Decreased shoot stature and grain α-amylase activity following ectopic expression of a gibberellin 2-oxidase gene in transgenic wheat

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Abstract
Ectopic expression of a gibberellin 2-oxidase gene (PcGA2ox1) decreased the content of bioactive gibberellins (GAs) in transgenic wheat, producing a range of dwarf plants with different degrees of severity. In at least one case, a single transformation event gave rise to T1 plants with different degrees of dwarfism, the phenotypes being stably inherited over at least four generations. The dwarf phenotype, which included dark-green leaves, increased tillering and, in severe cases, a prostrate growth habit, was replicated by the application of a GA biosynthesis inhibitor to the wild type. Ear rachis length, grain set, and grain size were also decreased in the wheat transformants, compared with an azygous (null) line. The extent of post-germination α-amylase production in grains reflected the severity of the shoot phenotype of the transformants and both developmental processes were restored to normal by the application of gibberellic acid (GA3). Expression of two GA biosynthesis genes (TaGA20ox1 and TaGA3ox2) was up-regulated, and that of two α-amylase gene families (α-Amy1 and α-Amy2) down regulated, in scutella of semi-dwarf lines, compared with controls. The marked decline in transcript abundance of both α-amylase gene families in aleurone was associated with a decreased content of bioactive GAs in grains of the semi-dwarf lines.

Key words: α-Amylase, ear development, GA 2-oxidase, gene expression, gibberellin, grain size, paclobutrazol, shoot height, tillering, transgenic wheat.

Introduction
Genetic and biochemical analysis of certain dwarf and overgrowth mutants of several mono- and dicotyledonous species has provided compelling evidence that gibberellins (GAs) are important regulators of stem internode growth determining final plant height. In cereals, the breeding of semi-dwarf varieties of wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.), coupled with the increased use of nitrogen fertilizers and herbicides, resulted in increased yields worldwide during the so-called 'green revolution' of the 1960–1970s. The importance of decreasing shoot stature also led the agrochemical industry to develop various classes of plant growth retardants that act by inhibiting different steps on the GA biosynthesis pathway. For many years, it has been routine agronomic practice to treat intensively managed wheat crops with growth retardants in order to prevent the damaging effects of wind and rain that cause stem collapse (lodging) in untreated crops.

The reduced height (*Rht*) in hexaploid bread wheat was derived mainly from the Japanese semi-dwarf variety, Norin 10, containing the *Rht-B1b* and *Rht-D1b* mutant alleles (formally *Rht1* and *Rht2*) (Gale and Youssefian, 1985; Börner *et al.*, 1997). These semi-dominant *Rht* mutations are associated with an inability of shoot tissues to respond fully to applied GA, suggesting an interference with the normal GA signal transduction pathway. DNA sequence analysis of *Rht-B1b* and *Rht-D1b* genes showed that they were orthologues of maize (*Zea mays* L.) *dwarf-8* and *Arabidopsis thaliana* L. *GAI* (Peng *et al.*, 1999), mutant

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genes that also confer GA-insensitive dwarf phenotypes. The wild-type genes encode N-terminal DELLA-domain proteins that repress plant growth and GA relieves growth repression by targeting their degradation via the ubiquitin–proteosome pathway (reviewed in Thomas and Sun, 2004). The mutant wheat semi-dwarfing genes contain nucleotide substitutions that create stop codons in the N-terminal region that, by reinitiation, are assumed to result in the formation of truncated proteins that retain a capacity to inhibit growth, but are resistant to GA-induced degradation (Peng et al., 1999).

Near-isogenic wheat lines containing different GA non-responsive Rht dwarfing alleles have decreased rates of elongation and final lengths of leaves (Keyes et al., 1989; Pinthus et al., 1989) and associated stem internodes (Hoogendoorn et al., 1990), compared with GA-responsive tall lines. The primary effect of these Rht genes is to decrease cell expansion in the elongation zone of vegetative stem tissues without affecting final ear size. As a consequence, more assimilates are partitioned to developing ears thus supporting greater floret survival at anthesis and increased grain set, compared with GA-responsive, tall wheats (Youssefian et al., 1992b). Interestingly, the Rht semi-dwarfing genes do not appear to affect the timing of the transition of shoot apical meristem from a vegetative to a reproductive state (Youssefian et al., 1992a), a response that is hastened by applied GA and delayed by treatment with plant growth retardants that block GA production (Hutley-Bull and Schwabe, 1982; Evans et al., 1995).

In contrast to wheat, the commercially successful varieties of rice are GA-responsive semi-dwarfs, suggesting the presence of mutations in the biosynthetic pathway leading to the formation of bioactive GA. Recently, the basis of semi-dwarfism in four rice varieties that contributed to the ‘green revolution’ has been attributed to different alleles at the recessive sd-1 locus that are independent mutations in the OsGA2ox2 gene. This gene, a member of a small gene family, is expressed mainly in stems and is an important regulatory step on the pathway to active GA (reviewed in Hedden, 2003). Specific suppression of another member of the gene family, OsGA20ox1, caused semi-dwarfism, suggesting that it is also involved in the regulation of shoot stature in rice (Oikawa et al., 2004). In addition, different alleles of the GA-responsive dwarf rice, d18, are associated with mutations in the OsGA3ox2 gene that is also expressed highly in stems (Itoh et al., 2001).

In wheat, one member each of the GA 20-oxidase and GA 3-oxidase gene families (TaGA20ox1 and TaGA3ox2) has been cloned and shown to be highly expressed in elongating stems and developing and germinated embryos supporting their involvement in GA-regulated processes in these tissues (Appleford et al., 2006). In stem tissues, expression was highest in the nodal region subtending a rapidly expanding internode. At present, expression of the orthologue of the rice sd1 gene (TaGA20ox2) has not been studied in wheat.

Induction of α-amylase and other hydrolytic enzymes in the aleurone layer of germinated cereal grains is dependent on embryo-produced GAs (reviewed in Fincher, 1989). GA-deficient mutants of barley (Hordeum vulgare L.) and rice produce less post-germination α-amylase than corresponding wild types and a normal phenotype is restored by GA application (reviewed in Appleford and Lentont, 1997). In germinating wheat, high levels of expression of both TaGA20ox1 and TaGA3ox2 genes in the scutellum (Appleford et al., 2006) supported evidence that this organ is the main site of de novo GA biosynthesis (Appleford and Lentont, 1997). In rice, expression of two members each of the GA 20-oxidase and GA 3-oxidase gene families was confined to the scutellar epithelium showing that this specialized tissue is an important site of GA production (Kaneko et al., 2003) and mutant analysis confirmed that normal expression of OsGA3ox2 was essential for initiation of expression of R Amy1A in aleurone (Kaneko et al., 2002).

