

RESEARCH PAPER

Genetic and physiological analysis of *Rht8* in bread wheat: an alternative source of semi-dwarfism with a reduced sensitivity to brassinosteroids

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Abstract

Over the next decade, wheat grain production must increase to meet the demand of a fast growing human population. One strategy to meet this challenge is to raise wheat productivity by optimizing plant stature. The Reduced height 8 (*Rht8*) semi-dwarfing gene is one of the few, together with the Green Revolution genes, to reduce stature of wheat (*Triticum aestivum* L.), and improve lodging resistance, without compromising grain yield. *Rht8* is widely used in dry environments such as Mediterranean countries where it increases plant adaptability. With recent climate change, its use could become increasingly important even in more northern latitudes. In the present study, the characterization of *Rht8* was furthered. Morphological analyses show that the semi-dwarf phenotype of *Rht8* lines is due to shorter internodal segments along the wheat culm, achieved through reduced cell elongation. Physiological experiments show that the reduced cell elongation is not due to defective gibberellin biosynthesis or signalling, but possibly to a reduced sensitivity to brassinosteroids. Using a fine-resolution mapping approach and screening 3104 F₂ individuals of a newly developed mapping population, the *Rht8* genetic interval was reduced from 20.5 cM to 1.29 cM. Comparative genomics with model genomes confined the *Rht8* syntenic intervals to 3.3 Mb of the short arm of rice chromosome 4, and to 2 Mb of *Brachypodium distachyon* chromosome 5. The very high resolution potential of the plant material generated is crucial for the eventual cloning of *Rht8*.

Key words: Adaptation, brassinosteroid, height, *Rht8*, wheat

Introduction

Wheat genetics has the opportunity of understanding and improving a crop that has profound social and economic importance across countries and cultures. It is estimated that over the next decade, grain production must increase by 15% to meet the global demand and consumption of wheat as a result of a growing human population (Edgerton, 2009). One strategy to meet this challenge is to increase wheat productivity by optimizing plant architecture (defined by tillering, stature, and leaf and ear

morphology). Plant architecture is of major agronomic importance as it determines the adaptability of a plant to cultivation, harvest index, and potential grain yield (Reinhardt and Kuhlmeier, 2002).

A decisive component of plant architecture is stature, mainly determined by stem elongation. Wheat (*Triticum aestivum* L.) is an annual crop with round, hollow, and jointed culms (stems). There are usually five elongated internodes in fully grown culms,

with each internode progressively longer towards the ear. The last internode, the peduncle, is the longest. Numerous data from rice, barley, and *Arabidopsis* mutants indicate that internode elongation, which determines final plant height, is regulated by genes involved in brassinosteroid (BR) and gibberellin (GA) biosynthetic or signalling pathways (reviewed in Wang and Li, 2008).

Mutants defective in BR biosynthesis or signalling display characteristic growth-deficient phenotypes such as dwarfism. In rice, the *Osdwarf4-1* mutant exhibits erect leaves and slight dwarfism without compromising grain yield (Sakamoto *et al.*, 2006). This phenotype is due to loss of function of a cytochrome P450 (CYP90B2) involved in BR biosynthesis. In barley, the *uzu* semi-dwarfing allele of a gene encoding the putative BR receptor HvBR11 is being introduced into all hull-less barley cultivars in Japan (Chono *et al.*, 2003). These results provide a strategy for genetic improvement of crop production by modulation of BR biosynthesis and signal transduction. With a better understanding of the hormonal regulation of culm elongation, a similar strategy would also be possible in wheat, where to date there are no cloned genes involved in the BR pathway.

On the other hand, the deployment of genes influencing plant height through the GA pathway was a major factor in the success of the Green Revolution, which created high-yielding cultivars with shorter and sturdier culms (Khush, 2001). In contrast to the recessive semi-dwarf *sd-1* Green Revolution allele in rice, that is a loss-of-function mutation in one of the major GA biosynthetic genes (*GA 20-oxidase2* or *GA20ox2*) (Monna *et al.*, 2002; Sasaki *et al.*, 2002; Spielmeyer *et al.*, 2002), the wheat reduced height *Rht-B1b* and *Rht-D1b* Green Revolution alleles are dominant gain-of-function mutations causing impaired GA signalling (Peng *et al.*, 1999). Typically, mutants with decreased bioactive GA concentrations or impaired response are dwarf or semi-dwarf in stature, while elevated bioactive GA concentrations or increased signalling result in taller plants (Busov *et al.*, 2008). The wheat Green Revolution genes are orthologues of the *Arabidopsis* gibberellic acid-insensitive (*gai*), the maize dwarf-8 (*d8*) (Peng *et al.*, 1999), the rice *gai* (Ogawa *et al.*, 2000) or slender1 (*slr1*) (Ikeda *et al.*, 2001), and the barley slender1 (*sln1*) (Chandler *et al.*, 2002) genes. The wild-type genes encode a DELLA protein that acts as a negative regulator of GA signalling (Harberd *et al.*, 1998; Dill *et al.*, 2001). Although *Rht-B1* and *Rht-D1* have influenced major advances in varietal performance (Gale and Youssefian, 1985), they are not universally beneficial, and in certain hot and dry climates they can reduce rather than promote crop performance (Worland and Law, 1986; Rebetzke *et al.*, 2007). In addition to shortening plant stature, *Rht-D1b* and *Rht-B1b* also reduce coleoptile length and early vigour, which can reduce yield through poor seedling establishment when sown in unfavourable conditions (Rebetzke *et al.*, 1999).

The Reduced height 8 (*Rht8*) semi-dwarfing gene is one of the few, together with the Green Revolution genes, to shorten wheat culms and improve lodging resistance without penalizing grain yield (Worland and Law, 1986). *Rht8* is well adapted to southern and eastern European environments (Worland and Law, 1986) as it has no effect on coleoptile length or seedling vigour (Rebetzke *et al.*, 1999; Rebetzke and Richards, 2000). It was introduced from the Japanese variety Akakomugi into European wheats in the 1930s by the Italian breeder Strampelli together with the

photoperiod-insensitive, early flowering *Ppd-D1a* allele (Lorenzetti, 2000). Worland *et al.* (1990) described *Rht8* as a weak allele of a gene for height promotion on the short arm of chromosome 2D. Successively, Korzun *et al.* (1998) detected a closely linked microsatellite marker, *Xgwm261* that was mapped 0.6 cM distal to *Rht8*. The 192 bp allele of *Xgwm261* corresponds to a height-reducing phenotype of *Rht8* (Korzun *et al.*, 1998). Varieties carrying the *gwm261*_{192bp} allele showed a height reduction of 7–8 cm (typically 8–10%) in England and the former Yugoslavia, without pleiotropic effects on other agronomic characters except for a slight increase in spikelet fertility (Worland *et al.*, 1998). The *gwm261*_{192bp} allele was long proposed to be diagnostic for *Rht8* (Chebotar *et al.*, 2001; Ahmad and Sorrells, 2002; Bai *et al.*, 2004; Schmidt *et al.*, 2004; Ganeva *et al.*, 2005; Liu *et al.*, 2005; Zhang *et al.*, 2006). However, the marker allele is indicative of *Rht8* only for pedigrees derived from the original source variety Akakomugi (Ellis *et al.*, 2007). In fact Norin10, a source variety of the Green Revolution semi-dwarfing genes, carries a *gwm261*_{192bp} allele that is independent of the height reduction effect of *Rht8* (Ellis *et al.*, 2007). Due to the wide spread of the Green Revolution genes and the *gwm261*_{192bp} allele independent of the height reduction effect of *Rht8*, studies assessing the distribution of height-reducing alleles at the *Rht8* locus made by screening *gwm261* alleles can mispredict the presence of the height-reducing allele of *Rht8*.

In the present study, the characterization of *Rht8* was advanced by fine-resolution mapping and the identification of syntenic intervals in model genomes of *Oryza sativa* and *Brachypodium distachyon*. Furthermore, the relationships between *Rht8* lines and the phytohormones involved in stature regulation, GA and BR, were investigated. It is clear that the genetic potential of optimizing wheat stature has not been fully exploited and the majority of genes and alleles affecting height remain uncharacterized. The work presented here addresses fundamental questions of the regulation of stature control in hexaploid wheat, and provides new tools for the more efficient deployment of *Rht8* in international breeding programmes.

Materials and methods

Culm and total height measurements

Cappelle-Desprez, Cappelle-Desprez (Mara 2D) substitution line (SL), Mara, and the Cappelle-Desprez (Mara 2D) single chromosome recombinant inbred line (RIL) population of 89 lines (*Triticum aestivum* L.) were used to assess the effect of *Rht8* on the wheat culm, and check the original height scores for *Rht8* of Korzun *et al.* (1998). Seedlings were grown in a mixture of peat and sand and vernalized for 8 weeks under short days (10 h light/14 h dark). Vernalization growth chambers were maintained at 5 °C with 70% relative humidity and were illuminated by tungsten lamps to provide a light intensity of 250±50 μmol m⁻² s⁻¹ at the top of the canopy. Subsequently, plants were transferred to an outdoor soil house, and hand dibbed. Two plants of each genotype were arranged in a randomized block design with five replicated plots (10 data points for each genotype). No plant growth regulators or triazoles were applied to the plots throughout development. Main tillers were tagged for each plant at the beginning of culm elongation. Final plant height, measured from the soil to the tip of the ear in fully grown and yellowing main tillers, was recorded for each plant. Individual internode lengths and ear lengths of the main tiller were also recorded. Two-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference

(HSD) were performed to determine height differences among height classes. Statistical analyses were performed in R 2.10.1 (R Development Core Team, 2005). The same analysis was done for ear, peduncle, and internode lengths.

Microscopic analyses

For transverse sections, main tillers of five wild-type RIL33 (Cappelle-Desprez tall *rht8* allele) and five RIL4 (semi-dwarf *Rht8* allele) lines were compared at ear emergence (growth stage 50–59; Zadoks *et al.*, 1974). Sections were taken from the medial elongating zone of each culm segment (middle of peduncle, and middle of internode 1), and from the respective nodes. Cut tissues were immediately fixed (2.5% glutaraldehyde, 0.1 M phosphate buffer pH 7.0, 0.1% Triton X-100) and vacuum infiltrated overnight. Tissues were subsequently dehydrated in a graded ethanol series and embedded in Technovit 7100 resin (Kulzer-Technik, Germany) according to the manufacturer's instructions. Embedded tissues were sectioned with a Leica Autocut microtome (RM 2055) to 5–10 μm thick slices, stained with toluidine blue, and visualized on a Nikon Microphot-SA microscope fitted with a Pixera Pro600ES DiRactor™ camera. All pictures were taken at the same magnification, and cell number per unit area was calculated using an arbitrary but constant area of $550 \times 300 \mu\text{m}$ with ImageJ (Abramoff *et al.*, 2004).

Scanning electron microscopy (SEM) was used to compare longitudinal sections of five Cappelle-Desprez (tall *rht8* allele) and five RIL4 (semi-dwarf *Rht8* allele) main tillers from fully elongated internode 1 and peduncle at straw stage (growth stage 90; Zadoks *et al.*, 1974). Sections were taken from three different zones of each internode (basal, <1 cm after the basal node; medial, in the middle of the considered internode elongating zone; and distal, at >1 cm before the following node) under a dissecting microscope, and sputter coated with gold on an Agar, high resolution sputter-coater. Straw samples were observed directly using a FEI XL30 FEG scanning electron microscope, without the need of fixation and critical point drying. A minimum of 100 cells were measured for each sample using the instrument's software.

Both transversal and longitudinal cell measurements were evaluated with Student's *t*-test.

