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RESEARCH PAPER

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 T_1 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 (GA_3). 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 *RhtD1b* 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 1 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*., 1992*b*). 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*., 1992*a*), 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 *OsGA20ox2* 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 Lenton, 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 Lenton, 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 *RAmy1A* 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, GA_1 (to GA_8) and its precursor, GA_{20} (to GA_{29}), 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 *trangenic 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

3' untranslated sequence and polyadenylation signals of the nopaline synthase gene (*nos*) from the Ti plasmid from *Agrobacterium tumefaciens*. Plasmid CalNEO contained the neomycin phosphotransferase (*neo*) marker gene (conferring resistance to geneticin) under transcriptional control of the Cauliflower Mosaic Virus (CaMV) 35S promoter and the *nos* terminator. Scutella of immature embryos (14–16 d post anthesis) of spring wheat (cv. Canon) were co-transformed with plasmids pLAP2ox2 and pCalNEO using a particle delivery system (Sparks and Jones, 2004). Following induction of embryogenic calli, two rounds of selection in the presence of geneticin sulphate and rooting, putative primary transformants (T_0 generation) were transplanted into pots containing soil and grown to maturity in a greenhouse.

Grains from selfed ears (T_1 generation) from each of the six independent primary transformants were imbibed in the cold (5–6 °C) for 6 d before planting out in seed compost in modules. Seedlings at the 2-leaf stage were transferred to pots (100 mm diam, 1.0 l volume) containing loam-based compost with slow-release fertilizer and spaced widely in a greenhouse. All plants of Line 1, and some of Line 4, were prostrate dwarfs with dark-green leaves and produced many tillers. Line 1 plants 1–10 were treated with 2×10 µl (20 µg) GA_3 solution to the base of the rosette of shoots on six occasions to stimulate stem elongation, ear emergence, and grain set. Line 4 plants 2, 3, 6, 7, and 16 were also treated similarly but only on four occasions. Young leaf material was harvested directly into liquid N_2 and stored at –80 °C prior to DNA extraction. T_2 grains from selfed mainstem ears of T_1 plants that tested PCR positive for the *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 T_3 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′-TGTTCTTCACTGCTGTAATG-3′. Reactions, using the Reddymix™ 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 *Ssp*1, *Bam*HI or *Eco*RI. 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 (Biobond™ 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 5min 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 T_3 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 N_2 prior to RNA isolation and northern hybridization using methods described in Appleford *et al*. (2006). Briefly, poly(A)$^+$ RNA was extracted and purified on oligi-dT cellulose, size-fractionated by electrophoresis on formaldehyde-agarose gels, and transferred to nitrocellulose. Probes for hybridization to *TaGA20ox1* and *TaGA3ox2* were labelled with ^{32}P-dCTP using random primers and the full-length cDNAs as template whereas probes for α-*Amy 1* (high pI), α-*Amy 2* (low pI) used specific cDNA clones for the two α-amylase gene families (Lazarus *et al*., 1985). In a further experiment, T_4 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 N_2 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 T_4 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′-TAGCAAGAGGATTGGCC-CAAAC, antisense, 5′-GGTGTTGAGGA-GGAGGTATTCG; Actin: sense 5′-AGGCATCCTGACGCTCAAGTA, antisense 5′-GCTCGTTGTA-GAAGGTGTGGTG.

Growth conditions and phenotypic analysis of PcGA2ox1 *transgenic wheat*

T_3 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.

T_4 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 was 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*

T_4 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 GA_1 or 10 µM GA_3 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 2*S*,3*S*-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 T_4 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 GA_1 or 1.0 μM GA_3, as above, and α-amylase activity determined after 66 h at 20 °C.

Quantification of GAs in germinated grains

Grains of cv. Canon and T_3 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 N_2 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 [^{3}H]- and [^{2}H]-labelled GA standards added. Ethyl acetate-soluble acids, obtained from an aqueous phase, were purified by QAE Sephadex anion-exchange and C_{18} Sep-Pak cartridge chromatography prior to resolution by C_{18} reverse-phase HPLC. Grouped fractions, based on the retention times of the [^{3}H]GA standards, were methylated and the ethyl acetate-soluble neutrals passed through a NH_2 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 [^{2}H] 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 T_1 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 GA_3 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 T_1 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 T_1 PCR-positive plants of Lines 2 and 3 at the T_2 seedling stage. T_1 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 GA_3 to ensure ear emergence from the flag leaf and adequate grain set. Six putative homozygous sublines were recovered from Line 4 at the T_2 seedling stage. From the Line 5 primary transformant, 27 T_1 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 T_2 seedling stage. From the Line 6 primary transformant,