In the absence of single gene recessive mutants in hexaploid bread wheat, and as an alternative approach to the use of plant growth retardants, transgenic wheat was generated to determine the consequences of GA depletion on specific aspects of wheat development. We were interested in the range of phenotypes that might be produced compared with those of GA-‘non-responsive’, Rht semi-dwarfs and a GA-responsive tall line treated with an inhibitor of GA biosynthesis. The initial strategy involved ectopic expression of a full-length cDNA of a runner bean (Phaseolus coccineus L.) GA 2-oxidase gene (PcGA2ox1) (Thomas et al., 1999), fused behind a maize ubiquitin promoter. Based on the activity of the recombinant protein, it was reasoned that this gene product would cause a more rapid turnover of GAs by inactivation of the main biologically active GA of wheat tissues, GA1 (to GA3) and its precursor, GA29 (to GA3), by 2-oxidation. While this work was in progress, it was reported that rice plants transformed with a GA 2-oxidase, OsGA2ox1, behind a ‘constitutive’ actin promoter produced severely dwarfed plants that failed to set grain (Sakamoto et al., 2001). However, a more targeted approach using the promoter of the shoot-specific OsGA3ox2 gene to drive the OsGA2ox1 open reading frame produced transgenic rice plants with more moderate decreases in shoot stature and normal reproductive development (Sakamoto et al., 2003).

Materials and methods

Generation of PcGA2ox1 transgenic wheat lines

Plasmid pLAP2ox2 contained a full-length cDNA of a GA 2-oxidase from runner bean (PcGA2ox1) under transcriptional control of the maize ubiquitin-1 (Ubi-1) promoter, including its 5′ untranslated exon and first intron, and terminated with the
Grains of cv. Canon (wild type) and T3 Line 4 plant 10 (null) and RNA isolation, northern hybridization, and qRT-PCR detection kit for nucleic acids (Roche Diagnostics GmbH) according to the manufacturer’s instructions. The presence of PcGA2ox1 transgene were sown out, as above, for phenotypic analysis and collection of leaf material for segregation analysis and determination of copy number of inserts. The segregation analysis was repeated on selected T3 seedlings.

DNA extraction, PCR conditions, and Southern blot analysis

For screening of the transgenic lines, DNA was extracted from leaf tissue using the Nucleon Phytopure kit (GE healthcare) according to the manufacturer’s instructions. The presence of PcGA2ox1 was determined by PCR: sense primer 5'-TCATAGTGAACGCCTG-TAGG-3'; antisense primer 5’-GTTTCTTCATGCTGATATG-3’. Reactions, using the ReddyMixTM PCR system (Abgene) with 200 ng DNA in a total of 25 µl, were heated at 95 °C for 5 min and then subjected to 30 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s.

For Southern blot analysis, DNA was extracted from young leaf tissue using the CTAB method (Isaac et al., 1995) and digested with SspI, BamHI or EcoRI. The digested DNA (20 µg) was separated by electrophoresis on a 1.0% (w/v) agarose gel at 35 V for 24 h and transferred by capillary blotting on to positively charged nylon membrane (Biodyne™ Nylon membrane, Sigma) (Sambrook et al., 1989). A digoxigenin-labelled probe was generated using a PCR DIG probe synthesis kit (Roche Diagnostics GmbH) and the above PcGA2ox1 primers with plasmid pLAP2ox2 as template. The cycle conditions were 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min 30 s, and the extension of 72 °C for 10 min. Hybridization and detection of probe was carried out using a non-radioactive, DIG luminescent detection kit for nucleic acids (Roche Diagnostics GmbH) according to the manufacturer’s instructions.

RNA isolation, northern hybridization, and qRT-PCR

Grains of cv. Canon (wild type) and T3 Line 4 plant 10 (null) and plants 8 and 16 (semi-dwarfs) were germinated at 20 °C for 72 h and scutella dissected out and plunged into liquid N2 prior to RNA isolation and northern hybridization using methods described in Appleford et al. (2006). Briefly, poly(A)+ RNA was extracted and purified on oligo-dT cellulose, size-fractionated by electrophoresis on formaldehyde-agarose gels, and transferred to nitrocellulose. Probes for hybridization to TaGA20ox1 and TaGA3ox2 were labelled with 32P-dCTP using random primers and the full-length cDNAs as template whereas probes for α-Amy 1 (high pl), α-Amy 2 (low pl) used specific cDNA clones for the two α-amylase gene families (Lazarus et al., 1985). In a further experiment, T4 grains of Line 4 plants 10 (null) and 8 (semi-dwarf) were imbibed at 5–6 °C for 30 h before transfer to 20 °C. Scutella and aleurone layers were dissected from grains after 42 h and 66 h and plunged into liquid N2 prior to isolation of total RNA and northern hybridization (Lenton et al., 1994) using labelled probes, as above. Total RNA for qRT-PCR was extracted from young leaves, ears, peduncles, and nodes of greenhouse-grown T4 plants according to Verwoerd et al. (1989). Real-time qPCR was carried out on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Green as described in Griffiths et al. (2006) and employing actin as the sole reference gene. The primers for PcGA2ox1 and actin were as follows: PcGA2ox1: sense 5’-TAGCAAGAGGATTGGCCCAAAC, antisense 5’-GTTGTTGAGGA-GAGATTTCCG; Actin: sense 5’-AGGCATCTCTGACGCTCAAGTA, antisense 5’-GCTCGTGTGA-GAAGTGTGGTG.

Growth conditions and phenotypic analysis of PcGA2ox1 transgenic wheat

T3 grains of Line 4 plants 10 (null), 8 and 16 (semi-dwarfs), 2 (dwarf), and Line 6 plant 21 (severe dwarf) were germinated directly in modules and potted on, as above. Seedlings (15 per genotype) were transferred to a growth room under standard summer growing conditions (19/15 °C day/night, 16 h photoperiod, 488 µmol m−2 s−1 at 400 mm from pot base). Mainstem leaves 3 and 6 were tagged after emergence of the lamina and the time of emergence of the first anthers of the ear (anthesis) was recorded. Final lengths of leaf 3 (measured from the soil surface to the tip of the lamina) were measured after eight days in the growth room. Plants were grown to harvest ripeness in the growth room and dried off in a greenhouse. Final lengths of mainstem internodes plus ears were measured and grain numbers and weights recorded.

T4 grains of Line 4 plants 10, 8, and 2 were germinated and potted up, as above, and grown under summer greenhouse conditions. Mainstem leaves were tagged after emergence and total and lamina lengths measured twice weekly on a subset of randomly selected plants (12 plants per genotype) from the central area of plots of blocks of relatively densely spaced pots containing the three genotypes. Numbers of primary and secondary tillers in the axils of leaves 1–3 were recorded 6 weeks after sowing as the timing of anthesis on mainstem ears. On another subset of randomly selected plants apical development stage was determined following careful dissection of shoot apices and examination under a binocular microscope. Four plants per genotype were harvested at weekly intervals 4–7 weeks after sowing for this purpose.