GA assays

The endogenous GA content of elongating main culms of Cappelle-Desprez (*rht8*) was compared with that of RIL4 (*Rht8*). Leaf sheaths covering the main culm were removed and developing ears discarded before collecting culm tissue from 6-week-old plantlets (35–45 cm tall plants, flag leaf just visible, growth stage 37; Zadoks *et al.*, 1974). Cut samples were immediately frozen in liquid nitrogen and freeze-dried. Each variety was replicated five times, and 4–5 main tillers were pooled per sample. GA extraction and analysis was performed as in Griffiths *et al.* (2006), and values were compared with the Student's *t*-test.

To assess the culm response to exogenous GA_3 , Cappelle-Desprez (*rht8*), RIL4 (*Rht8*), and the Mercia *Rht3* near isogenic line (NIL) were used. Prior to treatments, wheat seedlings were grown in a mixture of peat and sand and vernalized as before. Plantlets were then transferred to 1 litre pots containing cereal mix, randomized, and grown under long days (16 h light/8 h dark) at 18 °C with 70% relative humidity with a light intensity of $350 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the top of the canopy. GA_3 (63492 Sigma) was dissolved in absolute ethanol at 1 mM, and treatments were prepared in fresh aqueous solutions. Test solutions (5 ml per seedling of 100 μM of GA_3) were applied with spray bottles to the main culm surface every 3 d until anthesis. Control plants were sprayed with 100 μM ethanol. The experiment was repeated twice, with five plants for each treatment. Responses (final plant height) were compared with ANOVA followed by Tukey's HSD test in R 2.10.1 (R Development Core Team, 2005).

BL assays

The root response of Cappelle-Desprez (*rht8*), and RIL6 (*Rht8*) plants to *epi*-brassinolide (*epi*-BL, E1641 Sigma) was measured and compared

with an adapted method from Chono *et al.* (2003) and Hong *et al.* (2003). Seeds were surface sterilized with 70% ethanol for 30 s and 10% NaClO for 10 min, and germinated for 2 d in Petri dishes on wet filter paper at 24 °C in the dark. Germinated seeds were transplanted to Magenta boxes containing 1% agar, half-strength Murashige and Skoog (MS) medium, and the appropriate *epi*-BL concentration (0, 0.01, 0.1, and 1 μM *epi*-BL). Samples were incubated at 23 °C under long days (14 h light/8 h dark, $350 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 4 d. The cotyledon and shoot length response of Cappelle-Desprez (*rht8*), and RIL6 (*Rht8*) plants to 1 μM *epi*-BL was measured and compared at 10 d post-germination. To determine root lengths, primary roots were stretched by forceps, photographed, and measured with ImageJ software (Abramoff *et al.*, 2004). Root dry mass was measured with an analytical scale after all roots were removed from the plantlets and dried for 3 d at 60 °C. The experiment was repeated three times, with a minimum 10 plants for each treatment. Data were analysed with ANOVA followed by Tukey's HSD test in R 2.10.1 (R Development Core Team, 2005).

The wheat leaf unrolling assay was performed according to Wada *et al.* (1985) on Cappelle-Desprez (*rht8*), RIL4 and RIL6 (*Rht8*), and selected homozygous F_4 recombinants. After plants were grown in the dark for 6 d at 25 °C, the first 1.5 cm long segment from the leaf tip was discarded, and the following 3×1.5 cm long leaf segments were excised and incubated in 2 ml of distilled water containing an appropriate *epi*-BL concentration. All excisions were conducted under a dim green safelight. Five *epi*-BL concentrations (0.0002, 0.002, 0.02, 0.2, and 2 μM) were tested against the control. A minimum of six leaf segments from six individual plants were incubated in each concentration, and each concentration was replicated three times. Leaf segments were allowed to unroll for 72 h in the dark at 25 °C and photographed at 0, 24, 48, and 72 h after excision. Unrolling of leaf segments was determined by measuring their width using the software ImageJ (Abramoff *et al.*, 2004). The unrolling percentage was calculated as described in Wada *et al.* (1985) with additional normalization for the initial leaf width and for the background (unrolling of the untreated control over time). Specifically, at a given time point (i) and treatment (j), 'unrolling %' = $[(W_{ij}/W_{0j}) - (W_{i0} - W_{00})]$, where W_{ij} is the mean leaf width of the sample considered, W_{0j} is the mean leaf width of the same sample at time '0', W_{i0} is the mean leaf width of the control at time 'i', and W_{00} is the mean leaf width of the control at time '0'. Statistical analysis (ANOVA and Tukey's HSD test) was performed with R 2.10.1. (R Development Core Team, 2005). The experiment was repeated twice. Homozygous F_4 recombinants were tested only at 0, 0.02, and 2 μM *epi*-BL.

Marker development

Gene-based markers were designed from wheat expressed sequence tags (ESTs) mapping to the chromosome 2DS1-0.33 deletion bin (Endo and Gill, 1996; http://wheat.pw.usda.gov/cgi-bin/westsq1/map_locus.cgi), from selected rice unspliced gene sequences (Rice MSU Rice Genome Annotation Project Database v6.1, ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_6.1/all.dir/; RAP database v5, <http://rapdb.dna.affrc.go.jp/download/index.html>), and from selected *B. distachyon* unspliced gene sequences (release 1.0, www.modelcrop.org; ftp://ftp.ensemblgenomes.org/pub/plants/release-3/fasta/brachypodium_distachyon/cdna/). Wheat assembled ESTs were downloaded from the TIGR Plant Transcript Assemblies database (ftp://ftp.tigr.org/pub/data/plantta/Triticum_aestivum/). For all data sets, a local BLAST database was produced using the command formatdb. A local similarity search (blastall -p blastn) (Altschul *et al.*, 1990) was performed on wheat ESTs against the unspliced gene databases to identify the corresponding unspliced gene. Wheat EST sequences with a first-hit similarity score of >150 were aligned to the genomic sequence of the identified gene using the est2genome algorithm (EMBOSS). To reduce false positives, results were filtered for repetitive elements (Triticeae Repeat database: TREP <http://wheat.pw.usda.gov/ITMI/Repeats>), and for wheat ESTs that hit genes outside the desired genomic interval when re-BLASTed. Primers amplifying products

of ~500–600 bp were designed on EST sequence-spanning predicted introns, and when possible on 3'-untranslated regions (UTRs), with Primer3 (<http://frodo.wi.mit.edu/>, Rozen and Skaletsky, 2000). Wheat ESTs from which markers were designed were also BLASTed against the SWISSPROT database for predicting the putative function (ftp://ftp.uniprot.org/pub/databases/uniprot/knowledgebase/uniprot_sprot.fasta.gz).

Polymorphic markers (Table 1) between parents of the coarse mapping population (89 RILs; Korzun *et al.*, 1998) were amplified from all RILs. Genomic DNA was extracted from 2-week-old seedlings with an adapted method of Pallotta *et al.* (2003). Amplification was conducted in a 20 μ l volume containing 50 ng of genomic DNA, 1 μ M of each primer, 1.25 mM dNTPs, 1 \times PCR buffer (Invitrogen), and 0.4 U of *Taq* DNA polymerase (Invitrogen). After an initial denaturation at 94 °C for 1 min, 35 amplification cycles were performed: 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. Single-stranded amplification products of newly developed gene-based markers were separated according to their conformation by single strand conformation polymorphism (SSCP) analysis as described by Bertin *et al.* (2005). Linkage of molecular markers on chromosome 2DS was analysed with JoinMap version 3.0, with a logarithm (base 10) of odds (LOD) score >5.0, and the Haldane mapping function.

Fine mapping population development

To develop the fine-resolution mapping population for map-based cloning of *Rht8*, selected semi-dwarf lines of the 89 RIL population (Korzun *et al.*, 1998) carrying *Xgwm261*_{192bp}, *Rht8*, and sensitive to photoperiod (*Ppd-D1b*), i.e. lines RIL4 and RIL6, were crossed with Cappelle-Desprez (*Xgwm261*_{172bp}, *rht8*, and *Ppd-D1b*). The resulting F₁ plants were selfed to produce a segregating F₂ population. F₂ plants were screened using high-throughput fluorescent genotyping with *Rht8*-flanking SSR markers *gwm261* and *cfid53* to retain recombinants. *Gwm261* and *cfid53* forward primers were 5' labelled with four different dyes: 6-FAM, VIC, NED, and PET (Applied Biosystems), and their amplification multiplexed in a single reaction. Amplification was conducted in a 10 μ l volume containing 50 ng of genomic DNA, 0.2 μ M of each of the four primers, 1 \times Hot Star MasterMix (Qiagen), with the following conditions: denaturation at 94 °C for 15 min, 40 cycles of 94°C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 1 min. Following amplification, four different samples, each labelled with a different dye, were pooled in the first 1:50 (H₂O) dilution at a ratio of 1:2:1:2 for 6-FAM:VIC:NED:PET. Each sample of the first dilution plate was further diluted 1:10 with 8.95 μ l of denaturing agent HiDi Formamide (Applied Biosystems), and 0.05 μ l of size standard LIZ500 (Applied Biosystems). Samples were separated by capillary electrophoresis on an ABI3730 (ABI PRISM 3730 DNA sequencer; Applied Biosystems), and semi-automated SSR allele sizing was performed using GeneMapper v.4 software (Applied Biosystems). This procedure allowed the analysis of eight data points per capillary run: two markers for each of the four DNA samples labelled in a different dye.

Identified recombinant F₂ plants (i.e. homozygous for one SSR locus and heterozygous for the other one, or homozygous at both loci with a different genotype from the crossing parents) were further genotyped with gene-based markers within the interval (*DG087*, *DG260*, *DG274*, *DG279*, and *DG371*), phenotyped for height, and selfed to produce recombinant F₃ families. The F₃ families were genotyped as before to identify homozygous *Rht8* recombinants (i.e. recombinants that have a different genotype from the crossing parents, and are homozygous at both loci of the flanking markers). Homozygous *Rht8* F₃ recombinants were selected, scored for height, and selfed to obtain homozygous F₄ recombinant lines. The segregation fit of genotypic frequencies was estimated with the χ^2 test, and the pair-wise recombination frequency (*r*) between *Rht8* and the other seven loci (*Xgwm261*, *DG260*, *DG087*, *DG274*, *DG279*, *DG371*, and *Xcfid53*) in the F₂ population was calculated as $r = [(\text{recombinant heterozygotes} + 2 \times \text{recombinant homozygotes}) / 2 \times F_2 \text{ plants}]$ to determine marker order and genetic distances.

F_{2:3} height analysis

Height data from homozygous F_{2:3} recombinants were used to back-score the original F₂ generation. F_{2:3} families deriving from recombinant F₂ plants, each consisting of 16 individuals, were genotyped for *Xgwm261* and *Xcfid53* by high-throughput fluorescent genotyping, for the selection of homozygous *Rht8* recombinants. Genotype frequencies were estimated with the χ^2 test. Homozygous F₃ recombinants were retained for each F_{2:3} family, and grown to maturity in glasshouse conditions in four randomized batches to produce homozygous F₄ recombinants. Mean height values of F₃ recombinants from each F_{2:3} family were used to determine a 'b' (short) or 'a' (tall) score, in a linear model expressed as the following equation: $H = \beta_0 + \beta_1 V + \beta_2 E + e$ ('H' is the plant height; ' β_0 ', ' β_1 ', and ' β_2 ' are the parameters evaluated by the model, 'V' is the variety, 'E' is the experiment, and 'e' is the error term). For each randomized batch, V_{Cappelle} (tall control) was imposed as 0, then β_0 became the average height of Cappelle-Desprez, β_1 the difference between Cappelle-Desprez and the other varieties, and β_2 the correction factor for the experiment. The model evaluates if the mean height values of F₃ recombinants from each F_{2:3} family have a statistically significant difference in height compared with Cappelle-Desprez (when β_1 differs from 0). Analyses were done with R 2.10.1 (R Development Core Team, 2005).

