RESEARCH PAPER

Differential coupling of gibberellin responses by Rht-B1c suppressor alleles and Rht-B1b in wheat highlights a unique role for the DELLA N-terminus in dormancy

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Abstract
During the Green Revolution, substantial increases in wheat (Triticum aestivum) yields were realized, at least in part, through the introduction of the Reduced height (Rht-B1b and Rht-D1b) semi-dwarfing alleles. In contrast to Rht-B1b and Rht-D1b, the Rht-B1c allele is characterized by extreme dwarfism and exceptionally strong dormancy. Recently, 35 intragenic Rht-B1c suppressor alleles were created in the spring wheat cultivar Maringá, and termed overgrowth (ovg) alleles. Here, 14 ovg alleles with agronomically relevant plant heights were reproducibly classified into nine tall and five semi-dwarf alleles. These alleles differentially affected grain dormancy, internode elongation rate, and coleoptile and leaf lengths. The stability of these ovg effects was demonstrated for three ovg alleles in different genetic backgrounds and environments. Importantly, two semi-dwarf ovg alleles increased dormancy, which correlated with improved pre-harvest sprouting (PHS) resistance. Since no negative effects on grain yield or quality were observed, these semi-dwarf ovg alleles are valuable for breeding to achieve adequate height reduction and protection of grain quality in regions prone to PHS. Furthermore, this research highlights a unique role for the first 70 amino acids of the DELLA protein, encoded by the Rht-1 genes, in grain dormancy.

Key words: DELLA, dormancy, gibberellin (GA), pre-harvest sprouting, suppressor alleles, wheat.

Introduction
Mutations in key regulators of the gibberellin (GA) pathway formed the genetic basis of a global agricultural revolution in the mid and late 20th century. During the so-called ‘Green Revolution’, yields of rice and wheat greatly increased due to intensified use of fertilizers and pesticides, combined with the introduction of semi-dwarfing alleles (Khush, 1999; Hedden, 2003). These alleles reduced plant height, which allowed higher fertilizer rates due to improved lodging resistance, and resulted in increased grain number per spike (Youssefian et al., 1992). In wheat, the Green Revolution semi-dwarfing alleles derive from a Della gene, encoded by the Reduced height (Rht-1) locus (Peng et al., 1999). In hexaploid bread wheat, Della is encoded by three homoeologous genes (Rht-A1, Rht-B1, and Rht-D1), with the wild-type alleles designated Rht-A1a, Rht-B1a, and Rht-D1a, respectively. The Green Revolution semi-dwarfing alleles of the B- and D-subgenome, Rht-B1b and

Abbreviations: BC, backcross; GA, gibberellin; KASP, Kompetitive Allele Specific PCR; ovg, overgrowth; PHS, pre-harvest sprouting; SRC, soil retention capacity; TGW, thousand grain weight.

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Rht-D1b (previously named Rht1 and Rht2, respectively), are now found in wheat cultivars grown worldwide. Both contain a mutation in the region of the DELLA protein’s eponymous N-terminal DELLA motif (Peng et al., 1999). This motif is crucial for degradation of the DELLA protein, which is triggered upon the detection of bioactive GAs by the GA receptor, GIBBERELLIN INSENSITIVE DWARF1 (GID1) (Davière and Achard, 2013). Although in planta biochemical evidence is still missing, yeast two-hybrid analysis has shown that the interaction between the DELLA protein and GID1 is reduced in the Rht-B1b mutant, presumably resulting in an accumulation of the DELLA protein (Pearce et al., 2011; Wu et al., 2011).

Besides elongation, DELLA controls the synthesis of hydrolytic enzymes in the grain aleurone layer (Richards et al., 2001). Hydrolytic enzymes, such as α-amylase, can occasionally be present in the mature grain, and affect the bread-making quality of wheat flour. Excessive α-amylase will degrade starch during the baking process, leading to a sticky and difficult to raise dough, and discolored, poorly structured loaves (Edwards et al., 1989). In practice, α-amylase activity is often indirectly assessed by the falling number, which is the time it takes an object to fall through an aqueous slurry of flour. The viscosity of the flour solution depends on the amount of long-chain carbohydrates. The lower the viscosity, which mainly results from the breakdown of long-chain carbohydrates by α-amylase activity, the lower the falling number (Perten, 1964). Elevated levels of α-amylase in the mature grain can originate from pre-harvest sprouting (PHS), which occurs when wet conditions prior to harvest break dormancy and induce grain germination while in the ear (Trethowan, 2001). Although PHS results in worldwide losses of more than US$1 billion per year (Black et al., 2006), the strong interaction between PHS and environmental and genetic factors often hampers selection for PHS resistance in conventional breeding programs (Trethowan, 2001; Groos et al., 2002; Mares and Mrva, 2014). Therefore, it is mandatory to study PHS resistance in different environments and genetic backgrounds.