Treatment of PcGA2ox1 transgenic wheat plants with GAs

T4 grains of Line 4 plants 10, 8, and 2 were pre-imbibed in Petri dishes at 5–6 °C for 48 h and planted out into pots (70×70 mm, 12 grains per pot, six pots per genotype) containing moist vermiculite. Pots were randomized into two blocks in a controlled environment cabinet set at 20 °C with a 16 h daylength (165 µmol m−2 s−1 at 50 mm above pot height). Two pots of each genotype were watered or treated with 10 µM GA3 or 10 µM GA4 solutions on days 2 and 5 (25 ml per pot, six pots per treatment). Nutrient solution (25 ml) was added to all pots on days 6 and 8 and pots were watered, as required, on days 7 and 9. Final lengths of the sheaths of leaf 1 were measured on day 12.
Treatment of wild-type wheat with paclobutrazol

Individual grains of wild type, cv. Canon, were planted in modules and at the 2-leaf stage transferred to pots (150 mm diameter) containing loam-based compost plus slow-release fertilizer. Pots were spaced relatively widely on benches under summer greenhouse conditions. Seedlings were treated at 3–4 d intervals with either water (control) or 10 μM 25,35-paclobutrazol (treated), applied to the soil at the base of the shoot, starting two days after transfer to the greenhouse. At the seedling stage, doses were less (5× 29.4 μg per pot) but increased as the plants grew larger (18× 58.7 μg per pot). Final lengths of leaf 8 (flag leaf), main stem and ears were measured for 15 plants for each treatment.

Measurement of α-amylase activity in germinated grains

Grains of cv. Canon (control) and T4 Line 4 plants 10, 8, 16, and 2 and Line 6 plant 21 were plated out (5×5 pattern) in Petri dishes (90 mm diameter) containing two layers of Whatman No. 1 filter paper moistened with 4.5 ml distilled water. Dishes were incubated in a cold room (5–6 °C) for 48 h prior to transfer to 20 °C for 72 h in darkness. Four replicate samples of three grains with shoots and roots removed were extracted for each genotype and assayed for α-amylase activity using Phadebas dye-bound starch substrate (Barnes and Blakeney, 1974). Units of activity were determined from a standard curve using purified barley malt α-amylase. Soluble protein was also assayed using Coomassie Brilliant Blue G-250 dye substrate and quantified using catalase to generate a standard curve. To determine the change in α-amylase activity over time, grains were set up, as above, and incubated for 30 h in the cold before transfer to 20 °C. α-Amylase activity was determined on grains harvested at 42, 66, and 90 h after transfer to the warm. To determine the effect of GA treatment, intact grains were incubated in water, 1.0 μM GA1 or 1.0 μM GA3, as above, and α-amylase activity determined after 66 h at 20 °C.

Quantification of GAs in germinated grains

Grains of cv. Canon and T4 Line 4 plants 10, 8, 16, and 2 were germinated in Petri dishes, as above, at 20 °C for 96 h. Uniformly sized shoot axes plus roots were dissected from grains (scutellum plus endosperm) and the separated tissues were plunged directly into liquid N2 prior to freeze-drying. The dry masses of the shoot plus root axes ranged from 1.73–2.27 g (308–368 items per genotype) and the grains from 7.33–9.14 g (296–360 items). Subsamples of shoot plus root axes (1.0 g) and grains (2.0 g) were removed for initial quantitative GA analyses using methods described in Webb et al. (1998). Briefly, samples were extracted with 80% methanol–water and [3H]- and [3H]-labelled GA standards added. Ethyl acetate-soluble acids, obtained from an aqueous phase, were purified by QAE Sephadex anion-exchange and C18 Sep-Pak cartridge chromatography prior to resolution by C18 reverse-phase HPLC. Grouped fractions, based on the retention times of the [3H]GA standards, were methylated and the ethyl acetate-soluble neutrals passed through a NH2 Bond Elut column, reduced to dryness, trimethylsilylated and analysed by GC-SIM using a BPX-5 capillary column. Amounts of endogenous compounds were determined from calibration curves. In a repeat analysis, consisting of the remainder of the axes and 2.0 g of grains, the amounts of [3H] GA standards added were similar to those determined for the endogenous GA content in the initial experiment. Results from the second analysis are presented.

Statistical analysis

The GenStat® statistical system, version 8.2, (Lawes Agricultural Trust, Rothamsted, UK) was used to analyse growth data and measurements of α-amylase activity. A natural log transformation of some data sets prior to analysis was required to account for heterogeneity of variance across treatments. For example, high variability was found within the paclobutrazol treatment of wild-type wheat, where a simple t test was applied to the log transformed data to make comparison. One-way Analysis of Variance (ANOVA) was applied for other measures of growth for the transformants, whilst a non-parametric Kruskal-Wallis one-way ANOVA was applied to the analysis of tiller production. The lack of variability in numbers precluded the use of standard one-way ANOVA or effective generalized linear modelling here. Hence, the ranks of the observations across the genotypes are used to provide a chi-squared test of significance between them. Two-way ANOVA was applied to growth data and natural log transformed α-amylase values from the experiments using control and transformant genotypes treated with GA. Following one-way or two-way (parametric) ANOVA, the relevant least significant difference (LSD) at the 5% level of significance was used to compare means of important biological interest.

Results

Molecular and phenotypic characterization of PcGA2ox1 transgenic wheat

Six independent primary transformants (Lines 1–6) expressing PcGA2ox1 cDNA were generated during 1999–2000 and subsequent generations from selfed ears were characterized in terms of presence of the transgene and shoot phenotype. Of the 20 T1 grains of Line 1 that were germinated, all tested positive for the transgene by PCR and produced extreme dwarf seedlings that were treated repeatedly with GA3 to encourage ear emergence and allow grain to set. As segregation ratios indicated that this line may have had transgene inserts at multiple loci it was not investigated further. From 20 grains each of Lines 2 and 3, several T1 plants had a relatively mild semi-dwarf shoot phenotype at anthesis. The tallest PCR-positives ranged from a decrease in shoot height of only 5–12%, compared with azygous talls, whereas the more dwarfed PCR-positives were up to 25% shorter. However, no putative homozygous sublines were recovered from the T1 PCR-positive plants of Lines 2 and 3 at the T2 seedling stage. T1 plants of Line 4 produced a wide range of shoot phenotype. Measurement of the lengths of leaf 2, as well as shoot height at anthesis, confirmed that the presence of the PcGA2ox1 transgene reflected a shorter shoot phenotype. Ten talls were PCR-negative and, of the 23 PCR-positives, 14 were classified as semi-dwarfs (33–49% decrease in shoot height, compared with nulls) and nine as more severe dwarfs, five of which were treated with GA3 to ensure ear emergence from the flag leaf and adequate grain set. Six putative homozygous sublines were recovered from Line 4 at the T2 seedling stage. From the Line 5 primary transformant, 27 T1 seedlings from 38 germinated grains tested PCR positive and produced both semi-dwarf and dwarf shoot phenotypes. However, no putative homozygous sublines were rescued at the T2 seedling stage. From the Line 6 primary transformant,
24 T1 seedlings from 40 germinated grains were PCR positives, again producing both semi-dwarf and dwarf phenotypes at maturity. Only one relatively severe dwarf putative homozygous subline was rescued at the T2 seedling stage.