Results

The effect of *Rht8* on wheat culms

The most evident effect of the semi-dwarf *Rht8* allele is to reduce wheat stature by shortening culm lengths (Fig. 1A, B). Worland *et al.* (1998) found that *Rht8* causes a 7–8 cm reduction in plant height, resulting in plants that are typically 10% shorter than their wild-type counterparts. However, it was not known whether the reduction in culm length is due to fewer or shorter internodes, and whether the reduction in culm length is concentrated in a particular internode or spread along all internodes of the culm. It is also important to separate clearly the effects of *Rht8* and *Ppd-D1a* which both influence culm length and are genetically linked. In varieties where *Rht8* is used, it normally occurs together with the photoperiod-insensitive allele *Ppd-D1a*.

In a randomized and replicated experiment, individual internode lengths of main tillers in the Cappelle-Desprez (Mara 2D) RIL population (89 lines; Korzun *et al.*, 1998) segregating for *Rht8* and *Ppd-D1*, and parental lines Cappelle-Desprez (tall) and Cappelle-Desprez (Mara 2D) SL (short) were measured. By mapping *Ppd-D1* using perfect markers developed by Beales *et al.* (2007), individual RILs were grouped into four classes according to their *Rht8*, *Ppd-D1*, and *gwm261* genotype: wild-type *Ppd-D1b+rht8* (33 lines, such as Cappelle-Desprez), *Ppd-D1a+rht8* (five lines), *Ppd-D1b+Rht8* (eight lines), and *Ppd-D1a+Rht8* [43 lines, such as Cappelle-Desprez (Mara 2D) SL].

Wild-type RILs were of the same height as the tall Cappelle-Desprez parent (112 \pm 4.3 cm), while *Rht8* RILs were 14 cm (13%) shorter than the tall parent. As previously reported (Borner *et al.*, 1993; Worland *et al.*, 1998), the *Ppd-D1a* allele also reduced the culm length of *Ppd-D1a* RILs by an average of 4 cm (3%). Under the long-day conditions used, an additive effect of *Rht8* and *Ppd-D1a* alleles to shorten stature was not detected. In fact, the culm length of *Rht8+Ppd-D1a* RILs was not significantly different from that of *Rht8* RILs or the short Cappelle-Desprez (Mara 2D) SL parent.

Analyses of internodal segments indicate that both *Rht8* and *Ppd-D1a* alleles reduce plant height by acting throughout the

Table 1. Polymorphic markers between parents of the Cappelle-Desprez (Mara 2D) RIL population developed in this study

Marker	Primer_F	Primer_R	Ta (°C)	Wheat EST	Rice (MSU) Hit	Brachy Hit
DG025	ACACGCACACATGAGCAAAT	ACGGGTTTCAGGAAGATGTTG	56	CD490659	LOC_Os04g02570	Bradi0026s00210
DG032	AGGAGGCAGATGCAGAAGC	CCTGATCAAGACACCGTAAGC	56	CD897865	LOC_Os04g02830	Bradi5g01720
DG035	CATATGGCAGGAGCAGGAGT	TCCATCAGTCATAACCTCTTCTG	56	DN949138	LOC_Os04g02870	Bradi5g01430
DG048	GGAATGGCTTTTTCCCTGTT	TGGCGATAAGCCTTGAATAAT	56	CD906478	LOC_Os04g04000	Bradi3g06030
DG057	TGGACTCAACCATTGGAGAA	CGATCACTTGTCTGTTTCA	56	TA27071_4565	LOC_Os04g05010	Bradi5g01180
DG062	GCAGGCATGGTTACTTCCAT	CCCTCTGACCTCCAGTTCC	56	TA24720_4565	LOC_Os04g05050	Bradi5g01130
DG072	CGTTCAATGTCTGGATCGAC	GGGTCACCTGAGTTTCGCAAT	58	TA43575_4565	LOC_Os04g11820	Bradi5g02870
DG086	TCAATGGCCATATTAAGGCTCTA	AGCAATCTTTGTGTCCATATCAA	58	TA43575_4565	LOC_Os04g11820	Bradi5g02910
DG087	GATCTGCACTGCTCCATCAA	TCCACTGCGACATAAAACCA	58	CD900476	LOC_Os04g12580	Bradi5g02980
DG118	GCCTTCCGGAACAGGTACT	GCAGCTAGGACCCCTCAAATG	58	BJ246143	LOC_Os04g14760	Bradi5g03700
DG236	CATCCAGACGCATGGATACT	CCATGCTTTCAGTTCTTCC	58	BE489611	LOC_Os12g16650	Bradi5g02490
DG241	TCCCTGCAGGCGTAAGTAAC	GGGTCACCTGAGTTTCGCAAT	58	TA43575_4565	LOC_Os04g11820	Bradi5g02870
DG244	GTTCAGATCAGGCGAGGAAG	GGAGGTCGTGATCGAGAAGA	58	TA11281_4565	LOC_Os12g17910	Bradi5g02890
DG260	ACCATTGGCTCCCTTCAGTA	TGGAGGCCTGATTCTGTTTC	58	CA629789	LOC_Os07g04160	Bradi5g02990
DG273	CTTGACGAGCTTGGAAATGG	GCAACAAGTGCTTCTGTCGT	58	CD877708	LOC_Os04g12960	Bradi5g03380
DG274	GGAGTCGACGCTTTGTTC	GCTCTCCATGTTAATCCATGTACTC	58	TA41391_4565	LOC_Os04g12720	Bradi5g03390
DG279	TGCTCAAGGGAAAGACCATC	AAAGCCTGAGCCTGCTTCTA	58	TA44444_4565	LOC_Os04g13210	Bradi5g03460
DG371	CCACTTGACAAGCAAATTAAGA	ATCACGAGGCTGGTGTCG	58	BJ307036	LOC_Os04g19140	Bradi5g04710

Ta (°C) is the optimum annealing temperature. Wheat EST, wheat ESTs retrieved from GenBank or from the TIGR Plant Transcript Assemblies database (ftp://ftp.tigr.org/pub/data/plantta/Triticum_aestivum/). Rice (MSU) Hit, wheat EST BLASTN hit to Rice Genome Annotation MSU database version 6.1 (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_6.1/all.dir/); Brachy Hit, wheat EST BLASTN hit to *B. distachyon* Genome Annotation Project database 1.0 (ftp://ftp.ensemblgenomes.org/pub/plants/release-1/fasta/brachypodium_distachyon/cdna/).

culm, and not by decreasing the number of elongating internodes and nodes. In fact, all RIL classes have the same number of internodes (peduncle+5) and visible nodes (5) as the wild-type Cappelle-Desprez parent (Fig. 1C). As previously reported (Worland *et al.*, 1998), the semi-dwarf *Rht8* allele does not affect ear morphology in this population. The largest measurable difference in segment length between semi-dwarf *Rht8* RILs and wild-type Cappelle-Desprez lines was observed in the peduncle (by an average of 15% length reduction) and internode 1 (by an average of 5% reduction), while other internodes were reduced proportionally. Internode 1 and peduncle are also the final two internodes to elongate, thus accounting for the majority of the final culm length.

Rht8 affects culm length by reducing cell elongation

In wheat, internode elongation is caused by cell division in the intercalary meristem, followed by cell elongation in the elongation zone. Shorter culms observed in *Rht8* lines could be caused by changes in cell division, cell elongation, or both. To investigate these possibilities, the internal cellular morphology of the peduncle and internode 1 was examined.

First, transverse sections of culm tissues at ear emergence (wheat growth stage 50–59; Zadoks *et al.*, 1974) in *Rht8* (*Ppd-D1b*) and wild-type lines was examined. This developmental stage provided the opportunity to study both cell expansion and cell proliferation by examining expanded parenchyma cells in internode 1 and node 2, and proliferation activity in the intercalary meristem of node 1. In both wild-type and *Rht8* lines, internode 1 was fully elongated and cell division at node 2 has ceased (Fig. 2I, J). The cellular structure of node 2 in both lines appeared very similar: tissues were fully differentiated with very thick cell walls, particularly the mechanically strong sclerenchyma cells

surrounding vascular bundles. Transverse sections of medial elongating zones of fully elongated internode 1 of wild-type and *Rht8* lines showed a similar anatomical organization of cell types (Fig. 2G, H). On the other hand, at the stage examined, the peduncle is still elongating and the meristem is active at node 1 until anthesis. A similar organization of cell types was also observed in medial elongating zones of the peduncle of wild-type and *Rht8* lines (Fig. 2A, B). Cell divisions were still very pronounced in both wild-type and *Rht8* lines (Fig. 2C–F). There was no apparent defect in cell proliferation in node 1 in the intercalary meristem of *Rht8* lines. This suggests that intercalary meristems do develop in *Rht8* mutants but perhaps cell elongation is reduced, resulting in shorter peduncles (and internodes).

Longitudinal sections of fully elongated peduncle and internode 1 at straw stage in wild-type and *Rht8* lines were further examined to see if shorter culms in *Rht8* lines are reflected in shorter parenchyma cells. Longitudinal sections were taken from three different zones of the internodes: basal, medial, and distal. In both wild-type Cappelle-Desprez and *Rht8* lines, parenchyma cells in the peduncle and internode 1 were longitudinally elongated and organized in longitudinal files (Fig. 3A, B). However, throughout the sections, cells in the *Rht8* lines were significantly shorter than those in Cappelle-Desprez plants (Fig. 3C, D, *t*-test $P < 0.05$). The observations show that the semi-dwarfing *Rht8* allele affects culm cell elongation.

Rht8 is not involved in gibberellin biosynthesis or signalling

Cell elongation in the culm is often regulated by gene products involved in the GA or BR pathways (reviewed in Wang and Li, 2008). Traditionally, *Rht8* has been classified as a GA-sensitive gene because plants respond to the exogenous application of

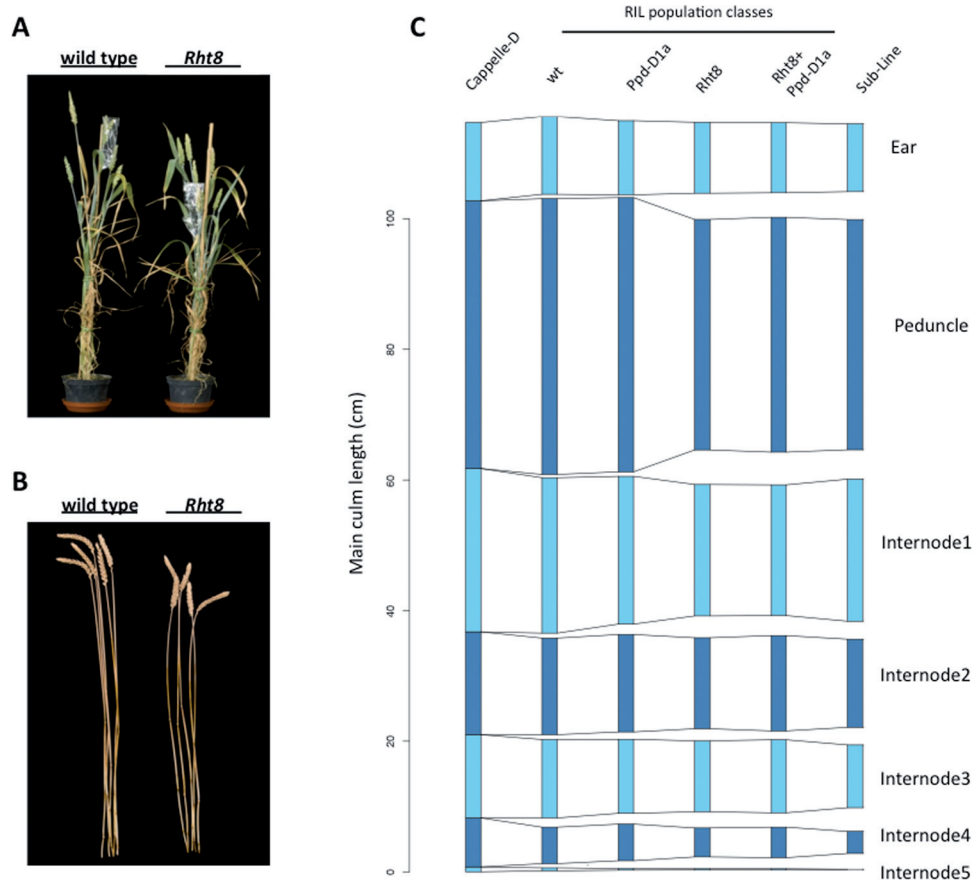


Fig. 1. Phenotype of hexaploid wheat semi-dwarf *Rht8* lines. (A) Gross plant morphology of wild-type (Cappelle-Desprez) and semi-dwarf *Rht8* [RIL4, from Cappelle-Desprez (Mara 2D) RIL population] lines. (B) Culm morphology of wild-type (Cappelle-Desprez) and semi-dwarf *Rht8* (RIL4) lines. (C) Schematic representation of internode elongation patterns in wild-type Cappelle-Desprez (Cappelle-D), semi-dwarf Cappelle-Desprez (Mara 2D) SL (substitution line), and the Cappelle-Desprez (Mara 2D) RIL population grouped in classes according to their *Rht8*, *Ppd-D1*, and *Xgwm261* genotype: wild-type (wt), *Ppd-D1a*, *Rht8*, and *Rht8+Ppd-D1a*.