24 T_1 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 T_2 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 *Ssp*1-digested DNA extracted from homozygous T_3 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 T_1 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 T_2 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

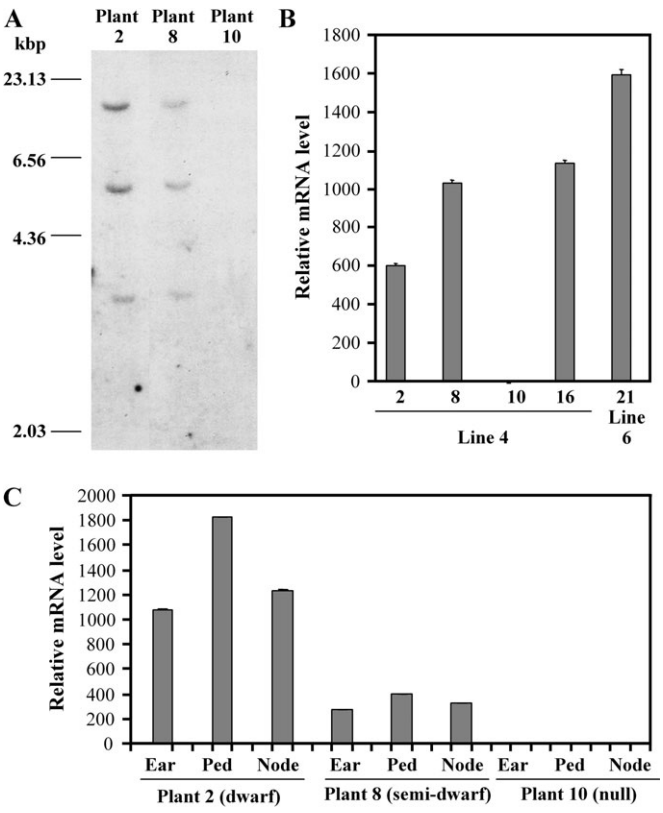

Fig. 1. Molecular analysis of *PcGA2ox1* transformants. Southern blot of *Ssp*I-digested DNA from selected T_3 *PcGA2ox1* transformants, Line 4 plants 2 (dwarf), 8 (semi-dwarf), and 10 (null) (A). Quantitative real-time RT-PCR analysis of relative *PcGA2ox1* transcript levels in: seedlings of Line 4 plants 2, 8, 10, and 16, and Line 6, plant 21 (B) and in ears, peduncles (Ped) and peduncle nodes of 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.