More detailed molecular and phenotypic characterization focused on the putative homozygous semi-dwarf and dwarf sublines from Line 4 and the more severe dwarf from Line 6. Southern blot analysis of Ssp1-digested DNA extracted from homozygous T3 seedlings of Line 4 showing extreme dwarf and semi-dwarf growth habits and probed with full-length PcGA2ox1 cDNA produced identical banding patterns (Fig. 1A). A tall azygous plant was analysed as a negative control and shown to produce no bands that hybridized to the probe. An identical banding pattern was obtained from several T1 plants from Line 4 showing different degrees of dwarfism, whereas an extreme dwarf plant from Line 6 gave a different pattern (data not shown). Furthermore, digestion of the DNA with other restriction enzymes also produced the same banding patterns for dwarf and semi-dwarf plants from Line 4 (data not shown). Thus, the Line 4 plants contained the same transgenic loci despite displaying differences in phenotype that were maintained for at least four generations. The abundance of PcGA2ox1 transcript was compared in the first leaf of Line 4 and 6 plants by real-time RT-PCR (Fig. 1B). Transcript abundance correlated with dwarfing severity (Figs 2A, 3), except for Line 4 plant 2, which contained less transcript than the semi-dwarf lines. However, when PcGA2ox1 mRNA abundance was measured in ears, peduncles, and peduncle nodes of the Line 4 plants 2 (severe dwarf), 8 (semi-dwarf), and 10 (azygous tall) there was a clear correlation between transgene expression and dwarfing severity (Fig. 1C).

In terms of shoot phenotype, selected T2 transgenic sublines from the original Line 4 showed decreased shoot height at anthesis and considerable variation in extent of prostrate growth habit, increased tillering, and delayed ear emergence, compared with a corresponding azygous plant (null) (Fig. 2A). When plant 10 (null) was at anthesis, plant 3 remained an extreme ‘cartwheel-type’ prostrate

Fig. 1. Molecular analysis of PcGA2ox1 transformants. Southern blot of Ssp1-digested DNA from selected T3 PcGA2ox1 transformants, Line 4 plants 2 (dwarf), 8 (semi-dwarf), and 10 (null) (A). Quantitative real-time RT-PCR analysis of relative PcGA2ox1 transcript levels in: seedlings of Line 4 plants 2, 8, 10, and 16, and Line 6, plant 21 (B) and in ears, peduncles (Ped) and peduncle nodes of the Line 4 plants 2, 8, and 10 (C). The lowest value is set to 1 in each case. Error bars represent standard error of the means of three technical replicates.

Fig. 2. Phenotypic variation in shoot architecture of widely-spaced T2 plants of the original Line 4 PcGA2ox1 transformant grown under spring greenhouse conditions with supplementary illumination. Plants from the left are 10 (null), 8 (semi-dwarf), 2 (dwarf), and 3 (severe dwarf) (A). Shoot phenotypes of wild-type cv. Canon (left) and paclobutrazol-treated (right) plants 8 weeks after sowing. Treated pots had received a total of 730 μg paclobutrazol over a period of 40 d at this time (B).
dwarf with large numbers of tillers and very few emerging ears. Plant 2 was another dark-green prostrate dwarf with many tillers and delayed ear emergence whereas plant 8 was semi-dwarf with fully emerged ears from the mainstem and several tillers. In this subline, there was also recovery of the natural orthogavitropic growth response of shoots during expansion of the peduncle, the last formed stem internode (compare plants 8 and 10 in Fig. 2A). Developmental plasticity was also observed within individual Line 4 sublines depending on plant density and light quality. In general, plants with a more prostrate growth habit and many more tillers were produced when plants were widely spaced and grown under artificial light.

Repeated applications of low doses of the GA biosynthesis inhibitor, 25S35-paclubutrazol, to relatively widely spaced wild-type plants grown under summer greenhouse conditions produced dark-green, prostrate dwarf plants with many tillers, phenotypically similar to the PcGA2ox1 transformants (Fig. 2B). In control plants, anthesis occurred between 59–63 d after sowing but was delayed to between 69–78 d in paclubutrazol-treated plants. The cumulative effect of increasing paclubutrazol dosage caused highly significant (P <0.001, t tests) decreases of 35–38% in the lengths of leaf 8 (flag leaf) and mainstem and of 23% in the size of the ear, compared with controls (Table 1).

Growth of the transformants was measured on T3 plants grown in a controlled environment simulating average August growing conditions. The decrease in length of leaf 3 (up to 38%) in sublines 2, 16, and 8 from Line 4, compared with a non-transgenic (null) segregant (Line 4, subline 10) was highly significant (P <0.05, LSD following ANOVA), but less severe than Line 6 plant 21 (66% decrease) (Fig. 3). In Line 4, plant 16 exhibited a slightly more prostrate growth habit than plant 8 whereas plant 2 and Line 6 plant 21 were even more extreme prostrate dwarfs. Main stem height of Line 4 plants 8 and 16 was decreased by 40–44%, compared with a null (plant 10), and corresponding highly significant (P <0.05, LSD) decreases of 54% and 61% were observed in Line 4 plant 2 and Line 6 plant 21, respectively. The lengths of the peduncle, the last-formed stem internode, reflected those of the main stem as a whole. The trans-formants also produced one extra main stem leaf, compared with the null, under these growing conditions.

Initially, the rate of floral development was much slower in the PcGA2ox1 transformants, but increased later so that timing to anthesis was delayed by only 1–2 d in Line 4, plant 8 and 5–7 d in Line 6, plant 21, compared with the null (plant 10). In Line 4 plants, there was a significant (P <0.05) 4–8% decrease in final ear length (Fig. 3) and ears also contained one additional spikelet (Table 2). Line 6, plant 21 produced a ‘club-like’ ear (30% decrease in length) which contained one less spikelet (Table 2). There was a significant (P <0.05) decrease in grain number per spikelet in Line 4 plant 2 and Line 6 plant 21 and the decrease in mean grain mass for these two lines was associated with the degree of severity of dwarfing of the main stem of the transformants, compared with the null (Table 2; Fig. 3).

