The main phenotypic effect of the sdw1/denso gene is a 10-20 cm reduction of plant height, depending on environmental conditions [10]. Besides, there are some deleterious effects associated with sdw1/denso gene, such as late heading and...
maturity, decreased thousand-grain weight and high screening [10–15]. As for the relationship between grain yield and sdw1/denso, both positive and negative were reported depending on different genetic backgrounds [10, 11, 15]. However, with its suitable semi-dwarf phenotype and potentially increased yield, sdw1/denso has been introduced to numerous cultivars. For example, newly released more than 150 European cultivars carried the denso allele [16].

There are at least four independent alleles based on the allelism test done up to date [12, 17]. One spontaneous mutant was derived from a Danish variety Abed denso in 1946, as named accordingly [17]. Two alleles induced by X-ray with different parents were named as denso and sdw1, respectively. Interestingly, the denso mutant has been used to develop malting barley, while the sdw1 has been limited to feed barley [10, 12]. The fourth sdw1 allele was found in a M2 generation from the variety Bomi, treated by neutrons in the Stockholm reactor and named as Riso no. 9265. Although these mutants are characterized by semi-dwarf phenotype, all of them are sensitive to GA3 [18].

In 1993, sdw1/denso was first mapped on the 3HL as a phenotypic trait [19]. In our previous study, GA20ox2 homolog was identified using PCR method with the primers designed from the conserved domain of rice sd1 and considered as a candidate gene of sdw1/denso [8]. Meanwhile, it was found that the expression of HvGA20ox2 in denso mutant was reduced by 4-fold, but almost 60-fold in the sdw1 mutant, compared to the control [15]. However, there was no direct evidence to prove the HvGA20ox2 as the functional gene. In addition, the function of HvGA20ox2 was predicted based on its functional domain, being highly similar with those of sd1 (OgGA20ox2) and ga5 (AtGA20ox2). No evidence showed its function in vitro or in vivo. In the present work we investigated the sdw1 allele of Riso no. 9265 and identified a complete deletion contig, which includes two genes Mloc_56462 (HvGA20ox2) and Mloc_56463 (a putative methyltransferase PMT26). The function of the HvGA20ox2 gene was further analyzed using genetically-transformed Arabidopsis. Furthermore, realtime PCR assays revealed that transcript level of GA synthesis-related genes were significantly different between Bomi and Riso no. 9265. The current results showed that the semi-dwarf phenotype of Riso no. 9265 is attributed to the deletion of HvGA20ox2.

**Results**

**Plant height, internode length and heading date of Bomi and Riso no. 9265**

At maturity, plant height and length of spike, culm and internodes of Bomi and its semi-dwarf mutant Riso no. 9265 were measured (Table 1). Plant height of Bomi was 83.1 cm, and Riso no. 9265 was 63.3 cm, being only 76.2 % of its wild type parent, as reported previously [17]. No obvious difference was found in spike length between the two genotypes. Obviously, the reduction of plant height for the mutant is completely attributed to the shorter culm length. Culm length of Riso no. 9265 was 53.5 cm, being 19.6 cm shorter than that of Bomi. In fact, all internodes of the mutant were shorter than those of the wild type (Table 1). Moreover, the difference between the two genotypes was smaller for the length of the top two internodes and larger for the basal four internodes. Therefore, it may be assumed that the effect of the semi-dwarf gene in Riso no. 9265 is mainly on the basal internodes. In addition, Riso no. 9265 headed three days later than Bomi.