GAs (McIntosh *et al.*, 2003), but its role, if any, in GA biosynthesis or signalling remains unknown. Ellis *et al.* (2004) found no difference in leaf elongation rate or responsiveness to GA using selected lines of the Vigour18 (tall)×Chuan-Mai 18 (semi-dwarf, *Rht8*) double haploid population. However, the response of semi-dwarf *Rht8* plants to the exogenous application of GA or the quantification of endogenous GAs has never been analysed in elongating culms.

The endogenous GA content was characterized in rapidly expanding culm internodes of 6-week-old wild-type and semi-dwarf *Rht8* lines (35–45 cm tall, flag leaf just visible, growth stage 37; Zadoks *et al.*, 1974). GAs belonging to the early 13-hydroxylation GA pathway (GA_1 , GA_8 , GA_{19} , GA_{20} , GA_{29} , and GA_{44}), the main pathway occurring in wheat, were quantified by full-scan gas chromatography–mass spectrometry. GA_{53} , GA_3 , and GA_{17} , also synthesized via the early 13-hydroxylation GA pathway, were below the level of detection or too low to quantify. If *Rht8* lines had a defect in GA biosynthesis, they would have lower levels of bioactive GA (GA_1) compared with the wild type, while a defect in GA signal transduction would result in an accumulation of C_{19} -GAs (Webb *et al.*, 1998). However, no significant difference in the amount of endogenous GAs compared with wild-type plants was found (Table 2), at

the developmental stage examined. The only significant difference was in the concentration of GA_{29} , which is the inactivated product of GA_{20} . As GA_{29} had the lowest concentration of all GAs measured, and levels of GA_{20} were the same in wild-type and *Rht8* lines, an explanation for the observed difference is that GA_{29} was too low to quantify accurately. The result indicates that *Rht8* is unlikely to be involved in GA biosynthesis or signalling.

To confirm that semi-dwarf *Rht8* lines do not differ from wild-type plants in GA content or signal transduction, wild-type and *Rht8* lines were sprayed with 100 μ M GA_3 throughout development and their response was measured as final culm length. The Mercia *Rht3* NIL containing the *Rht-B1c* GA-insensitive allele was used as a negative control. Both Cappelle-Desprez and *Rht8* lines showed an increase in culm elongation following the treatments, whereas Mercia *Rht3* NIL, as expected, did not (Supplementary Fig. S1 available at *JXB* online). Cappelle-Desprez and *Rht8* lines responded with a very similar increase in plant height (Tukey's HSD test $P < 0.05$): the wild type showed a 15% increase, whereas the semi-dwarf showed a 13% increase. Thus, *Rht8* lines had an elongation response comparable with that of the wild type. The data confirm that *Rht8* is unlikely to be directly involved in GA metabolism or signalling to reduce plant height.

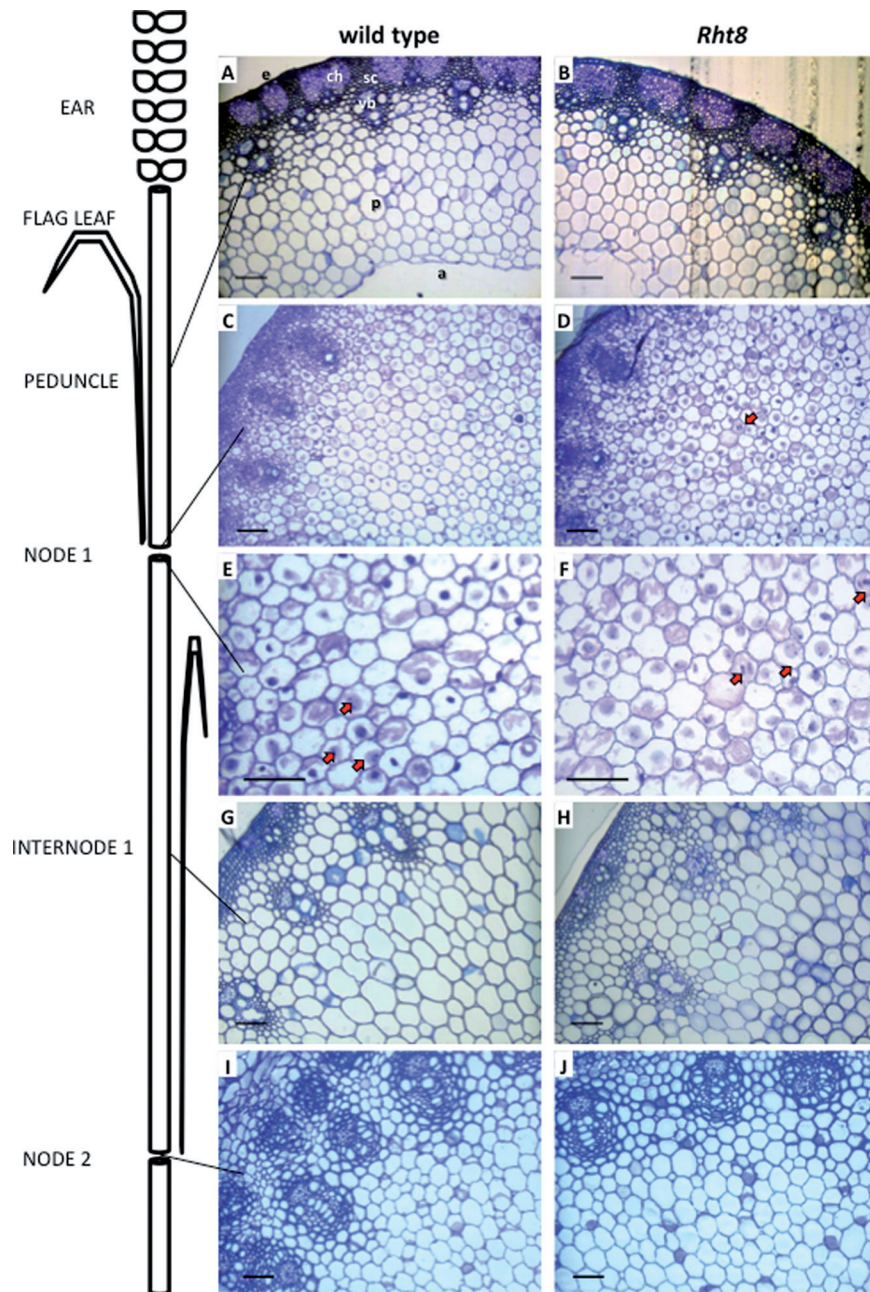


Fig. 2. Transverse culm sections of *Rht8* lines at ear emergence. Transverse sections of the medial elongating zone of the peduncle (A, B) and internode 1 (G, H), of node 1 (C, D, E, F), and node 2 (I, J) in wild-type [A, C, E, G, I, RIL33 from Cappelle-Desprez (Mara 2D) RIL population] and semi-dwarf *Rht8* lines [B, D, F, H, J, RIL4 from Cappelle-Desprez (Mara 2D) RIL population]. Sections were stained with toluidine blue; red arrows indicate cell divisions. Bars = 100 μ m. e, epidermis; ch, chlorenchyma; sc, sclerenchyma; vb, vascular bundle; p, parenchyma; a, aerenchymous centre.

Rht8 lines show an altered sensitivity to brassinolide

Typically, rice mutants with defects in BR biosynthesis or signaling are dwarfed, exhibit specific shortening of the second internode (equivalent to internode 1 of wheat), have very erect leaves, and show photomorphogenesis in the dark, also called the detiolated (DET) phenotype (Yamamuro *et al.*, 2000). The altered phenotypes can be restored upon BR application in deficient, but not in insensitive BR mutants. In temperate cereals, the only characterized BR mutant is *br1*, which encodes the brassinosteroid

receptor HvBR11, and was cloned using *uzu* barley (Chono *et al.*, 2003). To the authors' knowledge, it has not been reported whether *uzu* barley has an internode-specific reduction as in the case of rice BR mutants. To date, there are no characterized BR mutants in wheat, although precursors of the biologically active form BL are present in different parts of the wheat plant, and BRs are thought to influence wheat development at many levels (Takatsuto *et al.*, 1999; Feng *et al.*, 2007). *Rht8* lines show a semi-dwarf phenotype, pronounced shortening of the peduncle and first internode, and no direct or indirect involvement in GA

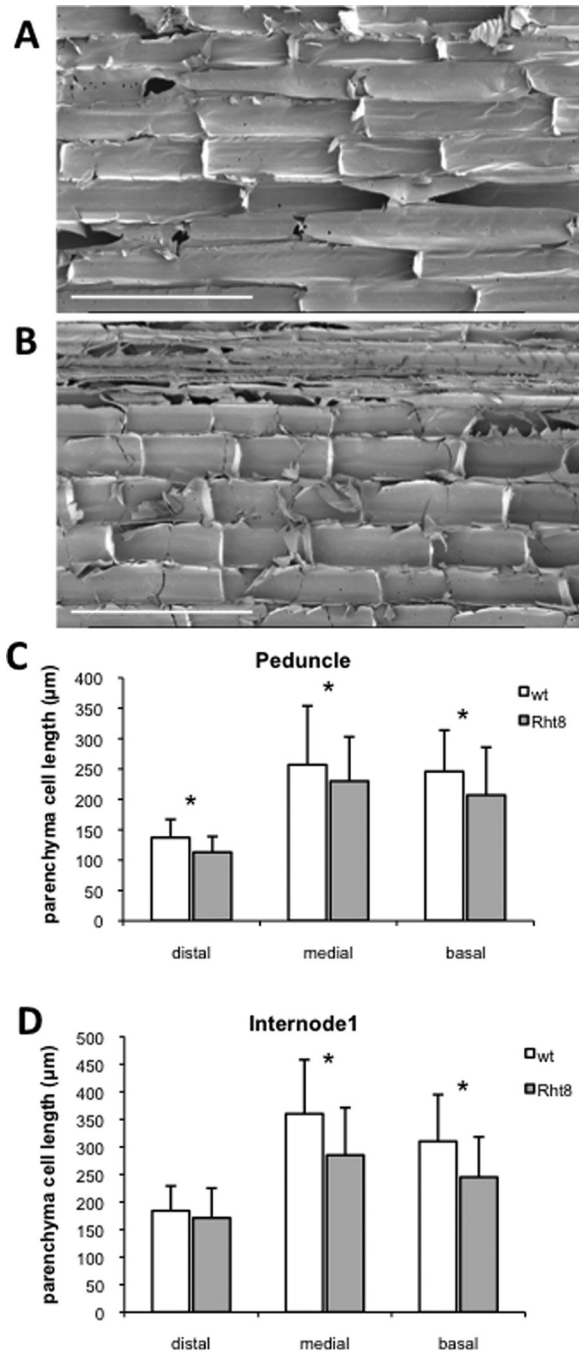


Fig. 3. Longitudinal culm sections of *Rht8* lines from straw. Scanning electron micrographs of the medial zone of fully elongated peduncle in (A) wild-type (Cappelle-Desprez) and (B) semi-dwarf *Rht8* lines [RIL4 from Cappelle-Desprez (Mara 2D) RIL population]. Bars = 200 μm. Comparisons of parenchymatic cell lengths (μm) in basal (<1 cm after the basal node), medial (in the middle the considered segment), and distal (>1 cm before the following node) sections of the (C) peduncle and (D) internode 1 between wild-type and *Rht8* lines. **t*-test $P < 0.05$. Bars represent the positive value of the standard deviation.

metabolism. These characters, in addition to the fact that BRs regulate cell elongation (Wang and Li, 2008), led to the decision to test the responsiveness of Cappelle-Desprez and isogenic *Rht8* seedlings to BL in two distinct assays.