A more detailed developmental analysis was conducted on selected Line 4 T4 plants grown under summer greenhouse conditions. As expected, lengths of main stem leaves 3 and 5 were shorter than the null (plant 10) with the effects of the transgene being greater on the sheath than on the lamina (Table 3). Although the lengths of the

Table 1. Mean lengths (mm ±SE) of wheat cv. Canon tissues after treatment with water (control) or 10 μM 2S,3S-paclubutrazol

T-tests on log transformed values showed highly significant differences (P <0.001) between control and paclubutrazol-treated tissues.

| Treatment       | Leaf 8  | Main stem | Ear   |
|-----------------|---------|-----------|-------|
| Water           | 888.5±4.7| 794.5±11.1| 102.0±0.63|
| Paclobutrazol   | 576.5±7.8| 491.7±7.4 | 78.5±1.41 |

Fig. 3 Final lengths (mm) of leaf 3, main stem and ear of selected T3 PcGA2ox1 transformants from Lines 4 and 6 grown in a controlled environment simulating average UK August growing conditions. (LSDs for comparison of lines within tissue, at the 5% significance level, are 7.6 mm for leaf 3, 12.9 mm for peduncle, 28.8 mm for main stem, and 3.6 mm for ear.)

Table 2. Yield components of main stem ears of selected T3 PcGA2ox1 transformants from Lines 4 and 6 grown in a controlled environment simulating August growing conditions in the UK

| Spikelet no. | Grain no. | Grains/ spikelet | Grain mass (mg) | Mass/grain (mg) |
|--------------|-----------|-----------------|-----------------|-----------------|
| Line 4 plant 10 (null) | 21.0 | 69.6 | 3.32 | 2539.0 | 36.5 |
| Line 4 plant 8 (semi-dwarf) | 21.9 | 70.7 | 3.22 | 2168.0 | 30.7 |
| Line 4 plant 16 (semi-dwarf) | 21.9 | 68.8 | 3.15 | 2029.0 | 29.6 |
| Line 4 plant 2 (dwarf) | 21.9 | 57.6 | 2.63 | 1470.0 | 25.5 |
| Line 6 plant 21 (dwarf) | 20.1 | 48.5 | 2.41 | 1251.0 | 25.8 |
| LSD (95%)(60 df) | 0.77 | 5.6 | 0.24 | 194.4 | 1.7 |
sheath of leaf 5 of the two transformants were significantly different ($P < 0.05$), this was not the case for the earlier produced leaf 3 ($P > 0.05$). Even in these relatively densely spaced plants, there was a highly significant increase in tiller numbers in the axil of leaf 2 and to a lesser extent of leaf 1 of the transgenic plants, compared with the null (Table 4), differences between lines being significant ($P < 0.05$, Kruskal–Wallis ANOVA).

Shoot apices were dissected from plants at weekly intervals to determine the timing of transition from the vegetative state to flowering. No genotypic differences in the timing to ‘double ridges’, the transition to flowering, were detected, although the subsequent rate of floral development was delayed in plant 8 and, to a greater extent, in plant 2, compared with the null. For example, at 35 d after sowing, apices of the null were at glume primordium stage, plant 8 was at terminal spikelet stage whereas plant 2 was only at the floret primordium stage. However, under these growing conditions, timing to anthesis was only delayed by 1–3 d in the transgenics, compared with the null.

Reversal of the dwarf shoot phenotype with applied GA

As expected, sheath length of the two Line 4 transformants was much shorter than the null and root application of GA$_1$ or GA$_3$ stimulated growth of all three sublines (Table 5). Genotype, GA treatment and the interaction of these two factors were highly significant ($P < 0.001$, ANOVA). The prediction was that GA$_1$ would be less effective than GA$_3$ in stimulating sheath length, but was much more effective in the transformants, as expected. In addition, GA$_1$ was less effective at restoring growth of the dwarf (plant 2), compared with the semi-dwarf (plant 8). Application of GA$_3$ to the transgenic plants restored sheath length almost completely to that of the treated null (Table 5).

| Transformant | Leaf 3 | Leaf 5 |
|-------------|-------|-------|
|             | Lamina | Sheath | Lamina | Sheath |
| 4.10 (null) | 233.6  | 71.8   | 335.2  | 152.7  |
| 4.8 (semi-dwarf) | 177.9  | 41.4   | 277.9  | 103.6  |
| 4.2 (dwarf)  | 158.2  | 36.2   | 242.8  | 88.5   |
| LSD (95%) (33 df) | 12.4 | 7.0 | 18.6 | 9.6 |

Table 3. Lengths (mm) of leaf components of selected T4 Line 4 PcGA2ox1 transformants grown under summer greenhouse conditions

| Transformant | Leaf 1 | Leaf 2 | Leaf 3 |
|-------------|-------|-------|-------|
| 4.10 (null) | 2.67±0.188 | 1.92±0.193 | 1.17±0.112 |
| 4.8 (semi-dwarf) | 3.17±0.122 | 2.73±0.131 | 1.42±0.149 |
| 4.2 (dwarf)  | 3.50±0.261 | 2.92±0.149 | 1.67±0.142 |
| $P$-value$^a$ | 0.024 | <0.001 | 0.05 |

$^a$ $P$-value from $\chi^2$ (2 df) test (Kruskal–Wallis one-way ANOVA).

Table 4. Mean numbers ±SE of tillers in selected T4 Line 4 PcGA2ox1 transformants 6 weeks after sowing under summer greenhouse conditions

| Transformant | Water | GA$_1$ | GA$_3$ |
|-------------|-------|-------|-------|
| 4.10 (null) | 57.4  | 85.9  | 90.0  |
| 4.8 (semi-dwarf) | 38.7  | 72.8  | 83.6  |
| 4.2 (dwarf)  | 32.5  | 60.6  | 82.5  |
| LSD (95%) (8 df) | 3.71  |

Germination capacity of grains of PcGA2ox1 transgenic plants

Germination tests on Line 4 T4 grains hand-threshed two weeks after harvest ripeness showed that the PcGA2ox1 transgene affected both embryo dormancy and seedling vigour. Whereas the null (plant 10) gave 76% germination after three days at 20 °C, the corresponding germination percentages were 40%, 32%, and 8% for plants 8, 16, and 2, respectively. Radicle lengths ranged from c. 20 mm in the null to 1–2 mm just rupturing the pericarp in plant 2. These results were not a consequence of poor embryo viability since all sublines gave 100% germination after five days at 15 °C. After a further three weeks of after-ripening in the warm, grains from all sublines showed 100% germination after three days at 20 °C, although differences in seedling vigour remained evident in material after-ripened up to seven weeks.