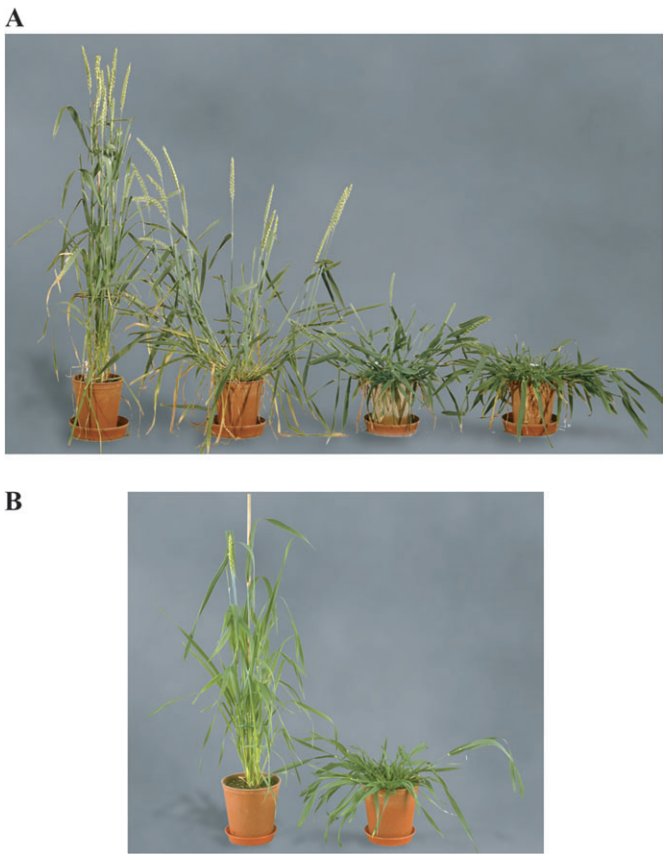

Fig. 2. Phenotypic variation in shoot architecture of widely-spaced T_2 plants of the original Line 4 *PcGA2ox1* transformant grown under spring greenhouse conditions with supplementary illumination. Plants from the left are 10 (null), 8 (semi-dwarf), 2 (dwarf), and 3 (severe dwarf) (A). Shoot phenotypes 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 orthogravitropic 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, 2*S*,3*S*-paclobutrazol, 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 paclobutrazol-treated plants. The cumulative effect of increasing paclobutrazol 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 T_3 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 transformants also produced one extra main stem leaf, compared with the null, under these growing conditions.

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

T-tests on log transformed values showed highly significant differences (P <0.001) between control and paclobutrazol-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

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 T_4 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

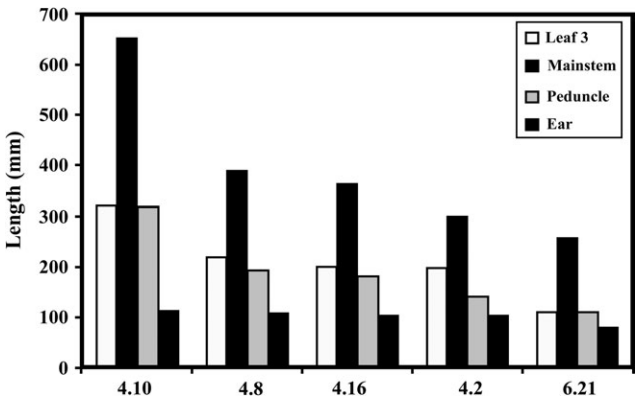

Fig. 3 Final lengths (mm) of leaf 3, main stem and ear of selected T_3 *PcGA2ox1* transformants from Lines 4 and 6 grown in a controlled environment simulating average UK August growing conditions. (LSDs for comparison of lines within tissue, at the 5% significance level, are 7.6 mm for leaf 3, 12.9 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 T_3* 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 tranformants 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 at restoring leaf growth of the transformants because of increased metabolism to an inactive GA, GA_8, whereas GA_3 is not a substrate for PcGA2ox1 (SG Thomas, AL Phillips, P Hedden, unpublished data). In the null line (plant 10), GA_3 was slightly more effective than GA_1 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).

Table 3. *Lengths (mm) of leaf components of selected T_4 Line 4* PcGA2ox1 *transformants grown under summer greenhouse conditions*

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 4. *Mean numbers ±SE of tillers in selected T_4 Line 4* PcGA2ox1 *transformants 6 weeks after sowing 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	2.75±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 χ^2 (2 df) test (Kruskal–Wallis one-way ANOVA).

Germination capacity of grains of PcGA2ox1 *transgenic plants*

Germination tests on Line 4 T_4 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.

α-Amylase production in germinated grains of PcGA2ox1 *transgenic plants*

After-ripened T_4 grains were imbibed in the cold for 48 h to remove any residual dormancy and germinated for 72 h at 20 °C. α-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 α-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 α-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

Table 5. *Length (mm) of the sheath of leaf 1 of selected T_4 Line 4* PcGA2ox1 *transformants after treatment with water, GA_1 or GA_3 (each 10 μM)*

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		

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 GA_1 or 1.0 μM GA_3 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 GA_1 was also ineffective in the transformants. Application of GA_3 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 GA_1-treated plants (P <0.05).

