Expression of fission yeast *cdc25* driven by the wheat ADP-glucose pyrophosphorylase large subunit promoter reduces pollen viability and prevents transmission of the transgene in wheat

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Summary

• Cell number was to be measured in wheat (*Triticum aestivum*) endosperm expressing *Spcdc25* (a fission yeast cell-cycle regulator) controlled by a supposedly endosperm-specific promoter, AGP2 (from the large subunit of ADP glucose pyrophosphorylase).

• Wheat was transformed by biolistics either with *AGP2::GUS* or *AGP2::Spcdc25*. PCR and RT–PCR checked integration and expression of the transgene, respectively.

• In cv. Chinese Spring, *AGP2::GUS* was unexpectedly expressed in carpels and pollen, as well as endosperm. In cv. Cadenza, three *AGP2::Spcdc25* plants, AGP2::*Spcdc25*.1, .2 and .3, were generated. *Spcdc25* expression was detected in mature leaves of AGP2::*Spcdc25*.1/.3 which exhibited abnormal spikes, 50% pollen viability and low seed set per plant; both were small compared with the nonexpressing and normal AGP2::*Spcdc25*.2. *Spcdc25* was not transmitted to the T_1 in AGP2::*Spcdc25*.1 or .3, which developed normally. *Spcdc25* was PCR-positive in AGP2::*Spcdc25*.2, using primers for a central portion, but not with primers for the 5′ end, of the ORF, indicating a rearrangement; *Spcdc25* was not expressed in either T_0 or T_1.

• The AGP2 promoter is not tissue-specific and *Spcdc25* expression disrupted reproduction.

Key words: ADP-glucose pyrophosphoryase, endosperm, fission yeast, *Spcdc25*, transgenic wheat (*Triticum aestivum*).

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Introduction

The fission yeast cell-cycle gene, *Spcdc25*, encodes a protein phosphatase (Russell & Nurse, 1986) that is the final all-or-nothing positive regulator of mitosis (O'Farrell, 2001). Homologues exist in all classes of organisms except plants, although a gene that encodes a protein homologous to the catalytic domain of *cdc25* has recently been identified (Landrieu *et al.*, 2004; Sorrell *et al.*, 2005). Overexpression (oe) of *Spcdc25* results in a short cell-length phenotype in both fission yeast and tobacco (Russell & Nurse, 1986; Bell *et al.*, 1993; McKibbin *et al.*, 1998). Spcdc25's substrate is a cyclin-dependent protein kinase (Cdc2) that drives the cell into mitosis (Nurse, 1990). For this to occur, Cdc2 must be dephosphorylated by Spcdc25 (Gould & Nurse, 1989); this yeast enzyme can also dephosphorylate plant cyclin-dependent kinase (CDK) (Zhang *et al.*, 1996). For most of interphase the CDK is phosphorylated by another protein kinase, Wee1 (McGowan & Russell, 1995), another regulatory enzyme that competes with Spcdc25 for the CDK substrate. Sp*wee1*^{oe} in fission yeast results in a long-celled phenotype; the exact converse of the phenotype observed with *Spcdc25*^{oe} (Russell & Nurse, 1986). Hence two key regulators of the transition from G2 to M-phase regulate cell size at division, although *wee1* is regarded as the main genetic element in this control (Sveiczer *et al.*, 1996).

In the absence of a full-length plant homologue to *cdc25*, we have expressed *Spcdc25* in tobacco plants and tobacco cell cultures; in each case a characteristic small mitotic cell size resulted (Bell *et al.*, 1993; McKibbin *et al.*, 1998). Further evidence suggesting that *Spcdc25* interacts with the plant cell cycle was the discovery that it binds with an *Arabidopsis* 14-3-3 protein that, in turn, can rescue cell-cycle checkpoint defects in fission yeast (Sorrell *et al.*, 2003).

In addition to cell-cycle effects, *Spcdc25* expression in plants leads to alterations in development. Constitutive expression of *Spcdc25* was accompanied by precocious flowering and twisted leaf lamina, while inducible expression in root cultures was followed by an increase in the number of laterals forming per unit length of primary tissue (McKibbin *et al.*, 1998). This phenotypic effect has also been observed with constitutive expression of *Spcdc25* in *Arabidopsis* (S. Li and coworkers, unpublished data). More recently, constitutive expression of *Spcdc25* in tobacco internode explants induced vegetative buds to form in culture in the absence of an exogenous supply of cytokinin (Suchomelova *et al.*, 2003).