**HvGA20ox2 was deleted in Riso no. 9265**

In this study, the complete genome sequence of HvGA20ox2 (Mloc_56462) was identified after blast against Gramene (http://www.gramene.org/) using the sequences of HvGA20ox2 from our previous study [8]. Two pairs of primers of HvGA20ox2 were designed to amplify its genomic sequence. In Bomi, both primer pairs produced single band with different sizes. One is 2974 bp and the other is 3165 bp. However, no PCR product was obtained in Riso no. 9265 (Fig. 1), indicating that HvGA20ox2 is lost in the mutant. Thus, the sequences of HvGA20ox2 were used to identify Contigs after blast against International Barley Genome Sequencing Consortium (http://webblast.ipk-gatersleben.de/barley/viroblast.php). One Morex_contig_40861, covering 21596 bp was identified, and it contains HvGA20ox2 and Mloc_56463 genes. Several primers covering Morex_contig_40861 were designed (Additional file 1: Table S1) and PCR amplification succeeded in Bomi, but failed in the mutant Riso no. 9265. Furthermore, we were able to amplify flanking genes (Mloc_3247, Mloc_45089,

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### Table 1 | Internode length and plant height of Bomi and Riso no. 9265

| Traits | Bomi | Riso no. 9265 |
|--------|------|--------------|
| Length (cm) | % | Length (cm) |
| Plant height | 83.2 ± 1.1 | 63.5 ± 2.4** |
| Spike length | 101 ± 0.4 | 100 ± 0.5 |
| Culm height | 73.1 ± 0.9 | 100 63.5 ± 2.1** |
| First-internode length | 25.2 ± 1.0 | 34.5 20.1 ± 0.7** |
| Second-internode length | 14.9 ± 0.3 | 20.4 13.1 ± 0.7** |
| Third-internode length | 10.5 ± 0.4 | 14.4 7.6 ± 0.6** |
| Fourth-internode length | 10.8 ± 0.4 | 14.8 6.7 ± 0.4** |
| Fifth-internode length | 8.2 ± 0.6 | 11.2 4.2 ± 0.3** |
| Sixth-internode length | 3.5 ± 0.6 | 4.8 1.8 ± 0.2** |

Values are means ± SE, N = 20. *significant difference at 0.005 level; **significant difference at 0.001 level
Mloc_51746 and Mloc_7311) of Morex_contig_40861, obtained using the latest barley assembly from Gramene and barley reference genome information from Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) (primers are listed in Additional file 1: Table S1). As a result, it was found that there is a large deletion spanned at least two genes HvGA20ox2 and Mloc_56463 in Riso no. 9265. A blast search for the Mloc_56463 protein sequence against NCBI identified a possible methyltransferase PMT26-like gene.

Overexpression of HvGA20ox2 caused more gibberellin production in Arabidopsis

To evaluate if HvGA20ox2 is responsible for the semi-dwarf phenotype, we examined transgenic Arabidopsis plants that over-express HvGA20ox2 in wild type (Col-gl1) and a semi-dwarf mutant ga5-3. T1 transgenic plants were selected by BASTA analysis and PCR method. We obtained eleven and six transgenic lines in Col-gl1 and ga5-3 background, respectively. All of them displayed a higher growth rate. Homozygous T3 plants of transgenic Col-gl1 lines (OE-1 and OE-2) and transgenic ga5-3 lines (OE-3 and OE-4) were taken randomly for further analysis. The four transgenic plants differed greatly in the expression levels of HvGA20ox2. However, transcripts of barley GA oxidase-encoding gene were not found in both wild type and semi-dwarf mutant ga5-3 (Fig. 2).

The two independent transgenic lines (OE-1 and OE-2) showed GA-overdose phenotype although they differed in the expression level of HvGA20ox2. There was no obvious difference in root length of 7-day-old seedlings between wild type and HvGA20ox2 over-expression plants, but the latter had 50% longer hypocotyl than the former (Table 2 and Fig. 3). In addition, the transgenic plants flowered relatively early.