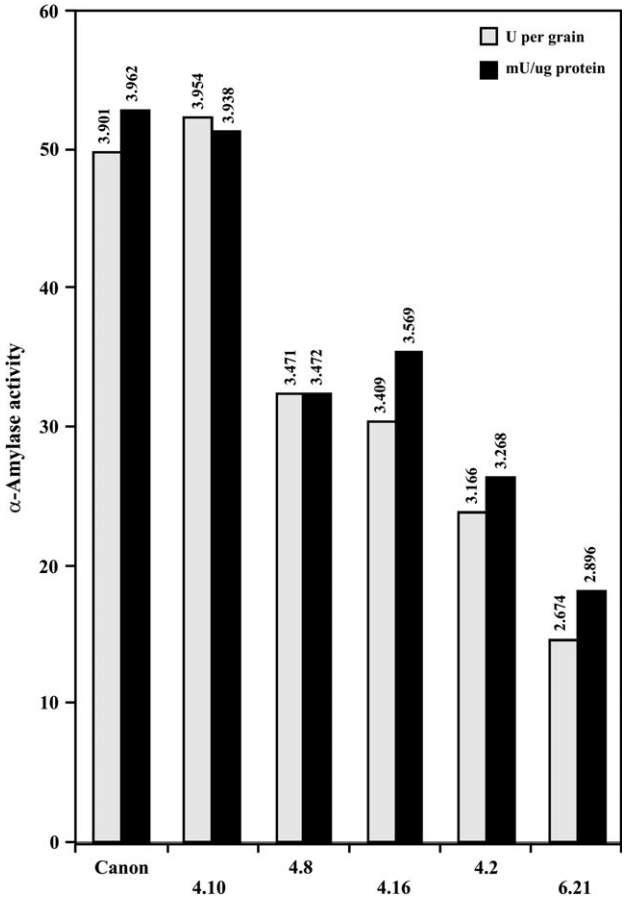

Fig. 4. α-Amylase activity in grains of wild-type cv. Canon and selected T_4 *PcGA2ox1* transformants after incubation at 5–6 °C for 48 h before transfer to 20 °C for 72 h. (Numbers above each column are the mean log-transformed values. LSDs for comparisons at the 5% significance level are 0.115 for Units per grain and 0.140 for mU per μg protein.)

Gibberellin content of PcGA2ox1 *transgenic plants*

Several GAs of the early 13-hydroxylation pathway were quantified in shoot and root axes of T_3 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* T_4 *Line 4* PcGA2ox1 *transformants at different times after imbibition*

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

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* T_4 *Line 4* PcGA2ox1 *transformants after treatment with water,* GA_1 *or* GA_3 *(each at 1.0* μ*M)*

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	GA_1	GA_3
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		

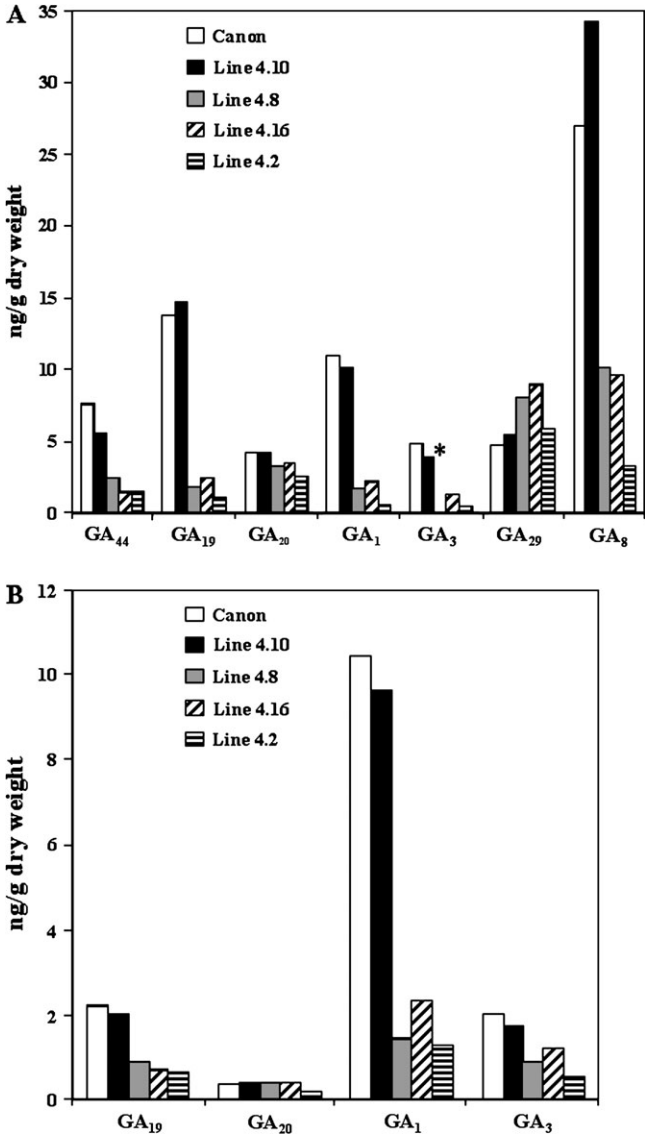

Fig. 5. Gibberellin concentration (ng g^{-1} dry mass) in shoot plus root axes (A) and grains (scutellum plus endosperm) (B) of wild-type cv. Canon and selected T_3 Line 4 *PcGA2ox1* transformants grown at 20 °C for 4 d in darkness. (Asterisk) Not determined due to interference.