As either *Spcdc25* or cytokinin treatment can dephosphorylate plant CDK (Zhang *et al.*, 1996), we begin to see a picture emerging of a link between *Spcdc25*-mediated changes to the cell cycle and development in dicots.

Cell cycles alter dramatically in the cereal endosperm where a synchronous wave of nuclear divisions is followed by a limited number of cellular divisions that, in wheat, generates >125 K cells in a 4–5 d period (Bennett *et al.*, 1975; Gao *et al.*, 1992). Thus our aim was to manipulate these cell cycles in wheat with *Spcdc25*, using a promoter that was strongly expressed in wheat endosperm, and examine how final cell number might be affected. To achieve this we placed *Spcdc25* under the control of the promoter of the large subunit of ADP-glucose pyrophosphorylase (AGP2), known to be highly expressed in wheat endosperm (Thorneycroft *et al.*, 2003). Here we report the unexpected results that this promoter is also expressed strongly in other reproductive tissues and in pollen. Consistent with this finding was that, whenever AGP2::*Spcdc25* was strongly expressed, perturbations in development were observed: malformed spikes and unexpectedly nonviable pollen, which prevented *Spcdc25* transmission to progeny.

Materials and Methods

Plant material

Allohexaploid bread wheat (*Triticum aestivum* L., var. Cadenza ($2n = 6x = 42$) was selected as the recipient for transformation (breeder, CPB Twyford UK). This variety was on the UK NIAB recommended list for commercial planting from 1993 to 1998, is normally winter-sown (although it has virtually no vernalization requirement) and is a hard, group 2 bread-making wheat. Donor plants were grown as five plants per 20 cm diameter pot filled with soil (prepared by Petersfield Products, Leicestershire, UK) with a composition of 75% L&P fine-grade peat, 12% screened sterilized loam, 10% 6 mm screened, lime-free grit and 3% medium-grade vermiculite, including 3.5 kg m^{-3} Osmocote (slow-release fertilizer, 15-11-13 NPK plus micronutrients) and 0.5 kg m^{-3} PG mix (14-16-18 NPK granular fertilizer plus micronutrients). Plants were grown in an environmentally controlled plant-growth room with a photoperiod of 16 h under irradiance of ≈300 μmol m^{-2} s^{-1} PAR, from 400 W sodium lamps (Royal Philips Electronics, Eindhoven, the Netherlands). Air temperatures were adjusted to 18 : 15°C (day : night) with a relative humidity of 50–70%.

GUS-staining protocol

Plant material was incubated in X-gluc solution (1 mM X-gluc, 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% v/v Triton X-100) for 16 h at 37°C. The material was viewed under a dissection microscope to assess GUS staining and imaged using an Olympus DP11 microscope digital camera system.

Constructs

Two constructs were used for this work: pAHC25 (Christensen & Quail, 1996) containing both the *uidA* reporter gene and *bar* selection gene, both independently controlled by the maize UBI1 promoter; and pAGP2cdc25, containing the fission yeast *cdc25* ORF driven by the *AGP2* promoter and first intron. pAGP2cdc25 was generated by restriction of the pAGP2::GUS plasmid (Thorneycroft *et al.*, 2003) with *Nco*I and *Xba*I to remove the gus and nos fragment, and replaced with the ORF of *Spcdc25* generated via PCR using primers CDC25NCO (ATCCCATGGATTCTCCTTTCTTCAC) and CDC25XBA (ATGCGGCCGCAATGGATTTCT-CCGCTTTCTTCAG) with *Nco*I and *Xba*I (3′) restriction sites. The nos fragment was PCR-amplified using primers (NOSXF = GCATTCTAGAGAAATTTCCCCGATTCGT TCA) and (NOSXR = ATGCTCTAGAGAGATCTAGT-AAACATAGATG) and then cloned in as an *Xba*1 fragment. Finally, the 54 bp *Nco*I fragment encoding the putative peptide sequence was gel-purified from an *Nco*I digest of pAGP2::GUS and inserted into the *Nco*I site (Fig. 1). The final construct was checked by sequencing all PCR products and across all junctions.