$\alpha$-Amylase production in germinated grains of PcGA2ox1 transgenic plants

After-ripened T4 grains were imbibed in the cold for 48 h to remove any residual dormancy and germinated for 72 h at 20 °C. $\alpha$-Amylase activity was determined in extracts of grains following excision of root and shoot axes. In addition, activity was also determined on a unit protein basis in order to account for genotypic differences in grain size. Using a natural log transformation to correct for variance heterogeneity across lines, overall highly significant differences ($P < 0.001$) in $\alpha$-amylase activity on both a per grain and unit protein basis were found (Fig. 4). All lines were significantly different from the wild type and the azygous (null) line, grains of which produced similar amounts of $\alpha$-amylase per grain, whereas activity in the two semi-dwarfs (plants 8 and 16) was decreased by 35% and in the dwarf (plant 2) by 50% (Fig. 4). The more
extreme dwarf, Line 6 plant 21, showed a 65% decrease in activity, compared with the wild-type and null controls. Even in dwarf sublines containing smaller grains (Table 2), genotypic differences in enzyme activity per unit protein remained substantial (Fig. 4).

In the time-course experiment, grains of Line 4 plant 10 (null), Line 4 plant 8 (semi-dwarf), and Line 4 plant 2 (dwarf) were imbibed in the cold for 30 h before transfer to 20 °C. α-Amylase activity was measured after 42, 66, and 90 h at 20 °C. There were highly significant main effects of both genotype and time (P < 0.001) and a significant interaction (P = 0.008) between these two factors. By 42 h, significant (P < 0.05, LSD) decreases of 31% and 48% in α-amylase activity per grain were observed in plants 8 and 2, respectively, compared with the null. The relative differences between genotypes became greater over time up to 90 h (Table 6).

In order to determine if the effect of the transgene could be rescued by GA treatment, grains of the three lines used above were imbibed in water, 1.0 μM GA1 or 1.0 μM GA3 solutions in the cold for 30 h before transfer to the warm and measurement of α-amylase activity after a further 66 h. When analysing the log transformed α-amylase activities, the main effects of genotype and GA treatment of were significant (P < 0.001, ANOVA) and there was a weak genotype by treatment interaction (P = 0.036 ANOVA). As expected, when using the LSD (5%) values for comparisons, there were highly significant decreases in activity in the two transgenic lines incubated in water, compared with the null (Table 7). Neither GA caused a significant (P < 0.05) increase in enzyme activity in the null and GA1 was also ineffective in the transformants. Application of GA3 restored amylase activity in the semi-dwarf (plant 8) to that in the water-treated null line (plant 10) and caused a large increase in the dwarf (plant 2), compared with water or GA1-treated plants (P < 0.05).

Gibberellin content of PcGA2ox1 transgenic plants

Several GAs of the early 13-hydroxylation pathway were quantified in shoot and root axes of T3 grains germinated for 4 d at 20 °C in darkness. Comparisons were made between two independent ‘controls’ (Canon and null, Line 4 plant 10), two independent semi-dwarfs (Line 4 plants 8 and 16), and a single more severe dwarf (Line 4 plant 2). Similar results were obtained from two separate analyses, but data from only one experiment are presented in Fig. 5.

### Table 6. α-Amylase activity in grains of selected T4 Line 4 PcGA2ox1 transformants at different times after imbibition

| Transformants | Log e α-amylase activity (back-transformed means, U grain⁻¹) | 42 h | 66 h | 90 h |
|---------------|------------------------------------------------------------|------|------|------|
| 4.10 (null)   | 2.470 (11.8) 3.531 (34.2) 4.168 (64.6)                   |      |      |      |
| 4.8 (semi-dwarf) | 2.093 (8.1) 2.909 (18.3) 3.449 (31.5)                   |      |      |      |
| 4.2 (dwarf)   | 1.791 (6.0) 2.678 (14.6) 2.867 (17.6)                   |      |      |      |
| LSD (95%) (27 df) | 0.214                                                     |      |      |      |

### Table 7. α-Amylase activity in grains of selected T4 Line 4 PcGA2ox1 transformants after treatment with water, GA1 or GA3 (each at 1.0 μM)

Grains were pre-imbibed at 5–6 °C for 30 h prior to transfer to 20 °C for 66 h. Statistical analysis was on the log-transformed data. The back-transformed means are shown in parenthesis.

| Transformant | Log e α-amylase activity (back-transformed means, U grain⁻¹) | Water | GA1 | GA3 |
|---------------|------------------------------------------------------------|------|-----|-----|
| 4.10 (null)   | 3.661 (38.9) 3.663 (39.0) 3.833 (46.2)                   |      |     |     |
| 4.8 (semi-dwarf) | 3.109 (22.4) 3.285 (26.7) 3.680 (39.7)                   |      |     |     |
| 4.2 (dwarf)   | 2.893 (18.1) 2.982 (19.7) 3.392 (29.7)                   |      |     |     |
| LSD (95%) (27 df) | 0.182                                                     |      |     |     |
because values for GA₃ and GA₈ were not obtained from
the first analysis. Overall, the concentrations of GA₄₄,
GA₁₉, GA₁, GA₃, and GA₈ were substantially reduced in
axes of the transgenic plants compared with the two
control lines (Fig. 5A). The largest differences were for
GA₁₉ and GA₁ concentrations, for which there were >5-
fold decreases in Line 4 plants 8 and 16 and a >6-fold decrease in the dwarf (Line 4
plant 2), compared with the controls (Fig. 5B). These
differences reflected 4.4-fold and 9.2-fold decreases in biologically active GA on a per grain basis, for the semi-
and dwarf plants, respectively.

Expression of two GA biosynthesis dioxygenase genes
was examined in T₃ grains of two independent semi-dwarf
lines and controls (Canon and Line 4 plant 10) that were
germinated for 3 d at 20 °C in darkness. In scutella,
expression of TaGA₃ox2 was higher than TaGA₂₀ox1 and
both were up-regulated in the semi-dwarf lines, compared
with controls (Fig. 6A). Interestingly, there was an
apparent decrease in abundance of transcripts for both
α-amylase gene families in scutella of the semi-dwarfs,
compared with controls.

In a further experiment, T₄ grains of the null (plant 10)
and semi-dwarf (plant 8) were preincubated in the cold for
30 h before transfer to 20 °C. Decreases in abundance of
35–40% for α-Amy 1 and 60–70% for α-Amy 2 transcripts
were observed in aleurone of the semi-dwarf, compared
with the null, after 42 h and 66 h in the warm (Fig. 6B).
There was also a slight decrease in α-Amy 1 transcripts in
scutella (data not shown). No transcripts for either of the
wheat GA biosynthesis genes (TaGA₂₀ox1 and TaGA₃ox2)
were detected in aleurone under these conditions. The
PcGA₂₀ox1 transgene was expressed in both scutellum and
aleurone of the semi-dwarf (plant 8) but, as expected, was
undetected in the null (plant 10) (data not shown).