because values for GA_3 and GA_8 were not obtained from the first analysis. Overall, the concentrations of GA_{44}, GA_{19}, GA_1, GA_3, and GA_8 were substantially reduced in axes of the transgenic plants compared with the two control lines (Fig. 5A). The largest differences were for GA_{19} and GA_1 concentrations, for which there were >5-fold decreases in Line 4 plants 8 and 16 (semi-dwarfs) and approximately 15-fold decreases in the same GAs in Line 4 plant 2 (dwarf). As a by-product of GA 3-oxidase activity, GA_3 contents reflected those of GA_1 but genotypic differences were much less as this GA is not a substrate for PcGA2ox1 activity. In addition, there was a 1.7-fold increase in the concentration of GA_{29} in the semi-dwarfs, compared with controls, but only a modest decrease in its immediate precursor, GA_{20}.

Quantification of several GAs in the scutellum plus endosperm of germinated grains of the transgenic plants was problematic due to the large amount of impurities present. Of those GAs that could be quantified reliably, there was an overall 4-fold decrease in the combined GA_1 and GA_3 concentration in the semi-dwarf (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 GA dioxygenase and α-amylase genes in germinated grains

Expression of two GA biosynthesis dioxygenase genes was examined in T_3 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, T_4 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 *PcGA2ox1* 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, ectopic expression of *PcGA2ox1* in wheat produced a range of dark-green, dwarf shoot phenotypes (Fig. 2A) similar to those in which *OsGA2ox1* 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 transformants contained the same number of copies of the

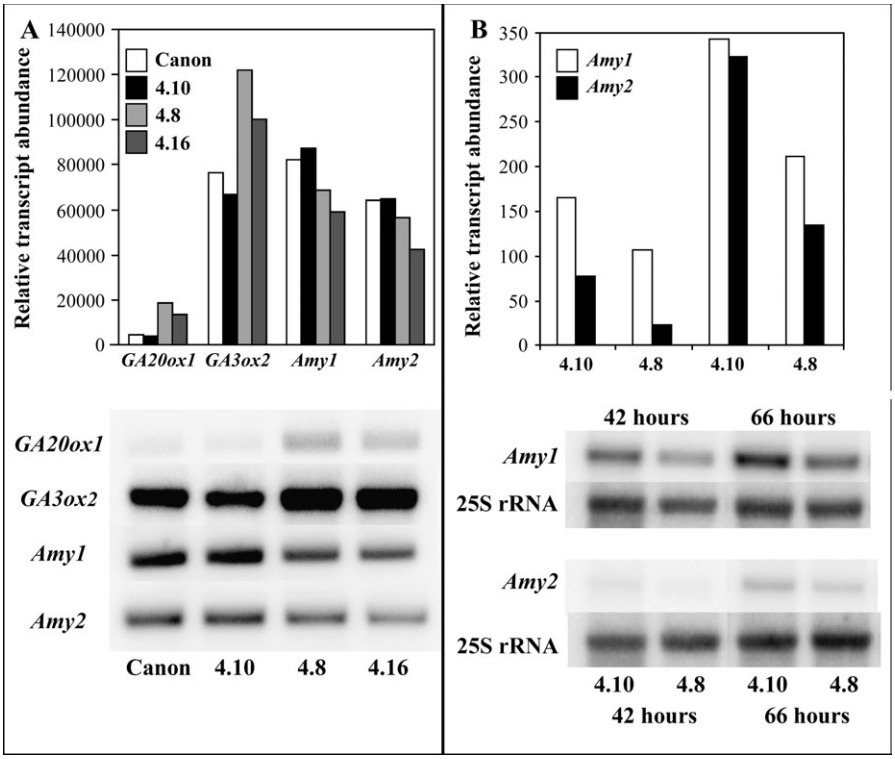

Fig. 6. Expression of GA biosynthesis and α-amylase genes in scutella of wild-type cv. Canon and selected Line 4 *PcGA2ox1* transformants germinated at 20 °C for 3 d. Relative transcript abundance was determined from northern blots of equal loadings of poly(A)$^+$ RNA and the signal detected and quantified using a phosphoimager (A). Expression of α-*Amy1* (high pI) and α-*Amy2* (low pI) α-amylase gene families in aleurone layers from T_4 grains of Line 4 *PcGA2ox1* transformants plants 10 (null) and 8 (semi-dwarf) pre-incubated at 5–6 °C for 30 h prior to transfer to 20 °C for 42 h and 66 h. Relative transcript abundance was determined from northern blots of total RNA (3 μg per lane). Blots were stripped and reprobed with a 25S rRNA probe and signals quantified using a phosphoimager.