Confirmation of insertion of the *Spcdc25* gene into wheat was tested by carrying out PCR on total DNA using two sets of primers. Primers P7 (TTAGGTCCCTTCTCCGATG) and P101 (TCAATGAGTCCTCCTTCACG) were used to amplify a 243 bp region of the *Spcdc25* ORF, and secondly using P1 (GCGCGAATTCATGGATTCTCCHCTTTC) and P3 (GCGTACGACGAGGAGTCGG) to amplify the 5′ 245 bp of the *Spcdc25* gene (Fig. 1). The conditions used were 35 amplification cycles of 94°C (30 s), 55°C (30 s) and 72°C (30 s). To detect whether any transcript was produced, RNA was

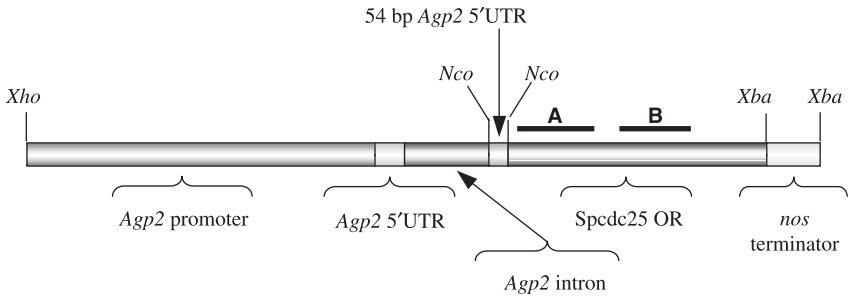

Fig. 1 Agp2::*Spcdc25* construct: restriction enzyme sites used for the cloning are indicated as are the Agp2 promoter, 5′ UTR, *Spcdc25* ORF and *nos* terminator. A, 5′ fragment of the *Spcdc25* ORF amplified with PCR primers P1 and P3, which is not amplifiable from AGP2.2 T_1 plants; B, central portion of the *Spcdc25* ORF amplified with PCR primers P7 and P101.

isolated using Tri-reagent (Sigma-Aldrich, Poole, UK) following the manufacturer's instructions. Before the generation of cDNA, the RNA was incubated with 10 U RNase free DNase for 20 min at 37°C. cDNA was generated using RMV(H–) reverse transcriptase (Promega, Southampton, UK) with 3 μg total RNA, following the manufacturer's instructions. To be entirely sure that the PCR products were RNA-derived, control-RT reactions were carried out using the PCR conditions stated above.

Plant transformation

Immature scutella were used as the target tissue for transformation by particle bombardment following Pastori *et al.* (2001) and Rasco-Gaunt *et al.* (2001). Wheat spikes containing immature seeds of ≈12–14 d post anthesis were collected and sterilized. Immature embryos were dissected aseptically and the embryonic axis discarded. The explants were placed abaxial (convex) side in contact with basal callus induction medium and cultured in darkness at 22°C for 1–2 d before bombardment. Gene delivery into plant cells was mediated by 0.6 μm diameter gold microprojectiles, which were accelerated by compressed helium gas in a PDS-1000/He device (Bio-Rad, Hemel Hempstead, UK). Before bombardment, foreign DNA was coated onto gold particles following Barcelo & Lazzeri (1995). The distance between the stopping plate and the target tissue was adjusted to 5.5 cm and the acceleration pressure used was 650 psi. Immediately after bombardment, scutella were distributed evenly over the callus-induction medium (typically 10 per plate) and incubated at 22°C in darkness for 3–4 wk to promote the formation of embryogenic calli. These were then transferred to shoot-regeneration medium and cultured at 25°C with a 12 h photoperiod. Selection of transformed tissue was applied using the herbicide formulation Basta (Bayer CropScience, Cambridge, UK) containing the active agent glufosinate ammonium at 10 mg ml^{-1}. The regenerated plantlets, which continued to grow under selection conditions, were considered to be putative transformants and transplanted in pots containing soil. They were grown to maturity in contained glasshouse facilities maintained at 18–20°C during the day and 16°C during the night under natural light supplemented with sodium lamps (irradiance ≈750 μmol m^{-2} s^{-1}) with a 16 h photoperiod.

Pollen viability staining

The viability of pollen was assessed as described by Zhang *et al.* (2001), by staining the pollen in 0.1% fluorescein diacetate (w/v acetone) in 0.4 M mannitol. The pollen was viewed under an Olympus BH2 research microscope using UV light with a 450–495 filter with viable pollen emitting a bright blue/green florescence.