Discussion
Genetic dissection of the GA biosynthesis (reviewed in
Sakamoto et al., 2004) and signal transduction pathways
(reviewed in Hartweck and Olszewski, 2006) has provided
compelling evidence for the involvement of GAs in the
regulation of plant stature in both Arabidopsis and diploid
cereals such as rice and barley. As might be predicted,
extopic expression of PcGA₂₀ox1 in wheat produced a
range of dark-green, dwarf shoot phenotypes (Fig. 2A)
similar to those in which OsGA₂₀ox1 was over-expressed
in transgenic rice (Sakamoto et al., 2001). In the present
wheat transformants it is difficult to reconcile why a range
of shoot phenotypes arose from a single transformation
event and has remained stable over four generations.
Southern blot analysis showed that the Line 4 trans-
formants contained the same number of copies of the
insert (Fig. 1A) and qRT-PCR clearly indicated different levels of expression of the transgene (Fig. 1B, C). We are not aware of previous reports of different degrees of transgene expression being stably inherited from a single transformation event, but assume it is due to differential transcriptional silencing in the T1 generation, as has been described during generation of a newly synthesized wheat allotetraploid (Kashkush et al., 2002). Furthermore, the degree of silencing appears to be tissue-specific since, although there was a clear correlation between transgene expression in ears, peduncles, and peduncle nodes with the severity of the mature plant phenotype in Line 4 sub-lines 2 and 8 (Figs 1C, 2A), expression in young leaves of these plants did not reflect the final phenotype (Fig. 1B). Tissue-specific transgene silencing has been noted previously (Kloti et al., 2002).

At the whole plant level, a similar severe dwarf shoot phenotype was also produced when wild-type wheat was treated with the GA biosynthesis inhibitor, paclobutrazol (Fig. 2B; Table 1). The decreased shoot stature of the present wheat transformants was relatively severe, ranging from 40–60% that of the null (Fig. 3), similar to that of the more extreme ‘GA-insensitive’ Rht dwarfs, Rht-B1c and Rht-D1b+Rht-B1c (Youssefian et al., 1992b; Flintham et al., 1997). By comparison, shoot height is decreased by only 15–20% when the commercially successful semi-dwarfing Rht-B1b or Rht-D1b alleles are introduced into tall wheat.

During the vegetative stage of development, leaf growth of the wheat transformants was decreased with a greater effect being on the sheath than on the lamina (Fig. 3; Table 3). Growth of the first leaf sheath of the transformants was restored by application of GA3, which is not a substrate for PcGA2ox1. However, growth was restored only partially by application of GA1 and to a lesser extent in the more dwarf line (plant 2), whereas both GAs stimulated growth of the null (Table 5). Although not measured directly, the extent of deactivation of applied (and endogenous) GA1 appeared to reflect the degree of shoot dwarfism (Fig. 3; Table 5). Similar results were observed when seedlings of transgenic rice expressing OsGA2ox1 cDNA were treated with the same GAs (Sakamoto et al., 2001).

Another feature of the present wheat transformants, when grown widely-spaced, was a prostrate growth habit with loss of apical dominance and increased outgrowth of lateral shoots (tillers) (Fig. 2A). This shoot branching pattern was replicated by application of paclobutrazol to wild-type plants (Fig. 2B) and is also characteristic of several ‘GA-insensitive’ Rht wheat varieties (Gale and Marshall,
slender barley mutant, stimulation. In contrast, the constitutive GA-signalling, and showed much reduced pulvinar bending upon gravistimulation. In Arabidopsis following ectopic

remains to be established. The production of one extra leaf at the shoot apex following GA depletion was consistent with previous observations on early applications of growth retardants to wheat during the vegetative growth stage of growth (Hutley-Bull and Schwabe, 1982). In the present experiments with plants growing under long-day conditions, no difference in the timing of floral transition was detected between the wheat transformants and the null line. Increased allocation of resources to support growth of the additional leaf and tillers, rather than the young developing ear, might explain the observed initial delay in floral development, which recovered later such that timing to anthesis was only delayed by a few days. More frequent sampling of material grown under different daylengths is required to clarify the precise role of GA in determining the rate of both leaf and spikelet primordium initiation at the shoot apex, as well as the timing of the transition from a vegetative to a reproductive state. It is, however, interesting to note that GAs have been implicated previously in the timing of vegetative phase change and reproductive maturity in maize (Evans and Poethig, 1995).

One unexpected aspect of the shoot phenotype of both the present wheat transformants and a wild type treated with paclobutrazol was a decrease in final ear size (Fig. 3; Table 1), a response not observed even in wheat lines containing the more severe ‘GA-insensitive’ *Rht-B1c* allele (Youssefian et al., 1992b; Webb et al., 1998). In the present experiments, induction of GA deficiency, either genetically or chemically, clearly indicated some degree of GA-dependency for normal ear growth. It is not immediately clear, therefore, why introduction of so-called ‘GA-insensitivity’ *Rht* dwarving alleles into wheat does not also decrease final ear size. If the growth inhibiting N-terminal deleted DELLA proteins are expressed in developing ears of *Rht* lines then factor(s) other than GA (possibly auxin) must be able to promote their degradation in order to produce an ear phenotype similar to that of a wild type. Alternatively, changes in the pattern of cell division during rachis development in the ear may involve a GA signalling pathway independent of DELLA proteins whose primary function may be to restrict cell expansion in the extension zone of vegetative tissues. Whatever the case, further work is required to elucidate the hormonal signalling pathways regulating ear development in commercially important *Rht* wheat lines.

Another subtle difference between the present wheat transformants and semi-dwarf wheats was the degree of grain set within an ear. Although grain numbers per ear were similar in the null and the two semi-dwarf wheat transformants (plants 8 and 16) they were significantly decreased in the more extreme dwarfs (Table 2). Decreased grain set of the more extreme dwarf wheat transformants may have been a consequence of more severe GA deficiency restricting pollen tube growth and resulting in male sterility, as occurs in *Arabidopsis* following ectopic
expression of a pea (Pisum sativum L.) GA 2-oxidase (PsGA2ox2) (Singh et al., 2002). By contrast, increases in grain number per ear accounted for higher yields of field-grown wheat containing different Rht dwarfing wheat (Flintham et al., 1997). In this instance, increased resource allocation to developing ears supported greater floret survival at anthesis and hence higher grain numbers per ear in ‘GA-insensitive’ Rht wheat (Youssefian et al., 1992b).