To determine whether the over-expression constructs would affect the phenotype of ga5-3, we characterized the GA-affecting traits in ga5-3 transgenic lines (OE-3 and OE-4), including hypocotyl length, internodes length and number, flowering time, and final plant height. The results showed that ga5-3 transgenic lines (OE-3 and OE-4) also had the GA-overdose phenotype, with longer hypocotyls and internodes, more internode number, flowering earlier in comparison with those of wild type.
Table 2 Phenotypic parameters for wild type, ga5-3 and HvGA20ox2 over-expressing transgenic Arabidopsis

| Traits                          | Col-gl1 | OE-1(Col-gl1) | OE-2(Col-gl1) | ga5-3  | OE-3(ga5-3) | OE-4(ga5-3) |
|--------------------------------|---------|---------------|---------------|--------|-------------|-------------|
| Hypocotyl length (mm)          | 2.05 ± 0.04 | 3.64 ± 0.10A  | 4.04 ± 0.01A  | 2.07 ± 0.04A | 3.64 ± 0.10AB | 3.75 ± 0.12AB |
| Root length (mm)               | 36.1 ± 0.7  | 38.9 ± 1.8    | 40.1 ± 1.2    | 37.5 ± 0.67 | 40.3 ± 1.4   | 36.8 ± 1.7   |
| Flowering time (d)             | 26.2 ± 0.35 | 24.6 ± 0.33A  | 23.0 ± 0.25A  | 26.7 ± 0.28 | 22.5 ± 0.23AB| 23.7 ± 0.24AB|
| Vegetative internode length (cm)| 1.9 ± 0.1   | 2.7 ± 0.1A    | 2.9 ± 0.1A    | 0.6 ± 0.1A  | 3.0 ± 0.2AB  | 2.7 ± 0.1AB  |
| No.vegetative internodes       | 3.4 ± 0.1   | 5.2 ± 0.2A    | 5.0 ± 0.2A    | 3.1 ± 0.1A  | 4.8 ± 0.4AB  | 4.9 ± 0.1AB  |
| Inflorescence internode length (cm)| 0.61 ± 0.01 | 0.68 ± 0.01A  | 0.71 ± 0.02A  | 0.38 ± 0.01A| 0.67 ± 0.02AB| 0.64 ± 0.02AB|
| No.inflorescence internodes    | 14.4 ± 1.1  | 47.3 ± 1.2A   | 43.9 ± 1.4A   | 31.5 ± 1.1A | 43.7 ± 1.6AB | 47.3 ± 1.9AB |
| Final plant (cm)               | 28.8 ± 0.5  | 46.8 ± 0.7A   | 44.8 ± 1.0A   | 14.6 ± 0.4A | 42.0 ± 1.7AB | 43.8 ± 1.1AB |

The values are the means ± SE
Capital letter A means significantly different from the wild type (Col-gl1); Capital letter B means significantly different from ga5-3 mutant

Fig. 3 Overexpression of HvGA20ox2 in Arabidopsis affects plants development. The pictures compared the growth of transgenic and untransgenic at 1-week (a) and 6-week (b)
or ga5-3 mutant (Table 2 and Fig. 3). It can be seen from Fig. 4 that there was the dramatic difference in plant height among the four transgenic lines, wild type and ga5-3 throughout the whole growth stage.

GA4+7 content were much higher than GA1+3 content in wild type. The transgenic plants had significantly higher GA1+3 content than both wild-type and ga5-3 plants, but the difference in GA4+7 content among them was much smaller. Moreover, GA1+3 content was lower in ga5-3 mutants than in wild-type plants, whereas both genotypes had the similar GA4+7 content (Fig. 5).

In order to determine whether the changes of phenotype and GA content observed in the transgenic lines are accompanied by the alteration in expression of GA biosynthesis and catabolism, three GA 20-oxidases, one GA 3-oxidase and one GA 2-oxidase, highly expressed in 7-day-old seedlings, were selected for further analysis. As shown in Fig. 6, the expression of AtGA20ox1 and AtGA3ox1 was greatly up-regulated in the AtGA20ox1 deficient mutant ga5-3, while the expression of AtGA2ox was significantly decreased in comparison with the wild type. No significant difference was found in the expression of AtGA20ox3 between ga5-3 and wild type. In the transgenic Arabidopsis with HvGA20ox2 over-expression, the expression of AtGA20ox1, AtGA20ox2 and AtGA3ox1 was distinctly decreased, however, AtGA2ox3 was dramatically increased relative to wild type and ga5-3 mutants.