The Rht-B1c allele contains a 2 kb insertion which, after splicing, results in an in-frame insertion of 30 amino acids, adjacent to the DELLA motif. This insertion completely disrupts the interaction between the RHT-B1C protein and GID1, resulting in GA insensitivity (Pearce et al., 2011; Wu et al., 2011). In contrast to Rht-B1b and Rht-D1b, the Rht-B1c allele is characterized by exceptionally strong dormancy and extreme dwarfism, the latter preventing its use in commercial cultivars (Flintham and Gale, 1982; Flintham et al., 1997b). However, a screen for second-site suppressor mutants in the cultivar Maringá identified 35 new Rht-B1c derivative alleles (Chandler and Harding, 2013, 2014). These alleles were termed overgrowth (ovg) alleles and contained, besides the 30 amino acid insertion, second-site mutations in other regions of the gene. The ovg alleles partially restore primary GA responses, such as stem length, coleoptile length, and dormancy.

Here, we assess the GA-related effects in different plant organs for 14 Maringá ovg alleles that result in agronomically relevant plant heights. To validate further the observed ovg effects and to evaluate the breeding potential of selected alleles, a subset of ovg alleles was introgressed into four spring wheat cultivars. Phenotyping in the greenhouse and field demonstrated the consistency of the ovg effects and, importantly, revealed no negative effects on grain yield or quality. In addition, Rht-B1c.23 and Rht-B1c.26 showed increased dormancy and improved PHS resistance. We conclude that these ovg alleles are valuable for breeding to achieve adequate height reduction and protection of grain quality in regions prone to PHS. Furthermore, the phenotypic effect of specific amino acid residues that are mutated in the ovg alleles aids our understanding of the regulation of GA responses by the DELLA protein in different plant organs. In addition, a unique role of DELLA’s first 70 amino acids in grain dormancy is highlighted.

Materials and methods

Plant material

In the Brazilian bread wheat cultivar Maringá, sequencing of the Rht-B1 gene confirmed the genotype of tall (Rht-B1a), semi-dwarf (Rht-B1b), and dwarf (Rht-B1c) backcross (BC) 7 isolines, and the second-site mutations in the 14 ovg mutants (see Supplementary Table S1 at JXB online; Chandler and Harding, 2013, 2014). Maringá ovg mutants containing Rht-B1c.22, Rht-B1c.23, and Rht-B1c.26 were backcrossed to the spring wheat cultivars KWS Sciocco and Faller, derived from Europe and North America, respectively. While both cultivars carry the wild-type Rht-D1a allele, KWS Sciocco contains wild-type Rht-B1a, whereas Faller carries the Rht-B1b semi-dwarf allele. BCF1 plants were allowed to self when at least 90% of the 384 single nucleotide polymorphisms (SNPs), tested via Kompetitive Allele Specific PCR (KASP), were inherited from the recurrent parent. To this end, four backcrossing generations were required, except for Rht-B1c.23 and Rht-B1c.26 in KWS Sciocco, which required only three backcrossing generations. KASP designed against Rht-B1c-specific SNPs (Pearce et al., 2011) allowed selection of sister lines of four different BCF3 families, homozygous for either the ovg or the Rht-B1b/Rht-B1a allele. Phenotypes were assessed at the F3 generation in the greenhouse and the F4 generation in the field.

Maringá ovg mutants containing Rht-B1c.23 and Rht-B1c.26 were backcrossed to the Australian spring wheat cultivars Crusader and EGA Gregory, both of which carry the Rht-D1a wild-type and Rht-B1b semi-dwarfing allele. Seedlings of at least three different BCF2 families were genotyped to identify those homozygous for either the ovg or the Rht-B1b allele, and they were selfed until BC2F3 grains were obtained. Since Maringá has red grains, while Crusader and EGA Gregory have white grains, grain color segregated in this BC population. Therefore, the BC2F3 grains were scored for grain color after a 30 min treatment with 0.1 M KOH. Only white-grained sister lines, showing <2 d difference in anthesis compared with the recurrent parent, were selected for further study.

Plant growth conditions

After 2–6 d of stratification at 4 °C, Maringá or BCF1, Fallers/ KWS Sciocco grains were sown in 4 liter pots filled with a perlite-based substrate and lamped in 16 h cycles (pre-harvest sprouting (PHS)). The pots were white to avoid excessive heating of soil and roots by irradiation (Passioura, 2006). Plants were grown on the conveyor belts of a LemnaTec 3D-Scanalyzer in a greenhouse (21–19 °C) with incident day light and day length extension to a 16 h photoperiod when necessary (400 W high-pressure sodium lamps, GE Lucalox™). During the first week, rain water was applied daily, while for the remainder of the
experiment the plants were watered daily with fertilizer solution up to a pre-defined individual pot target weight. This target weight was calculated as a percentage of soil water content at retention capacity (SRC), as described in Granier et al. (2006). After flowering, plants were transferred to a standard greenhouse compartment and watered daily with fertilizer solution via an intermittent subirrigation system (commercially referred to as an ebb-and-flow system). All experiments were conducted with at least 10 biological replicates in a randomized block design, without altering the position of the plants during the experiment, as recommended by Brien et al. (2013).

Field experiments involving KWS Scirocco and Faller (BCF2c) were run in Gatersleben (Germany). Plots of 1.54 m x 4 m were sown on 4 April 2015 in a randomized block design consisting of three replicated plots for each sister line. Phenotypic measurements were executed on six plants per plot; however, yield was determined per plot.

Field trials involving Crusader and EGA Gregory were executed in New South Wales, Australia. Grain multiplication and dormancy studies were carried out with BC2F2 grains from a field nursery at Canberra in 2014. In Yanco, BC2F2 plots of 1.62 m x 5 m were sown on 4 June 2015, and were irrigated until grain development.