Decreased grain size of main stem ears (Table 2) was associated with the reduction in shoot height of the wheat transformants (Fig. 3) and may have been a consequence of an overall decrease in total dry matter production. However, it is also possible that GA depletion has a more direct effect on grain development. For example, an analysis of the lh-2 mutant of pea showed that GA produced during early seed development was required for normal seed growth and survival (Swain et al., 1997). Moreover, ectopic expression of PsGA2ox2 in Arabidopsis also caused abortion of developing seeds, similar to that observed in the GA-deficient pea mutant (Singh et al., 2002). Besides affecting grain size, preliminary results with the present wheat transformants showed that GA-depletion also resulted in increased post-harvest embryo dormancy. In addition, post-germination seedling vigour was associated with decreased mobilization of endosperm reserves (Fig. 4; Table 6). Induction of GA deficiency, either genetically or chemically, in developing kernels of an ABA-deficient maize mutant prevented premature embryo germination on the cob (vivipary) (White et al., 2000). This suggests that the balance between ABA and GA production and signalling is important for the maintenance and loss of embryo dormancy in cereals. Further evidence for a direct involvement of GAs in grain development also comes from the observation that pre-maturity α-amylase production and grain shrivelling were associated with increased GA content in a barley mutant (Green et al., 1997).

Direct evidence for decreased content of active GA in the present wheat transformants came from quantitative GC-MS analysis of GAs in etiolated embryonic axes and partially digested endosperm of germinated grains that were shown previously to be a rich source of GAs (Lenton et al., 1994). In the present work, there was a >5-fold decrease in GA1 concentration in both these tissues for two independent semi-dwarf lines, compared with a null and wild type (controls), and a much greater decrease in a more severe dwarf line, confirming that the PcGA2ox1 transgene was functional in planta (Fig. 5A, B). The decrease in GA1 content in the transgenic lines was associated with a decrease in GA19 (Fig. 5), particularly in axes, consistent with up-regulation of TaGA20ox1 expression due to relief of feedback repression, as was observed in scutella (Fig. 6A). The abundance of GA3 and GA29 were, like GA1, reduced in the transgenic plants (Fig. 6), suggesting that a major consequence of the enhanced 2-oxidase activity may be increased turnover of their common precursor GA20, thereby reducing its availability for 3-oxidation to GA1 and GA3. However, GA29, the product formed from GA20 by 2β-hydroxylation, did not accumulate in the transgenic plants as much as might be expected, perhaps due to its further oxidation to GA29-catabolite (Thomas et al., 1999), for which no internal standard was available. Clearly, metabolic studies with labelled precursors are required to explore these possibilities. Ectopic expression of OsGA2ox1 cDNA in transgenic rice, as well as causing a 4-fold decrease in GA1 concentration in light-grown leaves, resulted in 2.5-fold and 6.7-fold increases in the concentration of GA8 and GA29, respectively, compared with wild type (Sakamoto et al., 2001).

In grains of the controls, GA19 and GA20 were much less abundant than GA1 (Fig. 5B), probably reflecting the high level of expression of TaGA3ox2 and, to a lesser extent, TaGA20ox1 in scutella (Fig. 6A; Appleford et al., 2006). Previous evidence suggested that the scutellum of wheat was a major site of de novo GA biosynthesis, based on the accumulation of ent-kaurene in grains germinated in the presence of an ent-kauren oxidase inhibitor, paclotubrazol (Lenten et al., 1994; Appleford and Lenten, 1997). More recently, using GA promoter–reporter constructs, expression of two GA 20-oxidases and two GA 3-oxidases was shown to be confined to the epithelial layer of the scutellum of germinated rice (Kaneko et al., 2003). In future, it will be interesting to determine if expression of all genes of the GA-biosynthetic pathway is confined to the scutellar epithelium and if this is also the case in temperate cereals, such as wheat and barley.

In the two semi-dwarf wheat lines, expression of TaGA20ox1 and TaGA3ox2 increased in scutella on day 3 (Fig. 6A) and was associated with a 4-fold decrease in active GAs in grains on day 4 (Fig. 5B). A similar up-regulation of expression of these GA biosynthesis genes was also observed in scutella of wild type incubated in the presence of paclotubrazol, compared with untreated plants (NEJ Appleford and JR Lenton, unpublished results). One interpretation of these results is that the decreased concentration of active GAs relieved, in part, a homeostatic mechanism whereby repression of these two GA biosynthesis genes normally occurs as a consequence of GA action (reviewed in Hedden and Phillips, 2000). An alternative, but not exclusive, possibility is that decreased carbohydrate availability in scutella may partially relieve a postulated negative feedback loop imposed by sugars on GA biosynthesis (Yu et al., 1996; Perata et al., 1997).

The decreased content of active GAs in grains of the transformants, compared with controls, (Fig. 5B) was associated with a decline in abundance of transcripts for both α-amylase gene families in aleurone (Fig. 6B) and in grain α-amylase activity (Fig. 4; Table 6). Previously, a decrease in GA content of endosperm was associated with a decline in abundance of transcripts for both the high- and low-pI
α-amylase gene families in aleurone of wheat grains imbibed in the presence of paclobutrazol (Lenton et al., 1994). Application of GA₃ largely reversed the decline in α-amylase production (Table 7). The reduced effectiveness of GA₁ was assumed to reflect increased catabolism as a consequence of the activation of the transgene. Taken together, these results provide strong evidence for the GA-dependency of α-amylase production in aleurone, the main source of the enzyme, in intact germinating wheat grains. Previous work with a GA-deficient barley mutant established the dependency of α-amylase gene expression in aleurone on embryo-produced GA (Chandler and Mosleth, 1990; Zwar and Chandler, 1995).

The decreased GA content of grains of the semi-dwarf wheat lines on day 4 (Fig. 5B) was also associated with a decline in abundance of transcripts for both α-amylase gene families in scutella on day 3 (Fig. 6A). This observation, like that for the high pI gene family in barley (Chandler and Mosleth, 1990), would also suggest that scutellum-produced α-amylase was GA-dependent. However, an alternative interpretation of these observations is also possible. For example, there is increasing evidence that sugar signalling can also cause repression of α-amylase gene expression in rice and barley embryos (Yu et al., 1996; Perata et al., 1997; Loreti et al., 2000). It is conceivable, therefore, that decreased mobilization of endosperm reserves may limit availability of sugars thus relieving repression of α-amylase gene expression in scutella of the wheat transformants (Fig. 6A). In fact, the present range of PcGA2oxI transgenic wheat provides useful material in which to study regulation of expression of both GA biosynthesis and α-amylase genes in relation to changes in GA and carbohydrate status in wheat scutellar tissues post germination.

In future, a more targeted approach is required to perturb GA production in specific tissues of stem internodes and/or developing grains, in order to manipulate processes that result in increased grain yield and quality. Support for such an approach comes from the generation of dwarf rice with normal grain production by restricting increased GA turnover to vegetative tissues (Sakamoto et al., 2003).

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