The expression of GA biosynthesis and catabolism genes were changed in Riso no. 9265 mutant

We determined the expression level of GA dioxygenase genes in stems of Bomi and Riso no. 9265 at the initial jointing stage. HvGA20ox1, HvGA3ox2 and HvGA2ox3 showed high transcript level. The relative mRNA expression of HvGA20ox3 and HvGA3ox2 were dramatically increased in the mutant Riso no. 9265 compared with those of Bomi. On the other hand, the mRNA expression of HvGA2ox3 was decreased in the Riso no. 9265 (Fig. 7).

Discussion

Previously, we reported that barley HvGA20ox2 (Mloc_56462) was a candidate gene of sdw1/denso and different expression level of HvGA20ox2 was observed between denso and sdw1 (Jotun) alleles [8, 15]. Here, we reported that around 21 kb DNA fragment, including both HvGA20ox2 and Mloc_56463 genes was completely deleted in the mutant Riso no. 9265. Because of the incomplete barley reference genomes and repetitive nature of the barley genome [20], the boundary of deleted segments was not determined precisely in Riso no. 9265. Gene annotation showed that HvGA20ox2 has gibberel-lin 20-oxidase activity and may be involved in GA biosynthetic pathway. Moreover, the sdw1/denso mutant is GA sensitive and exogenous application of 10 ppm GA3 restored its plant height [18], which indicates that sdw1/denso mutants are GA deficiency. On the contrary, Mloc_56463 is a putative methyltransferase and has not been reported or predicted as a GA-related gene. Thus, HvGA20ox2 is considered as the only candidate responsible for the semi-dwarfism in Riso no. 9265.

The GA biosynthetic pathway has been extensively investigated and the enzymes involved have been well characterized in plants [21]. The early GA-biosynthetic steps are encoded by single-copy genes, while the final steps catalyzed by GA 20-oxidase, GA 3-oxidase and GA 2-oxidase, are encoded by multigene families. GA20ox activity removes carbon-20 in the formation of C19-GA skeleton [22, 23]. In Arabidopsis, GA20-oxidase genes are differentially expressed and involved in different developmental processes controlled by GA, but the plants that constitutively express each of three GA20oxs (AtGA20ox1, AtGA20ox2 and AtGA20ox3) behave as the control treated with GA [22, 23]. The GA-over-

![Fig. 4 Stem heights of Arabidopsis over expressing HvGA20ox2 gene](image-url)
production phenotype was also found in HvGA20ox2 transgenic Arabidopsis (Table 2, Figs. 3 and 4). The phenotype was characterized by elongated hypocotyl and stems, early flowering, and higher growth rate. In addition, barley GA20ox2 could recover AtGA20ox1 lose-of-function mutant. These results suggest that HvGA20ox2 is the orthologous of AtGA20ox1 and acts as the oxidase at carbon-20 of GA biosynthetic pathway.

The bioactive GAs in plants are GA1, GA3, GA4 and GA7, but GA4 is a major active GA in Arabidopsis [24]. Both endogenous GA1,3 and GA4,7 in 7-day-old seedlings of the control and transgenic plants were quantified in this study. The results showed that transgenic plants had higher GA contents, especially for GA1,3 compared with the control. The increased GAs is associated with the changed phenotype (Figs. 3 and 4). Similar results have been reported that GA1 content was increased and GA4 content had little change in the 7-day-old Arabidopsis with AtGA2ox1 overexpressors [22]. In contrast, both GA1 and GA4 content had slight change in the shoot tip of Arabidopsis plant with over-expression AtGA20ox, while a 2- to 3-fold increase in GA4 content was observed in the rosette leaves of the transgenic lines [23]. Radi et al. [25] deduced that the dramatic difference in GAs content might be due to the variation in GA level among plant tissues and growth stage.