**Phenotypic measurements**

To quantify coleoptile length, grain stratification at 4 °C for 2 d was followed by germination in the dark in soil at a constant temperature of 20 °C. When 50% of the first or the second leaf was visible in Maringá or backcrossed lines (Faller and KWS Scirocco), respectively, etiolated coleoptile length was measured. Zadoks phenology stages were determined as described in Zadoks et al. (1974), and the first leaves were measured from the ligule to the leaf tip. Furthermore, the main stem was marked at spike emergence (Zadoks 50) and its length was measured after flowering, from the soil level to below the spike, whereas peduncle length was determined from above the highest node to below the spike. At flowering (Zadoks 65), the lengths of the flag leaf sheath and flag leaf lamina were measured from above the highest node up to the ligule and from the ligule to the flag leaf tip, respectively. Maximal flag leaf width was measured at the basal part of the leaf. Total plant height during growth was calculated as the average plant height of two side view images taken at a 90 ° horizontal rotation (Chen et al., 2014; Neumann et al., 2015). Mature spikes of individual plants were cut, dried for at least 3 d at 30 °C, and threshed (SRC SAS, Mayet, France). The organ length of the main stem generally correlated with the organ length of tiller 1 and 2 (Supplementary Table S2). Subsequently, all grains were counted (Contador, Pfeuffer, Kitzingen, Germany), weighed, and the thousand grain weight (TGW) of individual greenhouse-grown plants or field plots was calculated. Grain length and width of at least 150 grains per line were determined with VideometerLab 3 (Hørsholm, Denmark), as described in Hansen et al. (2015).

**Dormancy**

Physiologically mature spikes were harvested and dried for several days at 30 °C. Then, grains were hand threshed and stored at 30 °C. Hand threshing was required because machine threshing broke down dormancy completely. Each week, 100 grains were distributed on moist germination paper (AllPaper T10D li blue, 550 g m⁻²) in square Petri dishes (245 mm x 245 mm, Sigma-Aldrich®). After 7 d of incubation at 20 °C under constant fluorescent light (50 μmol m⁻² s⁻¹), grain germination was assessed by radicle protrusion, and the germination percentage was calculated. Machine-threshed wild-type grains stored at 10 °C for >6 months were included as positive controls. To improve statistical power, the dormancy test of back-crossed Faller and KWS Scirocco lines was executed in a randomized block design by sorting 96 grains on three round Petri dishes (15 cm diameter, Falcon®) filled with agar medium containing 0.2% GELRITE™ G1101 (Duchefa Biochemie BV, Haarlem, The Netherlands), supplemented with 0.94 g l⁻¹ MgCl₂ 6H₂O to improve coagulation. Prior to germination, grains were sterilized with 3% NaOCl.

**Pre-harvest sprouting**

Spikes were harvested during grain ripening, dried at 30 °C, and positioned upright in a growth chamber (16 h day length, 22/18 °C day/night temperature, 70 μmol m⁻² s⁻¹). Every 1.5 h or 2 h during day or night, respectively, nozzles simulated strong rainfall for 10–15 s. When spikes sprouted, pictures were taken.

**Falling number**

Grain samples were stored until germination tests indicated that dormancy was completely lost (i.e. grains germinated >95%). Subsequently, the falling number of 100 g of grains per replicate field plot or 30 g of bulked, greenhouse-harvested grains (split into three 10 g samples) was determined according to the international standard method, ICC-No. 1071.

**Data analysis**

Statistics were performed with R (R Development Core Team, 2008). Using a mixed model, the effect between the ovg alleles and the reference allele (Rht-B1a or Rht-B1b) was estimated, adjusting for irrigation treatment and block. The analysis model was also used to remove potential outliers that had a Studentized residual value >2. All effects with P<0.05 were considered significant.

**Results**

**Effect of ovg alleles on elongation**

From the 35 Maringá ovg alleles (Chandler and Harding, 2013, 2014), 14 ovg alleles that resulted in maximal 40% height reduction, relative to Rht-B1a, were selected as agronomically relevant. To compare these 14 ovg alleles with the widely deployed Rht-B1b allele, the final length of plant organs in different cultivars and environments was studied, and expressed relative to Rht-B1b (Table 1). In greenhouse-grown Maringá plants, the stem lengths of nine ovg alleles were 12–34% longer than those of Rht-B1b, while the stem lengths of five other ovg alleles were either ~10–15% shorter than, or not significantly different from Rht-B1b (Table 1). These ovg alleles were classified as tall and semi-dwarf alleles, respectively. Similar to their effect on stem length, tall and semi-dwarf alleles proportionally affected the final length of the coleoptile and peduncle (Table 1).