Results

AGP::GUS staining indicates that this putative endosperm-specific promoter is not tissue-specific

Our original aim was to express *Spcdc25* in the endosperm, and the *AGP2* promoter offered a potential way to do this because a preliminary expression analysis by Northern blots indicated that the *AGP2* transcript in cv. Chinese Spring was first detectable in the endosperm 5 d post-anthesis (dpa) (Thorneycroft *et al.*, 2003), which coincides with the transition from coenocytic to cellular phase of the wheat endosperm. As a follow-up, we monitored *AGP2::GUS* expression in cv. Chinese Spring through GUS staining. We could not detect GUS in leaves, anthers or styles, or in carpels that were 2 d preanthesis (Figs 2, 3a). However, surprisingly, GUS staining was very strong in pollen grains and was detectable in postanthesis carpels (Fig. 2e, f). In total, five independent transgenic lines were examined in the T_1 and T_2 generations. Segregation of GUS staining in the endosperm indicated that four plants were hemizygous while one was homozygous. In hemizygous plants, 50% of pollen grains were deeply stained, consistent with postmeiotic expression of *AGP2::GUS* in a hemizygous genetic background (Fig. 2e). We also detected *GUS* expression in the ovary walls before fertilization (Fig. 3b,c). Hence these observations indicated a surprising range of expression, as revealed by the GUS staining in tissues other than the endosperm. However, GUS expression in the endosperm was not detected until 4–5 dpa (Fig. 4b,c) and staining was stronger at 8 dpa (Fig. 4d), confirming the temporal expression of this promoter in wheat endosperm shown by Northern analysis (Thorneycroft *et al.*, 2003).

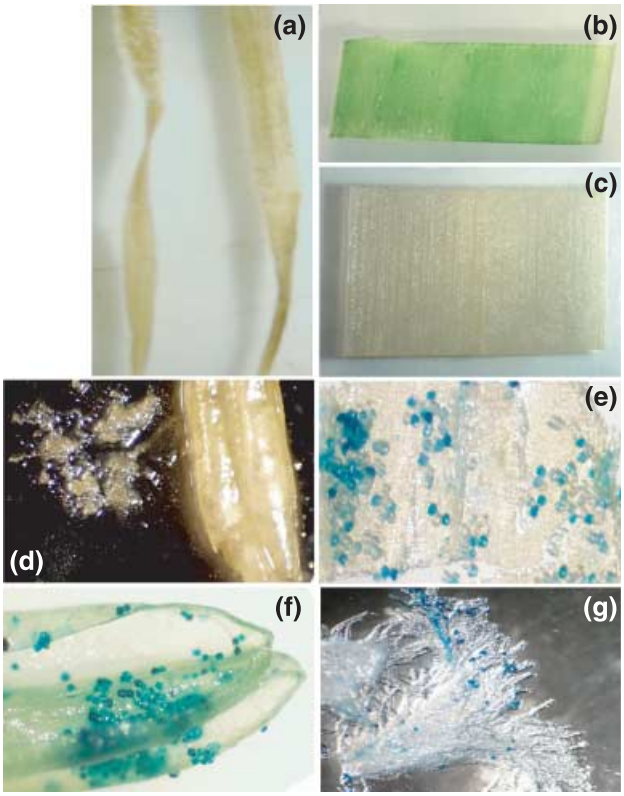

Fig. 2 Expression of *AGP2::GUS* reporter is restricted to pollen in transgenic wheat (*Triticum aestivum* cv. Chinese Spring). (a) Mature leaves; (b,c) leaf segments; (d) anthers; (e,f) mature pollen; (g) stylar tissue with adhering pollen grains.

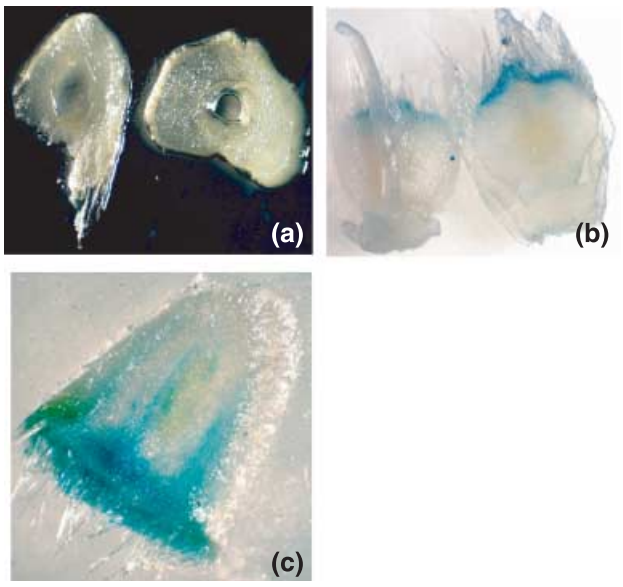

Fig. 3 Expression pattern of *AGP2::GUS* reporter in carpels of transgenic wheat (*Triticum aestivum* cv. Chinese Spring) plants. (a) 2 d preanthesis; (b) 1 d postanthesis with GUS staining in peripheral region; (c) 2 d postanthesis with strong staining at base.