As indicated in Fig. 6, the transgenic lines overexpressing barley GA 20-oxidases showed a decrease in AtGA20ox1, AtGA20ox2, AtGA3ox1 and an increase in AtGA2ox2 expression, while ga5-3 was just opposite in the expression of these genes, which could be attributed to feedback from an increased bioactive GAs in HvGA20ox2 over-expressing transgenic Arabidopsis or decreased bioactive GAs in ga5-3, respectively. A similar regulatory role has been proposed for AtGA3ox1, whose transcript level is also feedback-regulated in transgenic Arabidopsis of over-expressed GA20ox [23, 26]. No considerable change was detected in AtGA20ox3 level of transgenic Arabidopsis and the control, suggesting that AtGA20ox3 gene might be less sensitive than AtGA20ox1 and AtGA20ox2 to the alteration of bioactive GAs.

However, not all over-expressing GA20ox plants displayed GA-overdose phenotype. In the case of CmGA20ox1, over-expression of Cucurbita maxima GA 20-oxidase in Arabidopsis resulted in accumulation of inactive GA25 and GA17, and reduction of GA4 content, which caused a slight decrease in stem elongation [25, 27]. Accordingly, GAs accumulation and changed expression levels of GA-regulated transcripts confirmed that HvGA20ox2 should be involved in regulation of GAs production. In barley semi-dwarf mutant Riso no. 9265, lose of HvGA20ox2 caused its GA deficiency and reduced plant height.

It was reported that GA promoted stem elongation and was found in the young tissues [22, 23]. HvGA20ox2 is highly expressed in stem and possibly has effect on internode length [15]. Once jointing stage starts, the stems are young and internode region begins to elongate and grow rapidly. Therefore, the transcriptional levels of GA biosynthetic and catabolic genes were detected at the initial jointing stage. As a result, we found that GA biosynthesis genes (HvGA20ox1 and HvGA3ox2) were up-regulated and GA catabolic gene (HvGA2ox3) was down-regulated in Riso no. 9265 compared with Bomi, suggesting a type of feedback regulation from the low bioactive GAs because of the null mutation of HvGA20ox2. Feedback regulation of GA20ox and GA3ox and feed-forward regulation of GA2ox genes expression have been also shown in GA-deficient Arabidopsis mutants [27–29]. In rice sd1, the expression of OsGA20ox1 was increased in stem to compensate the sd1 effect [5, 30]. As a result, the increased expression of HvGA20ox1 could compensate the effect of HvGA20ox2 in the stem at least partially, and the feedback or feed-forward regulation of the GA dioxygenase

![Fig. 5](image-url) The content of active gibberellin in the 7-d-old seedlings of transgenic and control plants. Each column represents the mean of three repeats with ±SE bar. **means significant difference at 0.01 level.
Fig. 6 The effect of over expression HvGA20ox2 on expression of gibberellin biosynthetic and catabolic genes. *indicates significant difference at 0.05 level; **means significant difference at 0.01 level; nd means not detected.
helps maintaining a relative stable endogenous GA level. Consequently, Riso no. 9265 exhibited the observed plant height.