To validate the ovg effects in other genetic backgrounds, one tall (Rht-B1c.22) and two semi-dwarf (Rht-B1c.23 and Rht-B1c.26) alleles were introgressed into the spring wheat cultivars Faller and KWS Scirocco, developed for North America and Europe, respectively. KWS Scirocco contains the wild-type Rht-B1a allele at the Rht-B1 locus, whereas Faller contains the Rht-B1b allele. In both cultivars, homozygous sister lines carrying either the ovg or the wild-type (Rht-B1a or Rht-B1b, respectively) allele were generated from at least three BCF2 plants to assess the ovg effect rigorously. Herein, results of one sister line pair will be displayed, but all other pairs showed similar results. In greenhouse-grown Faller, the coleoptile, peduncle, and stem lengths of lines containing Rht-B1c.22 were 15–22% longer than those of
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Rht-B1b, while for the lines containing Rht-B1c.23 and Rht-B1c.26, these parameters were either up to 18% shorter than or not significantly different from Rht-B1b (Table 1, Fig. 1A, C). In KWS Scirocco, the length reduction of the coleoptile, stem, and peduncle was greater for semi-dwarfing alleles than for the tall allele: stem length reduction of Rht-B1c.23 and Rht-B1c.26 was 39% and 34%, respectively, while it was only 15% for Rht-B1c.22 (Table 1, Fig. 1B, D). For both cultivars, the differential effect on stem length was confirmed in field experiments (Table 1). Together, the observations show that the ovg effect on the elongation of vegetative organs is robust across different genetic backgrounds and environments.

To investigate further the effect of the ovg alleles, plant height and development were monitored on an automated phenotyping platform. In Maringá, Rht-B1c.22 grew taller than Rht-B1c.23 and Rht-B1c.26 from 40 d after sowing—the onset of stem elongation—while these effects were visible 15 d earlier in Faller and KWS Scirocco (Fig. 2). These findings suggest that the ovg effect on final stem length results from an effect on each internode. Accordingly, the final length of each stem internode was proportionally affected by Rht-B1c.26 in Maringá (Supplementary Fig. S1). Furthermore, the ovg alleles did not significantly affect timing of developmental events, indicated by the narrow window showing the onset of spike emergence (Fig. 2). Consequently, the ovg effect on final plant height resulted from a cumulative effect on internode elongation rate rather than from an effect on the total duration of elongation. In other words, internodes of semi-dwarf alleles always elongated more slowly than internodes of tall alleles. Interestingly, Rht-B1c.23 elongated more slowly than Rht-B1c.26 in all three cultivars, indicating subtle, reproducible differences between these two semi-dwarf alleles (Fig. 2).

**Effect of ovg alleles on yield-related traits**

The robustness of the ovg effect in controlling plant height offers a potential for breeding applications: when the extent of dwarfing with the standard semi-dwarfing alleles is slightly too strong or too weak for a specific target environment, respectively, a tall or semi-dwarf ovg allele could be used instead. To determine whether there were any negative pleiotropic effects of the ovg alleles on yield-related traits, flag leaf dimensions, spike length, grain size, and grain yield were measured in the greenhouse and field (Tables 1, 2; Fig. 3). In Maringá, KWS Scirocco, and Faller, flag leaf lamina length was generally reduced by tall alleles, but increased by semi-dwarf alleles.
The first 70 AAs of DELLA are important for the regulation of grain dormancy with a maximum increase of 24% in greenhouse-grown KWS Scirocco (Table 1). This finding is opposite to the effect of the ovg alleles on the elongation growth of coleoptile and stem. In addition, the flag leaf width was generally increased by all ovg alleles, linking this effect to the primary Rht-B1c allele, which showed the strongest increase of 25%, thereby confirming previous studies (Wen et al., 2013). Since the flag leaf provides photosynthetic assimilates to developing grains (Ghiglione et al., 2008), the increased flag leaf length and width in the semi-dwarf lines is generally regarded as a positive pleiotropic effect. Moreover, spike length was either increased up to 16%, or was not significantly different from Rht-B1b or Rht-B1a (Table 1). Longer spikes increase the potential to carry more grains (Zamski, 1995), provided sufficient photo-assimilates are available.

Grain size, assessed via TGW, of greenhouse-grown ovg lines of Maringá was lower than that of Rht-B1a, yet higher than that of Rht-B1c and Rht-B1b (Fig. 3). Relative to Rht-B1a, in greenhouse-grown KWS Scirocco, TGW was reduced ~35% by Rht-B1c.23 and Rht-B1c.26, and 15% by Rht-B1c.22, which corresponded to a proportionate reduction in both grain length and width (Fig. 1F; Table 2). While TGW only reduced ~5% in the field, the plot yield was higher because of a 10–22% increase in grain number per spike (Table 2). Interestingly, the number of spikelets per spike was not significantly affected, suggesting that this yield increase was caused by an increased grain number per spikelet (Table 2). Increased grain number has been previously described for Rht-B1b and Rht-D1b, and results from reduced floret abortion, a process during which developing florets enter a programmed cell death; hence it determines the final number of grains per spikelet (Youssefian et al., 1992; Miralles et al., 1998b). Although grain size was reduced relative to Rht-B1a, this negative effect was compensated by increased grain number, finally generating similar or larger yields than the wild type.

Relative to Rht-B1b, no negative ovg effects on TGW, grain length, grain width, or yield were observed in Faller, with the exception of field-grown Rht-B1c.22 (Table 2). To assess further the ovg effect, relative to Rht-B1b, in different genetic backgrounds and environments, Rht-B1c.23 and Rht-B1c.26 were introgressed in two Australian spring wheat cultivars, Crusader and EGA Gregory, and grown in an Australian field trial. Both cultivars carry the Rht-B1b allele, hence the ovg alleles either slightly reduced (7%) or did not significantly affect plant height (Supplementary Fig. S2). Similar to Faller, no significant, negative effects on yield or TGW were detected (Table 2). In conclusion, the ovg alleles did not reduce overall grain or plot yield, suggesting that the ovg alleles could be used for wheat breeding.