First, the root sensitivity to BL treatment was tested in 4-day-old wheat seedlings (wild-type Cappelle-Desprez and *Rht8* RIL6 lines). In wild-type rice seedlings grown on agar media containing BL, coleoptiles elongate abnormally, resulting in a twisted shape, leaves grow poorly and do not break through the coleoptile, root elongation is inhibited, and the roots develop in a wavy form (Yamamuro *et al.*, 2000). In contrast to rice, barley wild-type seedlings treated with BL show a clear response only in the roots, whereas BL effects on other tissues and body parts were inconclusive (Chono *et al.*, 2003). Similarly, at 1 μM BL, no treatment effect was observed on coleoptiles and shoots of both wild-type and *Rht8* lines (Supplementary Fig. S2 at *JXB* online). However, a differential root response after BL treatment was observed between the wild type and *Rht8*. In the wild type, the length of the primary root as well as the root dry mass decreased with increasing BL concentration ($P < 0.05$), while the semi-dwarf *Rht8* line showed a slight but significant increase in root length at lower BL concentrations (0.01 μM and 0.1 μM, $P < 0.05$) but no response at a higher dose (1 μM, $P > 0.05$) (Fig. 4). Furthermore, there was no significant change in root dry mass upon treatment in the *Rht8* line (Fig. 4, $P > 0.05$). Importantly, at the developmental stage examined, untreated wild-type and *Rht8* lines did not differ in their shoot, coleoptile, or root length and root dry mass (Fig. 4, Supplementary Fig. S2, $P > 0.05$).

To better characterize the differential BR sensitivity observed between the wild-type and *Rht8* seedlings, the established wheat leaf unrolling assay developed by Wada *et al.* (1985) was used. If *Rht8* lines have a different sensitivity to BRs, the degree of leaf unrolling should be distinct from that of wild-type Cappelle-Desprez plants. Etiolated leaf segments were incubated in different concentrations of BL for 72 h and photographed every 24 h, as shown in Fig. 5A. Leaf segments of both wild-type and *Rht8* lines did not respond to 0.0002 μM and 0.002 μM BL (Fig. 5B, test $P > 0.05$), but started to unroll at 0.02 μM BL (Fig. 5D), and the extent of unrolling (apparent width) increased over time at each concentration tested (Fig. 5D–F). For both lines the plateau was reached at 2 μM BL after 72 h (Fig. 5F), when leaf segments were totally flattened and of the same width. Although Cappelle-Desprez and *Rht8* lines reached the same final leaf width (Fig. 5B, test $P > 0.05$), a significant difference was observed in unrolling percentage (leaf segment width) over time at the higher BL concentrations tested (0.02, 0.2, and 2 μM BL). The extent of leaf unrolling at each concentration was always lower in *Rht8* lines ($P < 0.05$), suggesting that leaf sensitivity of *Rht8* lines to BRs is less than that of wild-type Cappelle-Desprez plants. A hyposensitivity of *Rht8* lines to BRs could explain the semi-dwarfed phenotype and the reduction in cell elongation.

Comparative mapping of the *Rht8* locus

Previously, *Rht8* was mapped between the *Xgwm261* (0.6 cM distal to *Rht8*) and the *Xgwm484* SSR loci (19.9 cM proximal to *Rht8*) by Korzun *et al.* (1998). In this study, the same mapping population of 89 Cappelle-Desprez (Mara 2D) RILs was used for improving the 2DS map in this interval. *Rht8* (plant height) was re-scored in the population according to the 95% confidence interval of the height controls: Cappelle-Desprez (Mara 2D) SL (short) or Cappelle-Desprez (tall). The 2DS map

Table 2. Endogenous GA concentrations (ng g⁻¹ dry weight) in uppermost expanding culm internodes of wild-type (wt, Cappelle-Desprez) and semi-dwarf *Rht8* lines [RIL4 from from the Cappelle-Desprez (Mara 2D) RIL population]

	GA ₄₄	GA ₁₉	GA ₂₀	GA ₂₉	GA ₁	GA ₈	GA ₃₈
wt	26.8±0.8	22.3±5.6	1.6±0.4	0.7±0.02	2.1±0.5	14.7±4.5	4.3±0.7
<i>Rht8</i>	26.4±0.8	26±5.6	1.7±0.1	0.8±0.03	2.2±0.4	11.5±3.9	3.4±0.9
t-test P-value	0.509682	0.460928	0.683785	0.000146	0.644931	0.267896	0.119346

Values are given as the mean ±SE.

was also improved by mapping four additional SSR markers (*wmc503*, *cfid53*, *wmc112*, and *gwm1418*), and the *Ppd-D1* perfect marker (Beales *et al.*, 2007). Amplification conditions and primer sequences were obtained from GrainGenes, <http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=marker>. To anchor collinear regions in model genomes, new gene-based markers were designed.

Initially, gene-based markers were designed from wheat ESTs mapping to the chromosome 2DS1-0.33 deletion bin. In fact, both *gwm261* and *gwm484* markers flanking *Rht8* in the 89 Cappelle-Desprez (Mara 2D) RILs have been assigned to this chromosomal deletion bin (<http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=marker>; Endo and Gill, 1996). In order to align the rice genomic sequence to the wheat *Rht8* locus, sequence information of the gene-based marker *COS2Q* was used. *COS2Q* was originally developed for the WGIN project as a conserved orthologous sequence (COS), and has been previously mapped distal to *Xgwm261* on 2DS using the Avalon×Cadenza

double haploid population (<http://www.wgin.org.uk>). The wheat EST BE444541 (GenBank ID), from which it was developed, is orthologous to the rice gene LOC_Os04g01590 (Rice Genome Annotation project MSU 6.1). This marker was monomorphic between parents of the mapping population used, but LOC_Os04g01590 was taken as an anchoring point on the rice genome for marker development. Markers were designed every 10 kb in both directions from the anchoring point, screened for polymorphism, and, if polymorphic, mapped in wheat to establish the direction of the wheat–rice collinearity. After anchoring the rice genome with polymorphic markers, syntenic rice (MSU 6.1) and *B. distachyon* (release 1.0 at www.modelcrop.org) sequence information was used to target and saturate the *Rht8* locus. Markers were named DG (for Dwarfing Gene) followed by three numbers. A total of 203 primer pairs were developed from collinearity with rice (from marker *DG001* to marker *DG203*), and 228 from the collinearity with *B. distachyon* (from marker *DG204* to marker *DG431*).

Following this approach, 18 newly developed gene-based markers (Table 1) were polymorphic and allowed mapping on the pre-existing genetic map (Korzun *et al.*, 1998), identifying two separate linkage groups for part of 2DS (Fig. 6). A small linkage group of 11.7 cM was defined by one restriction fragment length polymorphism (RFLP) marker (*Xpsr649*) and two gene-based COS markers (*DG048* and *DG032*). Four additional gene-based markers (*DG025*, *DG035*, *DG057*, and *DG062*) were tested on 24 lines of the mapping population, but were not genotyped on the remaining lines, as they map too distally from the target *Rht8* locus. The order and orientation of the two linkage groups were established by analysing other publicly available wheat maps from GrainGenes (<http://wheat.pw.usda.gov/GG2/index.shtml>). The two linkage groups did not join because the genetic distance between them probably exceeds 30 cM. Nevertheless, the major objective of the project was to improve the map around the *Rht8* locus.

A bigger linkage group of 48.1 cM, containing *Rht8*, comprises original SSR and RFLP markers mapped by Korzun *et al.* (1998). The mapping of *Rht8* was furthered by identifying a genetic interval of 2.5 cM containing the locus: *Rht8* is flanked 0.6 cM distally by two co-segregating SSR loci *Xgwm261* and *Xwmc503*, and 1.9 cM proximally by two co-segregating SSR markers *Xcfid53* and *Xwmc112*, and one co-segregating gene-based marker *DG371*. The *Rht8* genetic interval contains five gene-based markers that co-segregate with *Rht8* (*DG087*, *DG260*, *DG273*, *DG274*, and *DG279*). The closest gene-based markers that flank the *Rht8* locus were developed from *B. distachyon*: *DG244* is 1.8 cM distal and *DG371* is 1.9 cM proximal to *Rht8*. *DG244* corresponds to Bradi5g02890, and *DG371* to

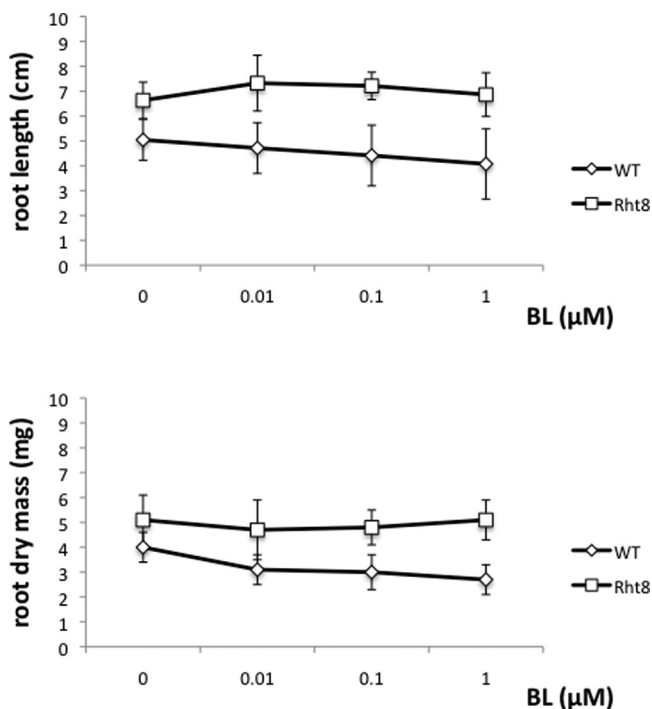


Fig. 4. Seedling root response to different BL concentrations. Root elongation and root dry mass in response to different BL concentrations of wild-type Cappelle-Desprez and semi-dwarf *Rht8* seedlings. Germinated seeds were transferred to media containing the indicated BL concentration and grown for 4 d. Bars indicate the standard deviation.