As mentioned above, we have characterized the barley semi-dwarf gene, *sdw1/denso*, and conclude that it encodes a GA biosynthetic enzyme, GA20ox, on the basis of the following results. Firstly, *sdw1/denso* mutant responds to exogenous GA3 on the basis of the late heading date. In contrast, *denso* allele and *sdw1* of Jotun shows different expression of *HvGA20ox2* [15]. Thirdly, the transgenic wild type *Arabidopsis* and semi-dwarf mutant *gas5-3* by *HvGA20ox2* gene showed GA over-dose phenotypes. Finally, GA-regulated transcripts were changed in Riso no. 9265 in the same way as those of some GA20ox alleles in Arabidopsis [5, 23, 26, 30]. It was reported that *sdw1/denso* was associated with later heading [11, 31]. Upon first mapped *denso* to the long arm of chromosome 3H, Barua et al. [19] found a quantitative trait locus for heading date, which could not be genetically separated from the *denso* locus. Previously identified QTLs of heading date was also located on the region around *denso* in the Blenheim × E224/3 DH population [14]. Furthermore, it was found that *sdw1* delayed maturity by around 3d based on eight populations [10]. We also found the QTL of development score was co-located with the *HvGA20ox2* eQTL on 3HL [15]. However, it is still difficult to distinguish if it is pleiotropy of *sdw1/denso* or a tight linkage between the gene and one controlling later heading. In the present study, over-expression of *HvGA20ox2* caused early flowering in *Arabidopsis*. The same is true for *AtGA20ox1* over-expressors [22, 23]. Both *AtGA20ox1* and *AtGA20ox2* act redundantly and affect many developmental processes, with *AtGA20ox1* making the great contribution to internode and filament elongation, and *AtGA20ox2* making the great contribution to flowering time and siliquae length in *Arabidopsis* [29]. It was also revealed that either *AtGA20ox1* or *AtGA20ox2* mutation delayed flowering under short-day condition, while only *AtGA20ox2* delayed flowering slightly under long-day condition. Thus it can be assumed that the deficiency of *AtGA20ox1* or *AtGA20ox2* together with low GA affects flowering time, because GA acts a particularly important developmental switch between vegetative and reproductive development [32]. In the same way, it might be the deletion of *HvGA20ox2* that causes later heading due to low GA concentration in Riso no. 9265. The hypothesis could be proved by the following facts. Firstly, GAs is involved in many developmental processes, including germination, stem extension, flowering and fruit set [22, 23, 29, 32]. Secondly, *HvGA20ox2* and Mloc_56463 are absent in Riso no. 9265, unlike its wild type, while it is *HvGA20ox2* that has gibberellin 20-oxidase activity and acts as a major determinant for GA production. Thirdly, GA deficiency was proved in *sdw1/denso* using GA sensitive experiment [15, 18]. Lastly, Riso no. 9265 mutant exhibited a late heading date. Similar results were found that semi-dwarf progenies with *sdw1/denso* matured generally later than their tall counterparts [10, 11, 31]. Considering all of these, it can be concluded that late heading in *sdw1/denso* could be the pleiotropy of *HvGA20ox2*.

**Conclusions**

The current study showed a complete deletion of over 21 kb DNA fragment including both *HvGA20ox2* and Mloc_56463 genes in the mutant Riso no. 9265. *HvGA20ox2* acts as GA 20-oxidase and is involved in gibberellin biosynthesis. The expression of the genes encoding GA biosynthesis (*HvGA20ox1* and *HvGA3ox2*) are up-regulated and the expression of GA catabolic gene *HvGA2ox3* is down-regulated in Riso no. 9265 in comparison with those in wild type Bomi, respectively. We conclude that *sdw1/denso* encodes one of the GA biosynthetic enzymes, GA 20-oxidase and the deletion of *HvGA20ox2* as well as the compensatory of *HvGA20ox1* and feedback regulation of gibberellin results in semidwarf phenotype of Riso no. 9265. We also deduced that *sdw1/denso* allele itself evoked later heading due to its reduced endogenous GAs concentration.