Fig. 1. Phenotypic effects of three ovg alleles, compared with the respective reference allele, in Faller and KWS Scirocco. From left to right: reference allele (Rht-B1b for Faller, Rht-B1a for KWS Scirocco), Rht-B1c.23, and Rht-B1c.26. (A, B) Flowering plants 3 months after sowing; scale bar=10 cm. (C, D) Etiolated coleoptiles when 50% of the second leaf was visible; all leaves were removed on the picture; scale bar=1 cm. (E, F) Ten grains; scale bar=1 cm. (G, H) Sprouted spikes, harvested at physiological maturity from plants grown in the greenhouse; scale bar=1 cm.
Effect of ovg alleles on pre-harvest sprouting

The exceptionally high dormancy of Rht-B1c has been suggested to be useful to increase PHS resistance (Flintham and Gale, 1982; Flintham et al., 1997b). To test whether the ovg alleles retained the resistance to PHS, spikes were exposed to germination-inducing conditions. All KWS Scirocco Rht-B1a spikes strongly sprouted in a humid environment (Fig. 1H). Spikes of Rht-B1c.23, and especially Rht-B1c.26, sprouted less than Rht-B1c.22 spikes (Fig. 1H). Similarly, Rht-B1c.23 and Rht-B1c.26 sprouted less than Rht-B1b in Faller (Fig. 1G), indicating that these alleles show improved sprouting resistance.

Since PHS is enhanced by a loss of dormancy, germination tests are currently regarded as the most accurate technique to quantify PHS resistance (Trethowan, 1995). We assessed germination of grains after harvesting each week, until germination reached at least 95% for all seed lots. Figure 4 shows germination at time points when the differences between Rht-B1a or Rht-B1b and ovg were at their maxima, yet all other time points showed a similar grouping of ovg alleles. In Maringá, the tall ovg alleles displayed a higher germination response than the semi-dwarf ovg alleles: when the differences between Rht-B1a and ovg were at their maxima, semi-dwarf alleles germinated <20%, while tall alleles, with the exception of Rht-B1c.15, germinated >40% (Fig. 4A). Although germination of the ovg semi-dwarfs was similar to the germination of Rht-B1c at this time point, Rht-B1c finally retained dormancy longer than the semi-dwarfing alleles (data not shown). In contrast to the effect of the ovg alleles on stem and coleoptile length, the dormancy of Rht-B1b was not intermediate between tall and semi-dwarf alleles, but rather similar to Rht-B1a (Fig. 4A). In KWS Scirocco, the differential ovg effect was confirmed in the greenhouse and field: Rht-B1c.23 and Rht-B1c.26 reduced germination by up to 65%, whereas Rht-B1c.22 resulted in a maximal 10% reduction in germination, relative to Rht-B1a (Fig. 4B).

Although Rht-B1a and Rht-B1b result in comparable dormancy levels (Fig. 4A), the dormancy effect of the semi-dwarf alleles was lower in the three Rht-B1b-containing cultivars (i.e. germination never dropped below 60%) (Fig. 4C, D). However, dormancy is strongly influenced by genetic and environmental factors (Trethowan, 2001; Groos et al., 2002; Mares and Mrva, 2014), which is demonstrated by the large differences in the time of after-ripening required for losing dormancy. The Australian cultivars lost dormancy more quickly than grains from the greenhouse or field trials in Germany: after 2 weeks, all Crusader and EGA Gregory grain samples showed >90% germination (data not shown). While Maringá, KWS Scirocco, and Faller have red grains, both Australian cultivars are white grained, which are generally less dormant (Mares and Mrva, 2014). However, besides these genetic factors, additional environmental factors cannot be excluded as causes for the rapid loss of dormancy. Although Faller and KWS Scirocco were grown in the same field conditions, the ovg effect on dormancy was lower in Faller than in KWS Scirocco. Since Faller has been developed for North America, this lower dormancy is probably a consequence of Faller having a different growth habit from KWS Scirocco (Figs 1A, B, 2), and being less adapted

Fig. 2. ovg alleles differentially affect plant elongation in different cultivars in the greenhouse. Plant heights are averages of at least 10 plants. The box indicates the period of first spike emergence (Zadoks 50). Black full line, Rht-B1a; black dashed line, Rht-B1b; black dotted line, Rht-B1c; full green line, Rht-B1c.22; full magenta line, Rht-B1c.26; dashed magenta line, Rht-B1c.23. Results were confirmed in two independent experiments. Representative data from the first experiment are shown.

Effect of ovg alleles on pre-harvest sprouting

The first 70 AAs of DELLA are important for the regulation of grain dormancy. Furthermore, high variation in dormancy has also been shown between Arabidopsis ecotypes (Alonso-Blanco et al., 2003). Interestingly, the dormancy of Rht-B1c.26 was twice as strong as that of Rht-B1c.23 in KWS Scirocco (Fig. 4B), which was reproduced across all genetic backgrounds, albeit to a smaller extent (Fig. 4C). In summary, Rht-B1c.23, and especially Rht-B1c.26, were more dormant than Rht-B1c.22 in different environments and genetic backgrounds. These findings provide robust and reproducible evidence of the ovg effect on dormancy, and demonstrate the breeding potential of Rht-B1c.23 and Rht-B1c.26 for increasing PHS resistance.