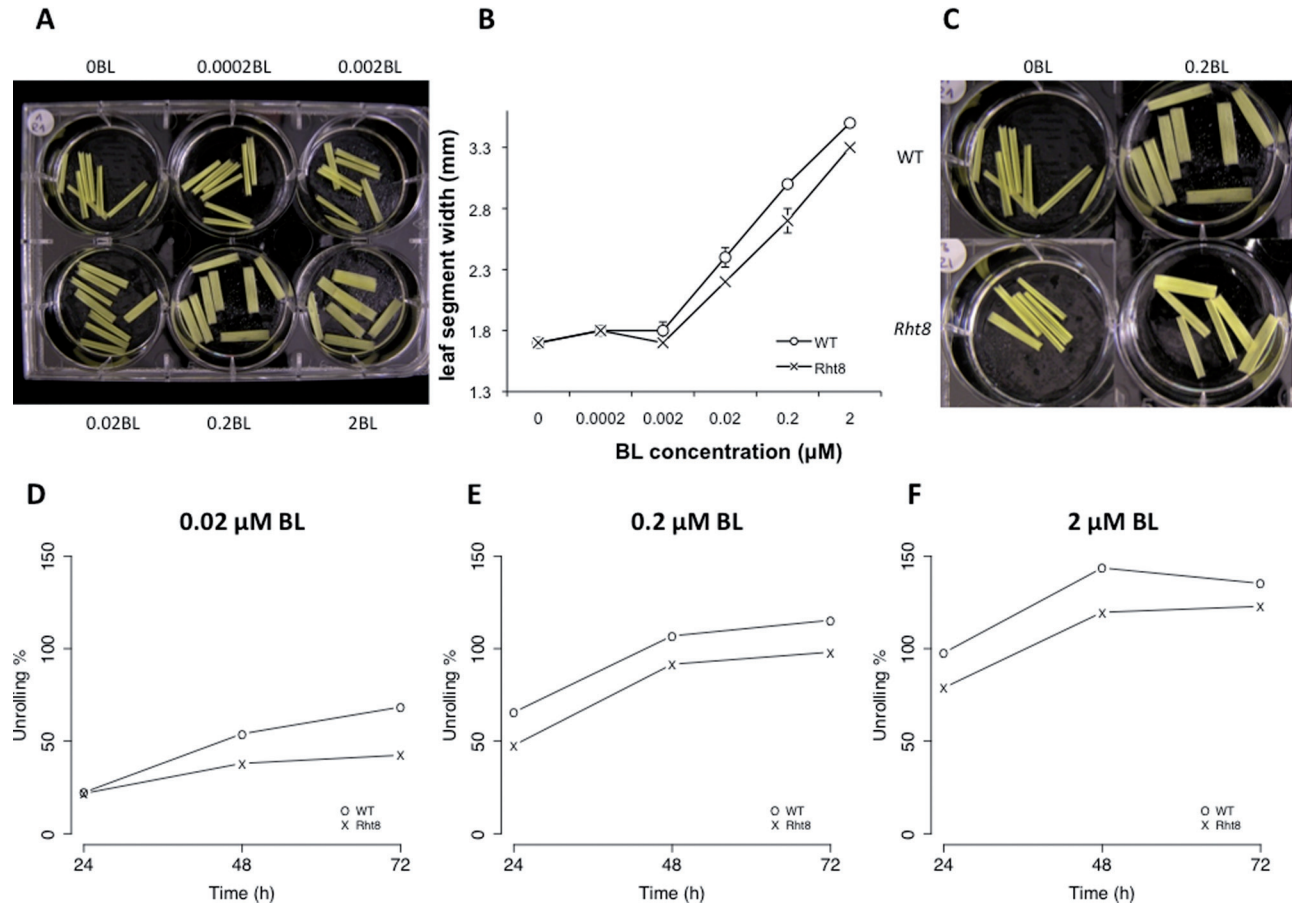


Fig. 5. Leaf unrolling response of the wild type (WT) and *Rht8* lines. (A) Example of the experimental outline: etiolated leaf segments of Cappelle-Desprez incubated in 0, 0.0002, 0.002, 0.02, 0.2, and 2 μM BL for 72 h in total darkness. (B) BL effect on the leaf unrolling of Cappelle-Desprez (WT) and *Rht8* lines after 72 h. Bars represent the standard error. (C) Visual comparison of Cappelle-Desprez (WT) and *Rht8* (RIL6) leaf unrolling after 72 h incubation in 0 and 0.2 μM BL. (D–F) Leaf ‘unrolling %’ at different BL concentrations over time of Cappelle-Desprez (WT) and *Rht8* leaf segments. (D) 0.02 μM BL, (E) 0.2 μM BL, (F) 2 μM BL. At a given time point (i) and treatment (j), ‘unrolling %’ was calculated as $[(W_{ij}/W_{0j}) - (W_{i0} - W_{00})]$, where W_{ij} is the mean leaf width of the sample considered, W_{0j} is the mean leaf width of the same sample at time ‘0’, W_{i0} is the mean leaf width of the control at time ‘i’, and W_{00} is the mean leaf width of the control at time ‘0’.

Bradi5g04710, identifying a physical interval of 2.88 Mb in *B. distachyon*. The *Rht8* proximal marker *DG371* also corresponds to rice LOC_Os04g19140, and with *DG086* (LOC_Os04g12480) defines a genetic interval of 5 cM containing *Rht8* and coinciding with a 3.72 Mb physical interval in rice.

Fine-resolution mapping of the *Rht8* locus

To delimit the *Rht8* location further and resolve the order of the co-segregating markers, a new F₂ fine mapping population was constructed. Selected short lines from the Cappelle-Desprez (Mara 2D) RIL population (Korzun et al., 1998) were crossed with Cappelle-Desprez (tall *rht8* allele) to allow recombination. The selected short RILs (RIL4 and RIL6) and Cappelle-Desprez are polymorphic for *Xgwm261* (192 bp and 174 bp) and *Xcfd53* (274 bp and 254 bp), the SSR loci flanking *Rht8*, but carry the same photoperiod-sensitive *Ppd-D1b* allele. The short phenotype of F₁ hybrids indicated that *Rht8* is not inherited recessively,

but that *Rht8* exhibits incomplete dominance towards the short parent (Supplementary Fig. S3 at JXB online).

F₂ seeds were collected from self-pollinated F₁ plants, and 3104 F₂ individuals were genotyped with SSR markers flanking *Rht8*: *gwm261* and *cf53*. Of the 3104 F₂ plants, 712 had the parental short genotype (*Xgwm261*_{192–192bp}, *Xcfd53*_{274–274bp}), 738 had the parental tall genotype (*Xgwm261*_{174–174bp}, *Xcfd53*_{254–254bp}), and 1502 were heterozygous (*Xgwm261*_{192–174bp}, *Xcfd53*_{274–254bp}). The two co-dominant SSR markers showed the expected Mendelian segregation ratio of 1:2:1 (χ^2 test value $P=0.5$), and their recombination frequency of 0.0248 is consistent with the genetic distance observed in the 89 RIL population of 2.5 cM (Fig. 6).

The screen identified 152 F₂ recombinant plants between *gwm261* and *cf53* that have a different genotype from the crossing parents (Supplementary Fig. S4 at JXB online). Recombinant F₂ plants were retained and genotyped further with co-dominant gene-based markers mapping within the *Xgwm261–Xcfd53* interval:

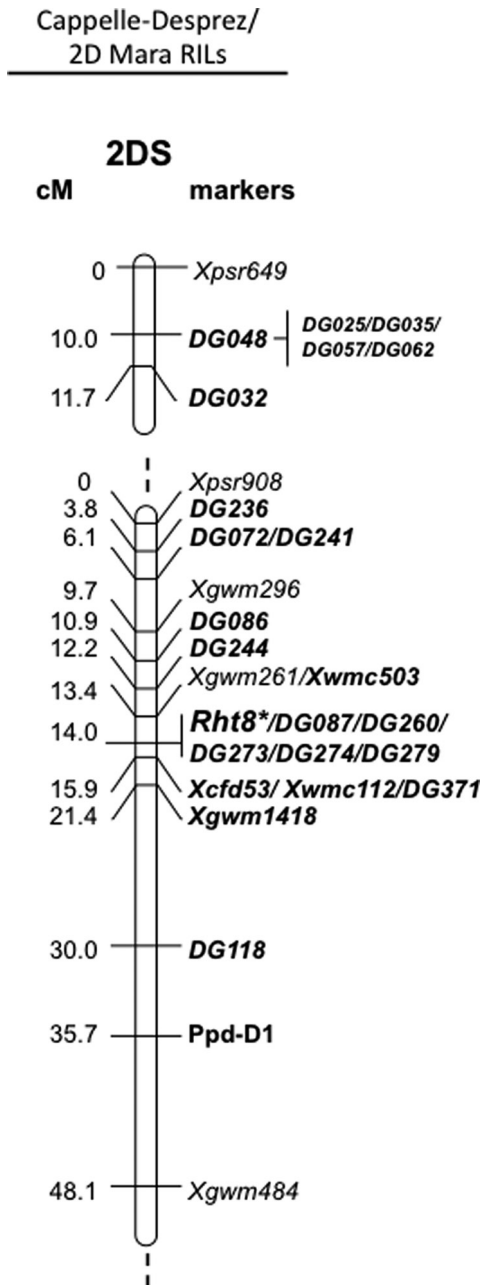


Fig. 6. Molecular linkage groups of part of wheat chromosome 2DS in the Cappelle-Desprez (Mara 2D) RIL population. Genetic distances are shown in cM at the left-hand side of each linkage group, while marker names are shown on the right-hand side. Newly developed gene-based markers are shown in bold and named DG followed by three numbers. Additional SSR and *Ppd-D1* markers mapped on the population are also shown in bold. *Rht8* was re-scored in the population as individual plant height in a replicated and randomized block design, and is marked with an asterisk.

DG087, *DG260*, *DG274*, *DG279*, and *DG371* (*DG273* does not exhibit a co-dominant amplification pattern and was therefore not used for further analyses). The recombinant plants were grouped in 14 recombinant classes according to their genotype at the seven marker loci (Supplementary Table S1 at *JXB* online; graphical

genotypes of selected homozygous $F_{2,3}$ recombinants). Segregation ratios for the five co-dominant markers were in agreement with the expected 1:2:1 ratios ($P=0.72$ for *DG260*, $P=0.76$ for *DG087*, $P=0.72$ for *DG274*, $P=0.84$ for *DG279*, and $P=0.80$ for *DG371*). The SSR and gene-based markers identify six genetic intervals of 0.69, 0.08, 0.18, 0.21, 1.29, and 0.04 cM in which *Rht8* could be mapped (Fig. 7).

To determine unequivocally the genotype at the *Rht8* locus and map the gene in one of the identified genetic intervals, $F_{2,3}$ phenotypic information was used. Sixteen individuals were genotyped with the two SSR markers for each segregating $F_{2,3}$ family, and only the homozygous *Rht8* recombinant lines (i.e. recombinants that have a different genotype from the crossing parents, and are homozygous at both loci of the flanking markers) were retained. Homozygous F_3 recombinants were grown in three replicated and randomized experiments, and analysed for height with a linear model to back-score *Rht8* in the F_2 generation (Supplementary Table S1 at *JXB* online). By back-scoring F_2 individuals with plant height observed in homozygous recombinant $F_{2,3}$, it was also possible to discriminate homozygous and heterozygous individuals at the *Rht8* locus. A segregation of 47 tall and 105 short plants was observed after back-scoring F_2 individuals with $F_{2,3}$ data. The phenotypic ratio fits the 1:3 segregation pattern ($\chi^2 P=0.092$), confirming F_1 data that *Rht8* exhibits incomplete dominance towards the short parent, and supporting the observation of Korzun *et al.* (1998) that the effect that *Rht8* has on height is due to a major gene inherited in a Mendelian manner.