**Methods**

**Plant materials and sampling**

Semi-dwarf mutant Riso no. 9265, kindly provided by Dr. Chengdao Li of Murdoch University, Australia and its wild parent Bomi, kindly provided by Dr. Jing Zhang of Chinese Academy of Agricultural Sciences, China were used in this study. Both genotypes were planted in 2 × 6 m plots. The heading date was recorded as the
number of days after sowed when 50 % of the spike emerged from the sheath. At maturity, 20 plants of each genotype were harvested at random for measurement of plant height, spike and internode length. Meanwhile, main stems of each genotype were taken at the initial jointing stage for measuring the expression levels of gibberellin biosynthetic and catabolic genes.

DNA isolation, PCR amplification and sequencing

Seedlings at tillering stage were sampled and DNA was extracted according to Murray and Thompson [33] with small modification. DNA samples were quantified using a Thermo Unicam UV300 and adjusted to a final concentration of 50 ng/μl for PCR analysis. PCR was performed on a Veriti 96 well thermocycler (Applied Biosystems). Sequencing of HvGA20ox2 isolated from barley was conducted using two-primers: (1) forward primer (sdwF10) 5' CTAGTCTACACACCTCTCA TCTCAT 3', and reverse primer (sdwR10) 5' GTTCCCG AAAAAATTCCGTGT 3'; (2) forward primer (sdwF9) 5' CTCTCCCGCACACCTCATGCAAC 3', and reverse primer (sdwR13) 5' GCGGTGAGGGGGCATGCATAT 3'. PCR reaction was comprised of 50 ng of template DNA, 0.3 μM of each primer, 1 × PCR buffer, 0.4 mm dNTP and 0.2 U PCR enzyme (KOD FX, TOYOBO) in a final volume of 10 μl. PCR cycling conditions consisted of an initial denaturation step of 94 °C for 3 min, followed by 30-35 cycles of 98 °C for 10 s, 60 °C for 30s, and 68 °C for 3 min.

Primers of Morex_contig_40861 and neighbor genes of HvGA20ox2 were listed in Additional file 1: Table S1. PCR reaction was comprised of 1 × Taq Mix (Bio life), 0.3 μM of each primer and 50 ng of template DNA. The following PCR amplification profile was used: denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 0.5-2 min depending on the size of amplilcons, and a final extension at 72 °C for 5 min. The amplification products were run in 1 % agarose gels and sequenced by Biosune Biotechnology Co. Ltd.

Plasmid construction and Arabidopsis transformation

For plasmid construction, full-length CDS of HvGA20ox2 was amplified from Bomi by PCR. The primers were designed as 5' AGTACTCGAGCTACACCATCTCAT CTCTCAT 3' and 5'CTATGGATCCATGCGGTGTCTG GAT 3' with Xhol and BamH I sites, respectively. The amplified PCR product was confirmed by sequencing and cloned into T4 vector for further manipulation. Insert was confirmed by sequencing. The CDS paragraph of HvGA20ox2 digested with Xhol/BamH I was cloned into the Xhol/ BamH I site located between the 35S CaMV promoter and ocs terminator of the pFGCS941 binary vector, named as pFGCS941- HvGA20ox2. The binary vector was transformed into Agrobacterium tumefaciens by electroporation, and the floral dip method was used to transform Col-gl1 (Columbia ecotype, different from Col-o only in its glabrous leaf. Hereafter refer to wild type) and a semi-dwarf mutant ga5-3 (a T-DNA inserted mutant of AtGA20ox1 in Col-o, Salk016701) [34]. T1 transgenic plants were screened by spraying bialaphos solution twice at an interval of three days at cotyledon stage, and confirmed by PCR using following specific primers: Forward, 5' GGAGCATCGTGAGAAAAAGAAC 3' (from CaMV 35S promoter sequence) and Reverse, 5' GAGGTCCAGGGCTGTGTGCAC 3' (from HvGA20ox2). The constructs were also verified by sequencing. For analysis of HvGA20ox2 gene expression level in transgenic plants, 7-day-old Col-gl1, ga5-3 and T3 transgenic seedlings were harvested for RNA extraction.