Effect of ovg alleles on falling number

PHS strongly reduces falling number, which is a key parameter for quality assessment of grains. Besides PHS, falling number is determined by other genetic and environmental factors influencing the starch reserve of the endosperm, such as the production of α-amylase during late grain development in the absence of rain (Mares and Mrva, 2008, 2014). To verify whether the ovg alleles affect falling number, independent of PHS, the ovg effect on falling number was investigated when grain dormancy was completely lost, in conditions that did not trigger PHS (Fig. 5). Therefore, sprouting was not induced in the greenhouse, and field-grown material did not experience significant rainfall prior to harvest (Supplementary Fig. S3). In greenhouse-grown Maringá, all ovg alleles had a falling number up to 15% higher than that of Rht-B1a, while Rht-B1b and Rht-B1c had similar falling numbers (Fig. 5A). In addition, the falling number was well above 300 s, which is associated with excellent baking quality (Gooding et al., 2012). Consequently, these data indicate that the ovg alleles have no negative effect on falling number.

In KWS Scirocco, some Rht-B1a sister lines had falling numbers <300 s. Interestingly, with the exception of greenhouse-grown Rht-B1c.22, all ovg alleles increased falling number in both greenhouse- and field-derived grains, with a maximum increase of 60% for Rht-B1c.26 in the greenhouse (Fig. 5B). In general, falling number negatively correlates with α-amylase activity, as was demonstrated for field-grown KWS Scirocco grains ($R^2=0.7$; Supplementary Fig. S5A). Consequently, a 20% increase in falling number by...
Rht-B1c.23 or Rht-B1c.26 was associated with a 37% reduction of α-amylase activity (Fig. 5B, Supplementary Fig. S5B). In contrast to KWS Scirocco, the effect of the ovg alleles in Rht-B1b-containing cultivars was limited (Fig. 5C, D). To summarize, the ovg alleles increased falling number compared with Rht-B1a, and correspond to that of the widely deployed semi-dwarf Rht-B1b allele, further strengthening their potential for wheat breeding.

Discussion

Novel second-site mutations in Rht-B1c show consistent GA-related effects across genetic backgrounds

Three ovg alleles (Rht-B1c.22, Rht-B1c.23, and Rht-B1c.26) exhibited strong and causal effects on vegetative organ growth, grain quality, and yield in different environments and genetic backgrounds (Figs 1–5; Table 1). First, these ovg alleles showed consistent effects in spring wheat cultivars with diverse growth characteristics. Secondly, the ovg effects were observed not only in independent greenhouse experiments, but also in Australian and German field trials, each representing very different climatic conditions. Thirdly, the backcrossed lines in four commercial spring wheat cultivars displayed phenotypes consistent with the original Maringá mutants, thereby ruling out that additional mutations might have caused the phenotype in the original mutated Maringá Rht-B1c lines.

Phenotypic ovg effects reveal different regulatory mechanisms in GA responses across organs

As summarized in Fig. 6, the second-site ovg mutations suppressed the dwarfism of Rht-B1c to different extents. This differential effect allowed classification of the 14 ovg alleles into nine slightly taller and five slightly shorter dwarfing alleles, relative to Rht-B1b (Fig. 6; Table 1). The grouping of ovg alleles into two groups that are shorter or taller than the standard semi-dwarfing Rht-B1b was similar for both coleoptile and peduncle length (Table 1). While similar grouping is still maintained when looking at the elongation of the first leaves, the ovg effect was weaker than for the other vegetative organs. For instance, in KWS Scirocco, Rht-B1c.23 reduced stem and peduncle length by >30%, whereas the length of the first leaves was only reduced ~20% (Table 1). The lower heritability of leaf elongation, as compared with that of the peduncle (Supplementary Table S3), suggests that leaf elongation is more strongly controlled by environmental factors, thereby buffering the phenotypic impact of the ovg alleles.

In contrast to the ovg effects on first leaves and internodes, the ovg alleles had an inverse effect on flag leaf lamina length: semi-dwarf alleles, which mildly restored the effect of Rht-B1c on stem length, had the longest flag leaves (Fig. 6; Table 1),
highlighting that different DELLA-regulating mechanisms are important in flag leaf elongation as compared with stem elongation. The deviating morphology of the flag leaf compared with the other leaves has been reported previously (Rawson et al., 1983; McCaig and Morgan, 1993; Calderini et al., 1996), and is probably linked to its role in providing photosynthetic assimilates to the developing grains (Ghiglione et al., 2008). These findings illustrate that the DELLA-driven elongation responses most probably depend on their interaction with development- and organ-specific factors. Since DELLA has been shown to function as an integrator of environmental and endogenous cues through direct interaction with a plethora of regulatory proteins, it is likely that different organs contain different sets of DELLA-interacting proteins (Davière and Achard, 2013; Locascio et al., 2013).

All ovg alleles showed a higher TGW than either Rht-B1b or Rht-B1c (Figs 3, 6). The smaller grain, hence lower TGW, of Rht-B1b is a known drawback of this allele. Miralles et al. (1998a) demonstrated that Rht-B1b and Rht-D1b reduced grain size compared with Rht-B1a by reducing cell division, whereas internodes were shortened by inhibiting cell elongation. Consequently, the mechanism by which different DELLA mutations regulate grain size needs further elucidation.