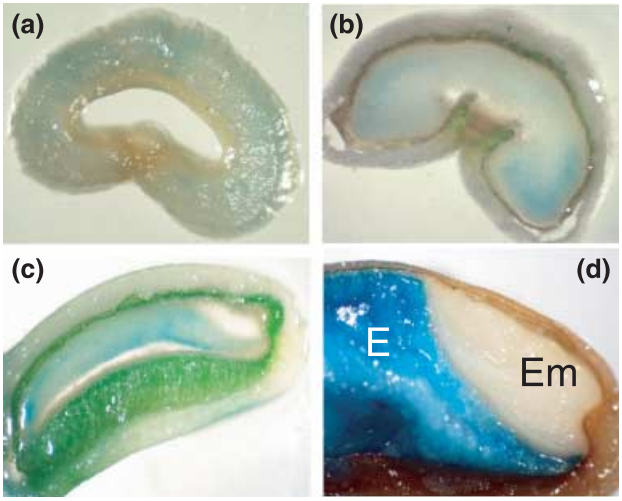

Fig. 4 *AGP2::GUS* expression pattern detectable in developing endosperms (E) but not embryos (Em) of transgenic wheat (*Triticum aestivum* cv. Chinese Spring) at (a) 2; (b) 4; (c) 5; (d) 8 dpa.

Expression of *AGP2::Spcdc25*

From the eight transformation experiments performed, only five plantlets survived the selection steps, and these were transferred to soil. The transformation efficiencies with these *AGP2::Spcdc25* were lower (0.29%) compared with typical transformations with other gene constructs (Pastori *et al.*, 2001; Rasco-Gaunt *et al.*, 2001). This may be due to a deleterious effect of *Spcdc25* expression on tissue culture of wheat. Only three surviving T_0 transgenic plantlets contained the *AGP2*::*Spcdc25* insertion; this was confirmed by genomic PCR using primers directed at a portion of the *Spcdc25* ORF (Fig. 5) and named AGP2::*Spcdc25*.1, AGP2::*Spcdc25*.2 and AGP2::*Spcdc25*.3. All three AGP2 plants set seed. Because of the lack of endosperm-specificity of the *AGP2* promoter, as revealed by GUS staining, we decided to test for expression of *AGP2*::*Spcdc25* in various tissues of the T_0 generation using RT–PCR. *Spcdc25* was expressed in mature leaves of AGP2::*Spcdc25*.1 and AGP2::*Spcdc25*.3, but not in AGP2::*Spcdc25*.2 (Fig. 6). For AGP2::*Spcdc25*.1 and AGP2::*Spcdc25*.3, RNA yields from the pollen and carpels were insufficient for RT–PCR,

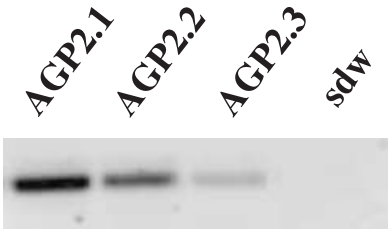

Fig. 5 PCR of genomic DNA demonstrating *Spcdc25* insertion in three transgenic wheat (*Triticum aestivum* cv. Cadenza) plants: AGP2::*Spcdc25*.1, AGP2::*Spcdc25*.2 and AGP2::*Spcdc25*.3. The final lane is a sterile distilled water (sdw) negative control.

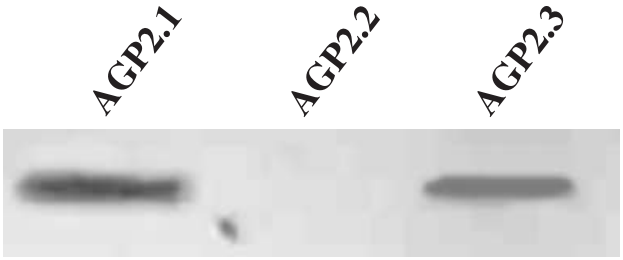

Fig. 6 Extent of *AGP*::*Spcdc25* expression as shown by RT–PCR in three T_0 transgenic AGP wheat (*Triticum aestivum* cv. Cadenza) plants.

and these plants neither produced abundant pollen nor set seed in quantity.