Rht8 was mapped within the 1.29 cM interval, between markers *DG279* (0.79 cM distal) and *DG371* (0.5 cM proximal). By increasing the size of the mapping population, a higher number of crossovers was observed between *Xgwm261* and *Rht8*. In fact, the genetic distance between *Xgwm261* and *Rht8* was found to be larger (1.95 cM) than previously published (0.6 cM; Korzun *et al.*, 1998). The gene-based markers identified the syntenic regions of the *Rht8* locus within 2 Mb in *B. distachyon* chromosome 5 (Bradi5g03460-04710, *Bd21* Genome Annotation 1.0) and 3.3 Mb in *Oryza sativa* chromosome 4 (LOC_Os04g13210-19140, MSU 6.1) (Fig. 7). The resolution potential of the F_2 mapping population described in this work, is extremely high as there are still 48 and 31 F_2 recombinants, respectively, on either side of the *Rht8* locus.

Altered sensitivity to brassinolide co-segregates with *Rht8*

To determine further the relationship between *Rht8* and the altered BR response observed in semi-dwarf *Rht8* lines and to test whether the BR response co-segregates specifically with the 1.29 cM *Rht8* interval, leaf unrolling tests were performed on selected homozygous F_4 recombinant lines segregating for *Rht8*. At 0.02 μM BL, it was possible to group the tall and short F_4 recombinant lines into two groups of BL sensitivity, according to the parental controls (Fig. 8A).

The semi-dwarf homozygous F_4 lines (*Rht8* allele) were less sensitive to BL, showing a minor leaf unrolling response when compared with the tall F_4 group (*rht8* allele) at 0.02 μM BL. The group of tall F_4 plants clearly unrolled more readily and grouped

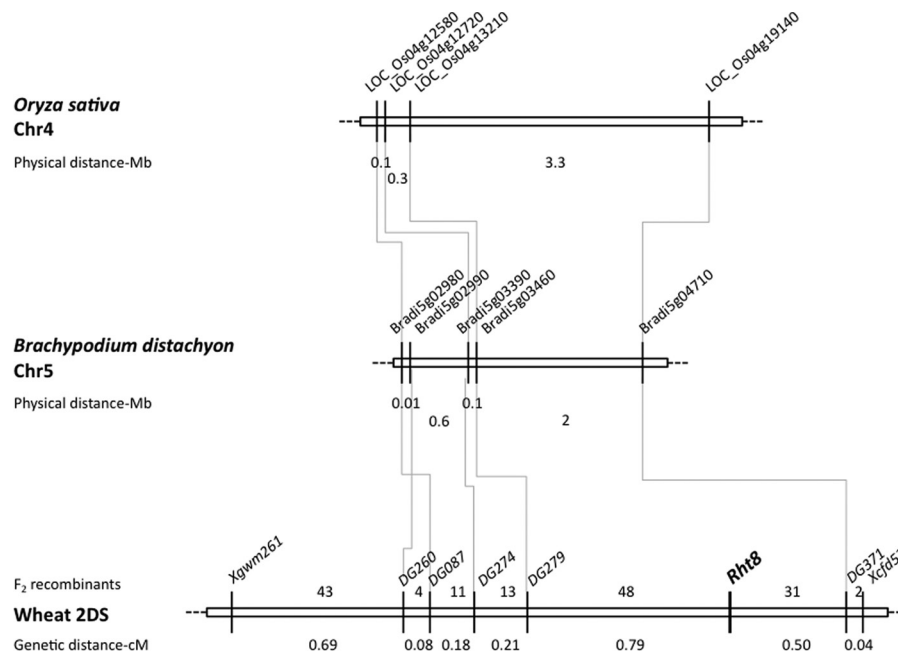


Fig. 7. *Rht8* fine map. Wheat 2DS genetic map around the *Rht8* locus, and comparative mapping with *B. distachyon* (*Bd21* Genome Annotation 1.0) and *O. sativa* (MSU 6.1) using gene-based markers developed for this project.

with the tall Cappelle-Desprez control. The leaf unrolling of all plants plateaued at 2 μ M BL after 72 h (Fig. 8B), and the final leaf width was the same, demonstrating that the difference in BL sensitivity is not due to a difference in leaf width. The differential unrolling response at 0.02 μ M BL in the F_4 lines tested co-segregates with the *Rht8* locus (Fig. 8C), indicating that the *Rht8* locus is possibly involved in modulating the BR response of the wheat plant.

Rht8 candidates in the rice chromosome 4 and *B. distachyon* chromosome 5 syntenic regions

The gene content in the physical intervals of the two model genomes was analysed to consider possible candidates for *Rht8*, as there was no genomic sequence information available for hexaploid wheat at this time. The identified rice interval (LOC_Os04g13210-19140, MSU 6.1) contains 584 loci. According to rice MSU 6.1 annotation, 379 of the total 584 loci are repetitive elements (65%). Similarity searches of the remaining 205 loci against the TREP and the rice annotation Rice Annotation Project database (RAP-DB, 5) revealed that 73 additional loci are similar to repetitive elements, leaving 132 non-transposable element (non-TE) loci. In the rice genomic region considered, there is a sequence assembly gap of 120 kb according to the RAP database (Chr4:9277690..9387689 missing data). The same genomic portion (LOC_Os04g16712-16896) is presumably misannotated in the MSU database. In fact, there are 57 fragmented predicted genes in a 123 kb genomic interval, mainly involved in photosynthesis (photosystem I and II proteins). Excluding the annotation/assembly gap, the *Rht8* equivalent region of rice contains 75 non-TE genic loci (Supplementary Table S2, worksheet 1 at *JXB* online). The same procedure was used to filter repetitive elements in the identified region of *B. distachyon* (2 Mb defined by Bradi5g03460-04710,

Bd21 Genome Annotation 1.0). A high content of repetitive elements was also observed in *B. distachyon*, leaving 62 non-TE genic loci (Supplementary Table S2, worksheet 2). Moreover, many predicted genes have unknown functions and have not been characterized.

Good conservation of gene content and order is observed between the two species (Supplementary Fig. S5 at *JXB* online). The only exception in gene order is between LOC_Os04g14510 and Bradi5g04580. The complexity, size, and gene content in the two model organisms represent a limiting factor for the description and analysis of functional candidate genes. However, the physiological assays indicate that a possible cause of the impaired cell elongation in *Rht8* lines is an altered BR sensitivity or response. Similarly, *Rht8* gene candidates could be involved in cell elongation and have a direct or indirect role in BR signalling. In the two intervals, loci with possible roles in cell elongation include: LOC_Os04g14510 and Bradi5g04580, LOC_Os04g16970 and Bradi5g04540, LOC_Os04g14110, LOC_Os04g 14190 (putative zinc-finger domain-containing proteins); LOC_Os04g15840 and Bradi5g04120 (putative expansin EXPA1). A gene cluster, present in the intervals of both model genomes, consisting of several adjacent protein kinases (PKs) was also identified. In rice, the PK cluster consists of a leucine-rich repeat (LRR)-RLK (LOC_Os04g15560), a Ser/Thr PK (LOC_Os04g15580), and three additional LRR-RLKs (LOC_Os04g15630-*Xa21*, LOC_Os04g15650, and LOC_Os04g15660). A similar gene content was also observed in the *B. distachyon* PK cluster, which consists of three Ser/Thr PKs containing N-terminal domains of unknown function (DUF) (Bradi5g03900, Bradi5g03930, and Bradi5g03940), two LRR-RLKs (Bradi5g03960 and Bradi5g04000), and an additional LRR-receptor like protein lacking the cytoplasmic kinase domain (Bradi5g03980). The LRR-RLKs present in the physical intervals of the two model genomes are particularly interest-

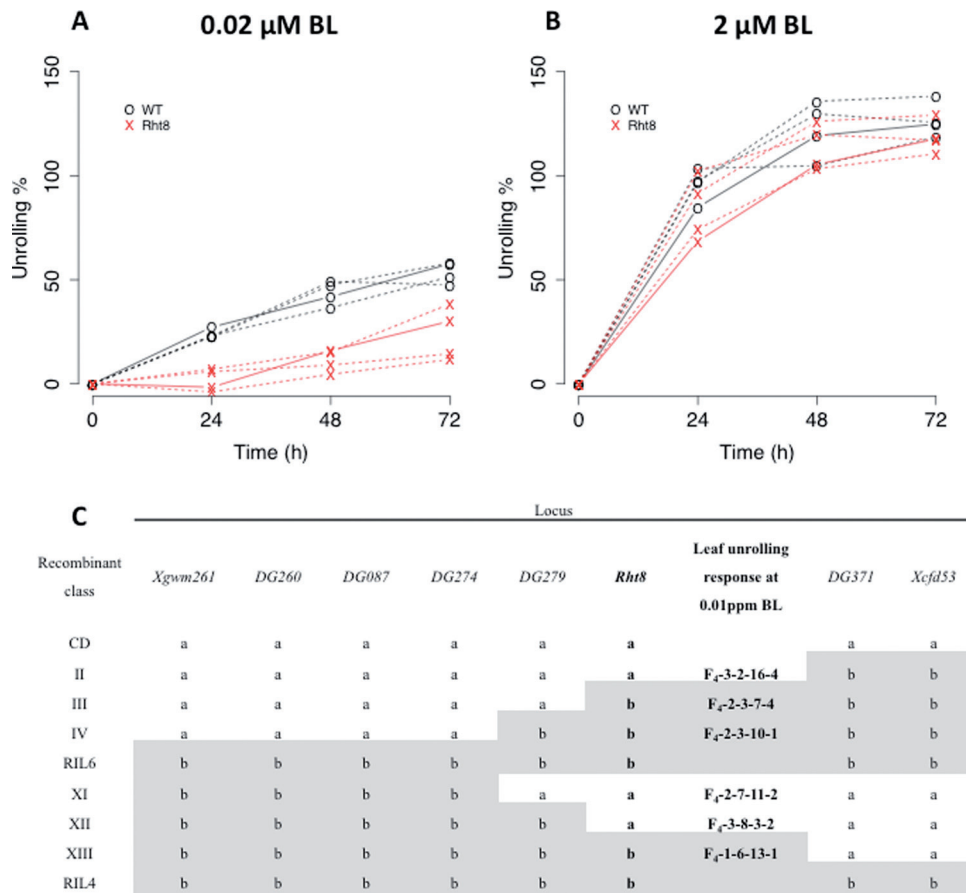


Fig. 8. Leaf unrolling response of parents of the fine-resolution mapping population, Cappelle-Desprez (WT) and RIL6 (*Rht8*), and selected homozygous F₄ recombinant lines around the *Rht8* locus. (A and B) BL effect on the leaf unrolling percentage over time in Cappelle-Desprez (WT), RIL6 (*Rht8*), and selected tall (dotted black lines) and short (dotted red lines) homozygous F₄ recombinant lines, at (A) 0.02 μM BL and (B) 2 μM BL. (C) Graphical genotypes of homozygous F₄ recombinant lines grouped in recombinant classes, and comparison of the leaf unrolling response of tall (unshaded, unrolling like tall wild-type Cappelle-Desprez) and short (shaded in grey, unrolling less than normal like the semi-dwarf parent RIL6) F₄ lines. The leaf unrolling response co-segregates with the height phenotype (*Rht8*).

ing putative *Rht8* candidates as BRI1, the BR receptor (Li and Chory, 1997), was shown to be an LRR-RLK.

Nevertheless, all of the genes present in the syntenic intervals need to be considered as *Rht8* putative candidates based on comparative genomics studies. A total of 152 primer pairs (from 47 different rice and *B. distachyon* loci) were developed to target and saturate the *DG279–DG371* interval, and refine the map location of *Rht8*. However, due to lack of polymorphism between parents of the fine-resolution mapping population it was not possible to map *Rht8* to a smaller genetic interval.