Dormancy and elongation are coupled in Rht-B1c and ovg mutants, but not in Green Revolution mutants

Due to the widespread use of Rht-B1b for reducing plant height, its pleiotropic effects relative to Rht-B1a have been characterized in detail over the past decades, and were confirmed in this study. Without changing the timing of developmental events (Fig. 2; Youssefian et al., 1992b), Rht-B1b reduces the length of vegetative organs down to 20–25% (Table 1; Fig. 6; Flintham et al., 1997b; Rebetzke et al., 2007), with the exception of the flag leaf (Table 1; Fig. 6; McCaig and Morgan, 1993; Calderini et al., 1996). Rht-B1b further leads to a 20% decrease of TGW (Figs 3, 6; Miralles and Slafer, 1996), while increasing grain number (Table 2; Youssefian et al., 1992; Miralles and Slafer, 1996; Miralles et al., 1998b). Remarkably, Rht-B1b does not affect dormancy; hence it germinates as readily as Rht-B1a (Figs 4, 6; Gooding et al., 2012). Similarly, increased expression of Rht-D1b in the Rht-D1c mutant also further increased dwarfism without affecting dormancy (Gooding et al., 2012). Although both elongation and dormancy are typical DELLA-regulated GA responses (reviewed by Graeber et al., 2012; Martinez et al., 2016), these findings suggest that the Green Revolution alleles affect elongation without affecting dormancy; in other words, Rht-B1b and Rht-D1b uncouple the elongation and dormancy responses.
In contrast to Rht-B1b and Rht-D1b, the extreme dwar-  


dism of Rht-B1c is associated with strong dormancy (Flintham and Gale, 1982; Flintham et al., 1997a; Gooding et al., 2012). Furthermore, the results presented here show that this associa-

tion persists when Rht-B1c partially loses its suppressive function: semi-dwarf ovg lines are more dormant than tall ovg lines (Figs 4, 6). Consequently, dormancy and elongation are coupled in Rht-B1c and its ovg derivatives, yet uncoupled in Rht-

B1b and Rht-D1b, thereby highlighting a remarkable difference between Rht-B1c and the widely deployed Green Revolution alleles. Although no biochemical evidence is available, DNA sequencing suggests that the DELLA proteins produced by Rht-B1b or Rht-D1b, and Rht-B1c strongly differ. Whereas RHT-B1C contains a 30 amino acid insertion following the DELLA motif, Rht-B1b and Rht-D1b encode a nucleotide substitu-

tion that introduces a premature stop codon in the LEXLE motif. It has been suggested that translational reinitiation, at one of the three AUG start codons that are immediately down-  


stream of this stop codon (Fig. 7A), could lead to the produc-

tion of N-terminally truncated proteins, lacking ~10% (66–70 amino acids) of the total protein (Peng et al., 1999; Pearce et al., 2011). Since these truncated DELLA proteins lack the DELLA motif, they are insensitive to the GA-induced degra-


dation mechanism and presumably accumulate (Peng et al., 1999; Pearce et al., 2011), thereby repressing elongation without affecting dormancy. Consequently, the first 70 amino acids of the DELLA protein have a unique role in regulating dormancy, but not in the control of elongation.

The first 70 amino acids of the DELLA protein have recently been shown to be important for the transactivation activity and post-translational modifications of the DELLA protein. For example, the deletion of the DELLA motif reduced the transactivation activity of the DELLA protein in rice (Hirano et al., 2010). In Arabidopsis, glycosylation also reduced the suppressive function of DELLA proteins, and occurred preferentially in the N-terminal half of the protein (Zentella et al., 2016). Interestingly, several transcrip-

tomic approaches demonstrated that there is only a marginal overlap of DELLA-regulated genes between different organs (Locascio et al., 2013). Therefore, the DELLA protein probably regulates a different set of genes in elongation from that in dormancy. Consequently, we hypothesize that the first 70 amino acids of the DELLA protein are involved in regulating gene expression in dormancy, but not in elongation.

**Application potential: improving pre-harvest sprouting resistance**

Although the high dormancy of Rht-B1c is beneficial for PHS resistance, its extreme dwarfism prevented its use in com-


mercial cultivars (Flintham and Gale, 1982; Flintham et al., 1997a). In this respect, this study reveals a unique application potential: Rht-B1c.23 and Rht-B1c.26 were more dormant than Rht-B1a or Rht-B1b, in different genetic backgrounds and environments, which correlated with improved PHS resistance (Figs 1G, H, 4). In addition, final height was within the range of standard semi-dwarfing alleles, whereas no negative effects on overall yield or falling number were observed (Table 2; Fig. 5). Consequently, Rht-B1c.23 and Rht-B1c.26 could be used to adjust plant height while improving PHS resistance in regions with frequent rainfall prior to harvest.