AGP2::*Spcdc25*.1 and .3 plants exhibit abnormal spike morphology and nonviable pollen

AGP::*Spcdc25*.3 exhibited an unusual double-ridged spike, and other spikes on both AGP2::*Spcdc25*.1 and AGP2::*Spcdc25*.3 plants were markedly smaller than wild type (Fig. 7a). We conclude that this abnormality was due to transgene expression, not to environmental stress, as tissue culture-derived non-transformants and AGP2::*Spcdc25*.2 plants flowered under the same conditions as the transgenic lines and exhibited normal morphology (Fig. 7b).

Given the high expression of *AGP2::GUS* in pollen of cv. Chinese Spring, we checked whether AGP2::*Spcdc25* expression affected pollen viability in cv. Cadenza. Using fluorescein diacetate, pollen viability was only 54% in AGP2::*Spcdc25*.1 (Fig. 8b) compared with 100% in wild type (Fig. 8a). Moreover, in AGP2::*Spcdc25*.1 and .3, only 30–50 seeds were set per plant, compared with ≈100–200 per wild-type plant.

AGP2::*Spcdc25*.2, in which *Spcdc25* had integrated into genomic DNA (PCR-positive), did not express *Spcdc25* (RT–PCR-negative), exhibited normal morphology with 100% pollen viability, and set seed in the same way as the wild type. Given the high level of AGP activity in the reproductive tissues of *AGP2::GUS* plants and in the pollen, we concluded that *Spcdc25* expression perturbed floral morphology, pollen development and subsequent seed set in AGP2.1 and AGP2.3.

Spcdc25 was not transmitted to the T_1 generation in the AGP2::*Spcdc25*.1 and ::*Spcdc25*.3 lines

Seeds from each of the primary (T_0) transgenic plants AGP2::*Spcdc25*.1 and AGP2::*Spcdc25*.3 were sown, and these T_1 plants flowered and set seed normally. Together with the segregation of viability in the pollen of the T_0 plants, and the segregation of GUS staining in the *AGP2::GUS* pollen, we suspected that *Spcdc25* was not transmitted to the next generation. This was confirmed by negative PCR reactions for these plants. PCR primers for the wheat fructose bisphosphatase (FBPase) gene were used as positive controls. Although the FBPase gene PCR product was detectable in most of the T_1 lines, neither AGP2::*Spcdc25*.1 nor AGP2::*Spcdc25*.3 T_1

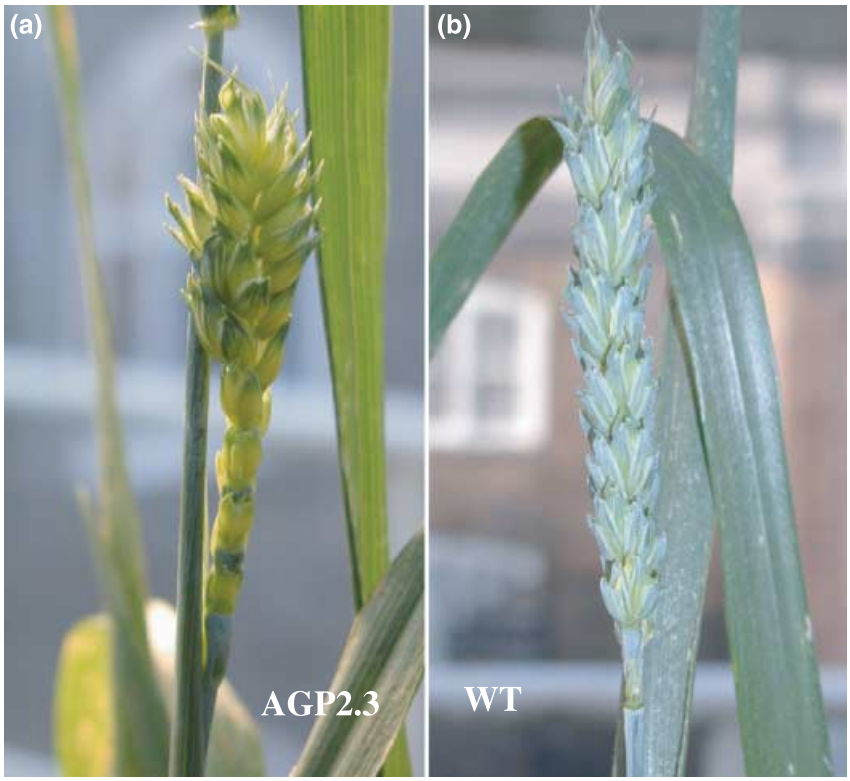

Fig. 7 (a) AGP2::*Spcdc25*.3 plant (*Triticum aestivum* cv. Cadenza) exhibiting an abnormal double-ridged spike compared with (b) wild type (WT).