Discussion

The reduced stature of varieties with the semi-dwarf *Rht8* allele confers a substantial agronomic impact in environments where the use of other semi-dwarfing genes is not advantageous (Law and Worland, 1985; Worland and Law, 1986; Rebetzke *et al.*, 1999; Worland *et al.*, 2001). In southern European and Russian areas, a selective advantage for preserving the linkage between *Ppd-D1a* and *Rht8* has been observed (Worland *et al.*, 1998). However, the use of *Ppd-D1a* is restricted to those environments conducive to

earlier flowering and a shorter life cycle. Most North European and American areas are associated with extended life cycles, so *Ppd-D1a* cannot be used to increase grain yield (Worland *et al.*, 1997). Probably, by selecting away from *Ppd-D1a* and thereby also losing *Rht8*, breeders in northern countries have never had the opportunity to test *Rht8* semi-dwarfing activity independently. Therefore, the identification of the gene underlying the semi-dwarf phenotype will not only broaden the knowledge of stature control in wheat, but will also facilitate the effective deployment of favourable *Rht8* alleles into modern breeding varieties in conjunction with other appropriate genes and their alleles.

The physiological analyses carried out show that the semi-dwarfed stature of *Rht8* plants is a consequence of shorter internodes throughout the culm, and the largest measurable difference lies in a shorter peduncle and the first internode (Fig. 1). Peduncle and internode 1 undergo the fastest and largest elongation process of all internodes in the culm (Kirby and Appleyard, 1981), thus accounting for the majority of the length reduction in the semi-dwarf line. Microscopic analyses revealed that the *Rht8* difference in internode length is due to impaired cell elongation in culm segments. *Rht8* lines have shorter parenchymatic cells in elongated peduncle and internode 1 (Fig.

3). No apparent defect in meristematic activity in intercalary meristems below elongating internodes was observed (Fig. 2). The extent of elongation depends on the cell type and is often regulated by environmental cues and endogenous hormones with crucial roles in cell expansion and elongation in culms and stems (Wang and Li, 2008).

Auxins have long been known to play a central role in orchestrating plant architecture during development, such as maintenance of apical dominance (Evans, 1985; Qi *et al.*, 2008; McSteen, 2009). Although auxins are involved in cell elongation (Wada *et al.*, 1968), no agriculturally beneficial rice, barley, or wheat auxin mutants are known to date, probably due to major pleiotropic effects other than plant stature. On the other hand, agriculturally beneficial mutants shortening plant stature are often involved in the biosynthesis or perception of GAs and BRs (Wang and Li, 2008). The semi-dwarf stature of *Rht8* lines is unlikely to be due to an altered GA biosynthesis or signalling as wild-type *rht8* and semi-dwarf *Rht8* lines responded similarly to the exogenous application of GA₃ (Supplementary Fig. S1 at JXB online), as well as containing the same amount of endogenous GAs (Table 2). Instead, a differential response to applied BL was observed between wild-type *rht8* and semi-dwarf *Rht8* lines. The whole plant response of wild-type wheat seedlings grown on media containing BL was similar to that of barley (Chono *et al.*, 2003) as opposed to that of rice: root elongation was inhibited, while aerial parts remained unaffected (Fig. 4; Supplementary Fig. S2). In contrast to wheat and barley, rice seedlings grown on BL media, show clear responses in both aerial and submerged parts of the plant (Yamamuro *et al.*, 2000). The observations strengthen the hypothesis that temperate cereals have a different BR uptake or transport mechanism when compared with rice (Chono *et al.*, 2003), and question the possibility of using rice as a physiological model to understand the BR pathway in wheat. A differential response in root length and root dry mass was also observed between semi-dwarf *Rht8* lines compared with the wild-type. While the wild-type line showed a decrease in primary root length and root dry mass with increasing BL concentrations, the semi-dwarf *Rht8* line showed a slight increase in root length at lower BL concentrations (0.01 μ M and 0.1 μ M) but no response at a higher dose (1 μ M; Fig. 4). The altered BR sensitivity in the semi-dwarf *Rht8* line could be due to a decreased BR sensitivity. To examine further the differential BR response between wild-type and *Rht8* seedlings and quantify the BR sensitivity, a more sensitive method established for temperate cereals (Wada *et al.*, 1985; Chono *et al.*, 2003) was used. The leaf unrolling response (Wada *et al.*, 1985) showed that although wild-type and semi-dwarf lines did not differ in their final leaf width, semi-dwarf *Rht8* lines showed a reduced rate of leaf unrolling (Fig. 5). The differential leaf unrolling response is likely to be a consequence of reduced BL sensitivity.

As well as providing novel physiological evidence for the mechanism of *Rht8* action, the present work has achieved fine mapping for the eventual map-based cloning of the gene and the provision of superior molecular markers for immediate use. Using gene-based markers co-segregating with *Rht8* in the coarse mapping population (Korzun *et al.*, 1998) and screening 3104 F₂ individuals of the fine-resolution mapping population developed for this project, it was possible to reduce the *Rht8* genetic interval from 20.5 cM (Korzun *et al.*, 1998) to 1.29 cM (Fig. 6). the

wheat *Rht8* syntenic region was identified and limited to 3.3 Mb of the short arm of rice chromosome 4 (from LOC_Os04g13210 to LOC_Os04g19140, MSU v6.1.), and to 2 Mb of *B. distachyon* chromosome 5 (from Bradi5g03460 to Bradi5g04710, genome assembly v1.) (Fig. 7). Moreover, following the observation of an altered BR sensitivity in parental lines of the fine-resolution mapping population, analyses of the leaf unrolling response in homozygous F₄ recombinant lines indicate a co-segregation between BR hyposensitivity and the height reduction of the *Rht8* locus (Fig. 8). Due to the compensatory dosage effect of the three wheat genomes, BR-deficient lines would be difficult to obtain, except in gain-of-function mutations. The semi-dwarfed phenotype and reduced culm length observed in *Rht8* lines could be explained by a reduced sensitivity to BRs and—unlikely—to a reduced endogenous BR concentration.

Although an obvious gene candidate involved in the regulation of the BR response was not found in the genomic interval of the two model genomes, and the large gene content makes it difficult to make assumptions, the intervals contain several candidates with possible roles in cell elongation.

Altered activity of zinc-finger (Zn-finger) transcription factors can influence a wide range of processes during plant growth and development, including reduced plant stature of *A. thaliana* (AtSHI; Fridborg *et al.*, 2001) and rice (OsLOL2; Xu and He, 2007). Several genes encoding putative Zn-finger proteins were found in the *Rht8* syntenic intervals of rice and *B. distachyon* (LOC_Os04g14510 and Bradi5g04580, LOC_Os04g16970 and Bradi5g04540, LOC_Os04g14110, and LOC_Os04g14190).

Expansins (EXPs) are cell wall proteins that induce wall extension (Cosgrove, 2000). Among EXP subfamilies (<http://www.bio.psu.edu/expansins>), EXPA and EXPB are known to induce cell wall loosening for the expansion of plant cells (Cosgrove *et al.*, 2002). The EXPA subfamily is composed of 26 genes in *Arabidopsis* and 34 genes in rice (Choi *et al.*, 2006). The diverse and specific expression patterns of the different EXPA transcripts suggest their distinct roles in plant growth and development (Choi *et al.*, 2006). The collinear genic loci LOC_Os04g15840/Bradi5g04120 encode a putative EXPA1 protein. Shin *et al.* (2005) showed that rice EXPA1 is specifically expressed in leaf blades, whereas transcript levels of EXPA7, 14, 15, 18, 21, and 29 were greater in stems. Recently, Park *et al.* (2010) found that AtEXPA5 is a growth-regulating gene whose expression is controlled by BR signalling downstream of BZR1 in *A. thaliana*.

The cytochrome P450 family is a large family of enzymes with important roles in lipid metabolism, metabolism of phytohormones including BRs, and defence responses (Nelson, 2006). None of the CYPs found in the physical intervals appears to be related to BR metabolism.

The LRR-RLKs present in the physical intervals or the two model genomes are particularly interesting putative *Rht8* candidates as several LRR-RLKs have been shown to play critical roles in development. Characterized LRR-RLKs include ERECTA that regulates organ shape (Torii *et al.*, 1996), CLAVATA1 which controls cell differentiation at the shoot meristem (Clark *et al.*, 1997), HAESA which regulates the floral abscission process (Jinn *et al.*, 2000), and BRI1 the BR receptor (Li and Chory, 1997). LRR-RLKs also play a role in disease resistance (XA21; Song *et al.*,

1995). LRR-RLKs comprise the largest class of plant RLKs, with 216 members in *A. thaliana* (Dievart and Clark, 2004). These have been divided into 14 subfamilies (LRR I–LRR XIV) classified on the basis of the organization of LRRs in the extracellular domain and the phylogenetic relationship between the kinase domains of subfamily members (Shiu and Bleecker, 2003). According to this classification BRI1, BRL1, BRL2, and BRL3 belong to the LRRX clade, whereas XA21 belongs to the XII clade (Morillo and Tax, 2006). To examine the phylogenetic relationships of the LRR-RLK present in the regions of interest with classified LRR-RLKs, a cladogram tree was constructed using the Neighbor–Joining method including at least one representative member of each LRR-RLK clade (data not shown). The five LRR-RLKs present in the region of interest (LOC_Os04g15560, LOC_Os04g15650, LOC_Os04g15660, Bradi5g03960, and Bradi04000) cluster to the LRR XII clade, comprising members involved in pathogen response such as XA21, FLS2, and EFR (Morillo and Tax, 2006), and not to the LRRX clade involved in developmental processes such as BR signalling and vascular differentiation. Therefore, both LRR-RLK clusters present in rice and *B. distachyon* seem to be involved in disease resistance.

Sequence information from rice and *B. distachyon* was used to saturate further the *Rht8* genetic interval, defined by *DG279* (0.79 cM distal) and *DG371* (0.5 cM proximal). However, all 152 primer pairs (from 47 different loci) were monomorphic between parents of the fine-resolution mapping population, and it was not possible to map *Rht8* to a smaller genetic interval. The low level of diversity found in the D-genome is expected from the evolutionary history of hexaploid wheat (Bossolini *et al.*, 2006; Dubcovsky and Dvorak, 2007). Nevertheless, the resolution potential of the F₂ mapping population is very high as there are 48 and 31 F₂ recombinants, respectively, either side of *Rht8*, and the population can be exploited further once polymorphic markers are identified (Fig. 7).

In addition to the fundamental scientific interest of the *Rht8* locus showing altered BR sensitivity, the work described presents exciting opportunities for wheat breeding and improved crop agronomy. The new gene-based *Rht8* flanking markers (*DG279* and *DG371*) can be used to generate a diagnostic haplotype for sources of *Rht8* where *gwm261* has been shown to be uninformative. The molecular marker-based prediction that a line carries *Rht8* can then be validated by the application of the BL leaf unrolling assay. This combined assay will arm breeders with an equivalent tool to the GA sensitivity test (McIntosh *et al.*, 1998) that has allowed the discrimination of GA-sensitive and GA-insensitive wheat varieties, marking the genotype for *Rht-B1b* and *Rht-D1b* types. The precise selection of *Rht8* in the same way will allow it to be efficiently and immediately deployed in the water-limited target environments where it has very high potential impact.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Exogenous GA treatment of semi-dwarf *Rht8* lines.

Figure S2. BL treatment.

Figure S3. The mode of inheritance of *Rht8*.

Figure S4. Electropherograms of 6-FAM-labelled *Xgwm261*

and *Xcfd53* amplification products from parental, heterozygous, and recombinant F₂ genotypes.

Figure S5. Graphical comparison of genomic segments of rice chromosome 4 with *B. distachyon* chromosome 5 around the wheat *Rht8* locus.

Table S1. Graphical genotypes of homozygous F₃ recombinant lines grouped in recombinant classes according to their genotype and *Rht8* phenotype.

Table S2. Worksheet 1—rice gene content. Worksheet 2—Bd gene content.

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