**Ovg mutations increase insight into DELLA protein function**

Together, the described phenotypes emphasize that the amino acid residues mutated in the ovg alleles are crucial for different GA responses in wheat. Figure 7A depicts the position of
The first 70 AAs of DELLA are important for the regulation of grain dormancy. The second-site ovg mutations on the RHT-B1C protein. At the N-terminal half, which consists of the DELLA, LExLE, TVHYNP, and poly S/T/V motifs, five ovg alleles probably result in a lower splicing efficiency of the Della transcript and, in turn, in less DELLA protein (Fig. 7A; Supplementary Table S1). The sixth N-terminal ovg allele, Rht-B1c.22, contains a stop codon within the non-spliced Rht-B1c insertion, thereby generating a low level of N-terminally truncated DELLA proteins. Taken together, the N-terminal ovg mutations probably reduce DELLA protein levels, which results in the tall ovg phenotype. Although validation of this hypothesis at the protein level is required, the absence of a suitable assay for detecting DELLA protein in wheat renders this experiment at least problematic (Pearce et al., 2011).

The other amino acid substitutions occurred within strongly conserved residues of the C-terminal GRAS domain of the DELLA protein (Fig. 7A; Chandler and Harding, 2013). This GRAS domain characterizes a plant-specific family of transcriptional regulators and contains five conserved subdomains: leucine heptad repeat I (LHR I), VHIID, leucine heptad repeat II (LHR II), PFYRE, and SAW (Pysh et al., 1999). Interestingly, some of those mutations were highlighted as important residues for DELLA function in other plant species. Substitutions identical to Rht-B1c.15 and Rht-B1c.24 in the PFYRE and LHRII subdomain, respectively, were identified in a suppressor screen in the GA-insensitive Shn1d barley mutant (Chandler and Harding, 2013). Alanine scanning in rice plants that overexpressed DELLA revealed that the residue mutated in the Rht-B1c.15 line is required for growth repression (Hirano et al., 2010). A suppressor screen in the Arabidopsis GA biosynthesis mutant ga1-3 identified a substitution of the amino acid adjacent to the residue mutated in the Rht-B1c.17 line, named rga-2 (Silverstone et al., 1998). Together, the amino acid residues substituted in the ovg alleles appear crucial for the DELLA-regulated growth mechanism in different plant species.

The recent characterization of the crystal structure of the rice SCARECROW-LIKE 7 (OsSCL7) GRAS protein further aids our understanding of the importance of specific amino acid residues for DELLA function (Li et al., 2016). Mapping the amino acid mutations of the ovg alleles on OsSCL7, based on homology between the GRAS domain of RHT-B1 and OsSCL7, reveals that the C-terminal ovg mutations are not clustered together, but presumably are located at different positions of the protein (Fig. 7B). Although the majority of mutated amino acids are positioned at the interior of the protein, RHT-B1C.26 (E579K) is positioned at the protein surface.
Therefore, the Rht-B1c.26 ovg substitution from glutamic acid to lysine, which alters the charge at this position from negative to positive, could directly affect interactions with other proteins. In addition, in OsSCL7, this glutamic acid residue is in close proximity to some residues of the PFYRE subdomain, potentially forming a binding pocket (Supplementary Fig. S6). However, this PFYRE region shows limited conservation in Rht-B1. In contrast, the aspartic acid at the Rht-B1c.23 position (D371) forms hydrogen bonds with three residues of the VHIID (G373 and W380) and PFYRE (N467) subdomain (Fig. 7B), which are conserved across monocot species. In turn, these residues form hydrogen bonds with other conserved residues of the VHIID (A353, N354, Q379) and PFYRE (T498) subdomain. The Rht-B1c.23 ovg substitution from aspartic acid to asparagine is likely to disrupt some of these hydrogen bonds through conformational changes. Interestingly, the importance of these hydrogen bonds for the functionality of the DELLA protein is supported by the observation that substitution of QWP379-381 by three alanine residues results in a loss-of-function phenotype in rice (Hirano et al., 2010). Furthermore, this alanine substitution reduced the interaction between the DELLA protein and GID1 in a yeast two-hybrid assay, suggesting that conformational changes of DELLA can reduce the affinity for specific interaction partners (Hirano et al., 2010). These findings further support the hypothesis that the binding affinity for specific proteins is differentially affected by semi-dwarf and tall ovg alleles. Alternatively, these conformational changes could increase the degradation rate of the DELLA protein.

In summary, the ovg amino acid substitutions partially released the growth repression caused by the N-terminal Rht-B1c insertion. Their functional importance in different GA responses and species highlights the value of the ovg alleles in elucidating DELLA function in different organs and growth responses. Furthermore, the coupling of dormancy and elongation in Rht-B1c and its ovg derivatives can shed light on the importance of the first 70 amino acids of the DELLA protein, and can now be agronomically exploited via Rht-B1c.23 and Rht-B1c.26.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Nucleotide and amino acid substitutions characteristic for each ovg allele.

Table S2. Phenotypic measurements on main stem correlated with measurements on the first and second tiller in Maringá.

Table S3. Reproducibility of ovg effects in KWS Scirocco.

Fig. S1. Rht-B1c.26 reduced the final length of each internode in Maringá.

Fig. S2. Effect of Rht-B1c.23 and Rht-B1c.26 on plant height in Crusader and EGA Gregory.

Fig. S3. Rainfall in Gatersleben (Germany) and Yanco (New South Wales, Australia).

Fig. S4. ovg alleles increased falling number in KWS Scirocco field trial.

Fig. S5. Negative correlation between falling number and α-amylase activity.

Fig. S6. Position of Rht-B1c.26 on the crystal structure of the OsSCL7 GRAS protein (Li et al., 2016).

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