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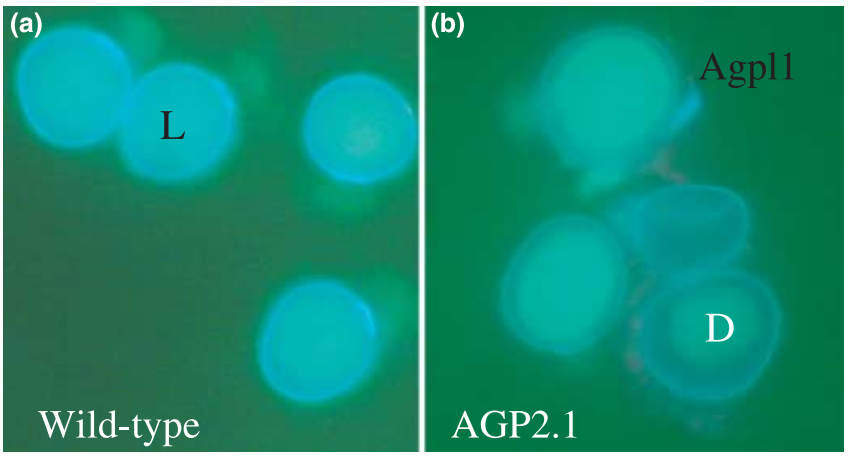

Fig. 8 Wheat (*Triticum aestivum* cv. Cadenza) pollen treated with FDA. Living cells (L) convert FDA to fluoroscein and stain bright green while dead cells (D) cannot. Staining indicated 100% viability for wild type but only 54% viability for AGP2::*Spcdc25*.1.

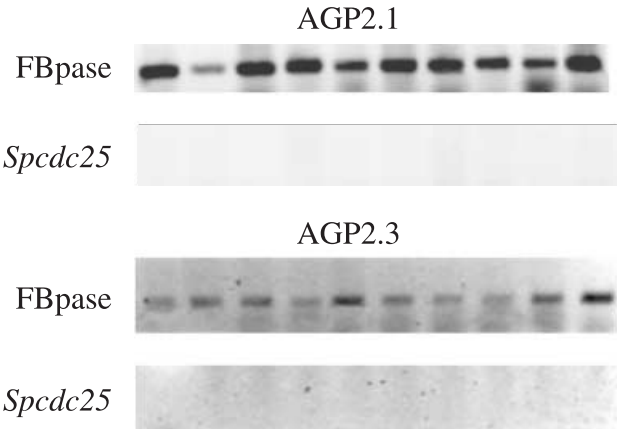

Fig. 9 PCR of genomic DNA indicating the presence of the housekeeping gene *FBpase* but the absence of *Spcdc25* in the T_1 generation of AGP2::*Spcdc25*.1 and AGP2::*Spcdc25*.3 plants (results of a representative sample of 10 T_1 plants).

plants were PCR-positive for *Spcdc25* (Fig. 9). Fourteen out of 18 of the T_1 AGP2::*Spcdc25*.2 plants were positive for *Spcdc25* genomic DNA using PCR primers that amplify a central portion of the ORF. When primers to the 5′ end of the *Spcdc25* ORF were used with T_1 plants, the expected PCR product was not seen. These results indicate a deletion or an insertional rearrangement of the transgene in the AGP2::*Spcdc25*.2 line which is consistent with the lack of transcriptional activity for the T_0 plants, their normal morphology and seed set. A summary of our findings is presented in Table 1.

Discussion

Our analyses indicate clearly that the AGP2 promoter's activity in hexaploid wheat cv. Cadenza is far less tissue-specific than originally thought. Histochemical analysis of AGP2::GUS in transgenic tobacco indicated an expression pattern in guard cells, leaves, and throughout the seed (Thorneycroft *et al.*, 2003). However, the same promoter activity was completely lacking in leaves of transgenic wheat (cv. Chinese spring), but was detected in developing endosperm, coming on at ≈5 dpa and the signal becoming progressively stronger in more mature endosperms (Thorneycroft *et al.*, 2003). Our AGP2::GUS analyses in cv. Chinese Spring confirm this staining pattern, although our positive GUS staining in pollen and the surface layers of carpels were unexpected and show a more variable activity of the *AGP2* promoter than originally thought.