Growth conditions and phenotypic characterization of Arabidopsis

The seeds of Arabidopsis were stratified at 4 °C for 1-2 d and sown on soil at 24 °C under LDs (16 h of light). Plants for hypocotyl and root length measurements were grown vertically on one half times Murashige and Skoog media (Sigma, M6899) containing 1 % MES, 3 % sucrose and 0.46 % Gelrite with pH 5.8 under LDs, being measured after 7 d. Flowering time was scored as the days when buds could be detected with naked eyes. Other measurements were performed on the plants that had stopped flowering.

Real-time quantitative RT-PCR

RNA was extracted from the stems of Bomi and Riso no. 9265, as well as 7-day-old seedlings of T3 transgenic lines, Col-gl1 and ga5-3 using Spin Column Plant total RNA Purification Kit(Sanggon Biotech (Shanghai) Co., Ltd). cDNA was prepared from 1 μg RNA using AMV First Strand cDNA Synthesis Kit(Sanggon Biotech (Shanghai) Co., Ltd). qPCR reactions were performed using SYBR Green (SG Fast qPCR Master Mix(High Rox), BBI ) and the Applied Biosystems Stepone plus Real-time PCR System. The Real-time PCR assays were performed in triplicate for each cDNA sample. For determining transcription levels of barley GA20ox2 and genes encoding final biosynthesis of GA, HvACTIN and HvGAPDH for barley, and At1g13320 and At4g26410 for Arabidopsis were employed as reference genes [5, 35, 36]. Additional file 2: Table S2 listed the oligonucleotide sequences used for quantitative RT-PCR.

ELISA assay of gibberellin (GA1,3 and GA4,7)

Approximately 0.5 g fresh Arabidopsis tissue (7-day-old seedling) was homogenized in liquid nitrogen and extracted at 4 °C in cold 80 % (v/v) methanol containing 1 mM butylated hydroxytoluene for 4 h. The extracts
were collected after centrifugation at 8000 g at 4 °C for 20 min. The residues were suspended in the same extraction solution and stored at 4 °C for 1 h, and then centrifuged again at 8000 g at 4 °C for 20 min. The two resulting supernatants were combined and passed through a C18 Sep-Pak cartridge (Waters, Milford, MA, USA). The efflux was collected and dried in N2. The residues was then dissolved in 0.01 M phosphate buffer solution (pH 7.5) and concentrations of GA1-3 and GA4-7 were determined in an indirect enzyme-linked immuno sorbent assay (ELISA) using GA3 and GA4 antibody, respectively. The ELISA KIT was obtained from Professor Baomin Wang (Chinese Agricultural University) and the methods were described in previous publications [37, 38].

Statistical analyses
Phenotypic differences of Bomi and Riso no. 9265 were tested by Student’s t test. In order to avoid heterogeneity of variance, a natural log transformation was applied to hypocotyl length and a square transformation was applied to flowering time (days), plant height, root and internode length. Least significant difference (LSD) at 5 % probability was used to assess the difference between genotypes. For statistical analysis of qPCR data, cycle threshold (Ct) values were used to determine Δ Ct values (Δ Ct = Ct_target − Ct_reference), and expression levels of target genes relative to reference gene were determined as 2^−ΔCt. For comparison of GA concentration and the expression levels of GA-regulated transcripts between transgenic and wild type plants, ANOVA was used. Analysis of qRT-PCR efficiency showed that all amplicons of the genes used in this study were within the optimal range of 98.7-102.4 %.

Ethics Statement
In this study we only used plant material including barley and Arabidopsis. Based on the rule of BMC Genomics, no ethics statement was required for the collection of genetic material.

Additional files

Additional file 1: Table S1. Primers used to detect flanking genes or sequences of HvGA20ox2. (XLS 18 kb)

Additional file 2: Table S2. Oligonucleotide sequences used in qRT-PCR assays. (XLS 17 kb)

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
QJ, CL and GZ wrote the manuscript, and all authors read and approved the final version.

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