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 T_1 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 sublines 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*., 1992*b*; 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 GA_3, which is not a substrate for PcGA2ox1. However, growth was restored only partially by application of GA_1 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) GA_1 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,

1973). Recent genetic evidence from rice showed that shoot dwarfism may arise from excessive tillering and that transport of apically-produced auxin is required to up-regulate a gene involved in the production of a carotenoid cleavage signal that normally suppresses outgrowth of lateral shoots (Zou *et al*., 2006). Outgrowth of tiller buds in wheat and barley is also influenced strongly by environmental factors such as light quality and nutrient status. Decreasing the far-red component of light in widely spaced plants resulted in increased tiller production (less apical dominance) in wheat (Barnes and Bugbee, 1991). Conversely, increased far-red light signalling at the base of a canopy decreased tiller numbers in densely spaced barley plants (Skinner and Simmons, 1993). Similarly, in the present experiments, a more upright growth habit with less tillering (more apical dominance) was observed when the wheat transformants were densely spaced, which relates to a normal shade avoidance response to increased far-red light reflected down the canopy (Table 4). Taken together, these results provide supporting evidence that besides a primary effect on shoot elongation, GAs are also involved, either directly or indirectly, in apical dominance of wheat shoots. Since auxin, cytokinin, and other hormonal signals are also known to affect axillary bud outgrowth, tillering in wheat is likely to be controlled, in part, by a complex interaction between hormone and phytochrome signalling pathways.

Auxin transport and signalling at the base of the leaf sheath (pulvinus) are known to be important components of the natural orthogravitropic growth response of cereal shoots when displaced from the vertical (Dayanandan *et al*., 1976). Wolbang *et al.* (2007) found a significant increase in IAA concentration in the lower half of barley p-node pulvini within 2.5 h of gravistimulation, whereas an increase in GA_1 content occurred later when bending was well underway. In addition, the GA_1-deficient, *grd2c* barley mutant was a prostrate dwarf, phenotypically similar to the present *PcGA2ox1* transgenic wheat (Fig. 2A) and wild type treated with a growth retardant (Fig. 2B), and showed much reduced pulvinar bending upon gravistimulation. In contrast, the constitutive GA-signalling, slender barley mutant, *sln1c*, lacking a functional DELLA protein showed greater pulvinar bending following gravitropic stimulation. Taken together, these results suggest that bending of the pulvinus following gravistimulation is driven primarily by an increase in auxin concentration on the lower side of the pulvinus, but that GA is also required for this response. It was assumed that differences in the set-point angle of the tillers in the present *PcGA2ox1* transgenic wheat (Fig. 2A) reflects the decreased GA_1 content of the different lines (Fig. 5A), as was the case for the *grd2b* and *grd2c* barley mutants (Wolbang *et al*., 2007).

Whilst a delay in ear emergence was evident in the present wheat transformants (Fig. 2A), and wild type treated with paclobutrazol (Fig. 2B), the precise cause(s) 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*., 1992*b*; 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* dwarfing 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 alleles (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*., 1992*b*).

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 GA_1 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 GA_1 content in the transgenic lines was associated with a decrease in GA_{19} (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 GA_3 and GA_8 were, like GA_1, 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 GA_{20}, thereby reducing its availability for 3-oxidation to GA_1 and GA_3. However, GA_{29}, the product formed from GA_{20} by 2β-hydroxylation, did not accumulate in the transgenic plants as much as might be expected, perhaps due to its further oxidation to GA_{29}-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 GA_1 concentration in light-grown leaves, resulted in 2.5-fold and 6.7-fold increases in the concentration of GA_8 and GA_{29}, respectively, compared with wild type (Sakamoto *et al*., 2001).

In grains of the controls, GA_{19} and GA_{20} were much less abundant than GA_1 (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*-kaurene oxidase inhibitor, paclobutrazol (Lenton *et al*., 1994; Appleford and Lenton, 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 paclobutrazol, 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 GA_3 largely reversed the decline in α-amylase production (Table 7). The reduced effectiveness of GA_1 was assumed to reflect increased catabolism as a consequence of the activity 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 *PcGA2ox1* 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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