Table 1 Summary of PCR and RT–PCR results together with floral morphology, percentage pollen viability and seed set for AGP2::*Spcdc*25.1, AGP2::*Spcdc25*.2 and AGP2::*Spcdc*25.3 T_0 and T_1 plants

	AGP2::*Spcdc25* (T_0)			AGP2::*Spcdc25* (T_1)		
Method/parameter	.1	.2	.3	.1	.2	.3
PCR (243 bp central segment of *Spcdc25* ORF)	+	+	+	– (0/23)	+ (14/18)	– (0/22)
PCR: 245 bp at 5′ end of *Spcdc25* ORF	**ND**	+	**ND**	ND	–	ND
RT–PCR: 243 bp central segment of *Spcdc25* ORF	+	–	+	ND	ND	ND
Floral morphology	**Abnormal**	Normal	**Abnormal**	Normal	Normal	Normal
Pollen viability (%)	**54**	100	**52**	100	100	100
Seed set	**Low**	Normal	**Low**	Normal	Normal	Normal

Numbers in brackets indicate the frequency of PCR positives for the T_1 generations. ND, not determined.

Consistent with the GUS staining patterns, the data presented here strongly indicate that *Spcdc25* expression under the control of the AGP2 promoter perturbs pollen development postmeiotically. Consequently, nonviable transgenic pollen precluded transfer of this transgene by male gametes to the T_1 generation. Clearly, *Spcdc25* must also have affected female gametogenesis, otherwise *Spcdc25* would be transmitted via the egg. Nontransmission through the female also suggests a postmeiotic effect on ovule development, as normal nontransgenic seeds were also recovered. The observation of intense GUS staining in pollen, but only 50% viability of the pollen in AGP2::*Spcdc25*.1 and AGP2::*Spcdc25*.3, is consistent with this view; tracking the early events of the female was not attempted although sporadic GUS staining was observed in carpel tissue.

We did detect a PCR signal for *Spcdc25* in the T_1 generation of AGP2::*Spcdc25*.2, but there is evidence of an insertional rearrangement and, moreover, we could not detect *Spcdc25* at the transcriptional level. Notably, both T_0 and T_1 AGP2::*Spcdc25*.2 plants were morphologically normal and flowered in the same way as the wild type. These plants are a testament to the transformation procedure *per se* not having an influence on the generated phenotypes. Our data are remarkably similar to those concerning the expression of antisense *SnRK1* with seed-specific promoters in barley. The T_0 generation of barley was unable to transmit antisense *SnRK1* to the T_1 generation because of unexpected expression of the transgene in the pollen (Zhang *et al.*, 2001). These authors concluded that the lack of viable pollen occurred because the plants were unable to metabolize imported sucrose. The data reported here, and those of Zhang *et al.* (2001), emphasize that 'tissue-specific' promoters can be expressed in other tissues, and that the gametophytic generation of wheat appears to be very sensitive to the ectopic expression of *Spcdc25.*

In wheat, male gametophyte development comprises two postmeiotic divisions. The first of these is a highly asymmetrical division of a haploid nucleus/cell into the generative and vegetative nuclei/cells (Esau, 1965). Hence polarity is induced at this stage, with dramatically different fates resulting for each of the daughter sibling cells/nuclei. Disruption of the asymmetrical division in the pollen in many species can be induced by colchicine or temperature treatments (Zhao *et al.*, 1996), both of which can result in the induction of haploid embryogenesis. Thus mild disruption of this asymmetrical division is tolerated, albeit with a change in developmental pathway. However, culturing of anthers or microspores at an optimal stage is required in order to regenerate haploid plants. Due to lack of material, it was not possible to determine whether culturing of the AGP2.1 or AGP2.3 anthers could result in embryonic development.

In tobacco BY2 cells, *Spcdc25* expression induces a smaller cell size at division, and can also induce longitudinal divisions where transverse divisions are the norm (C.B. Orchard, unpublished data). Hence *Spcdc25* expression may have disrupted the normal polarity of division in the tobacco BY-2 cells, and the same may have been true for the AGP2::*Spcdc25*.1 and AGP2::*Spcdc25*.3 pollen. Likewise, normal female gametophyte development may have been disrupted in the same way. Ovule development is dependent on a precise series of nuclear divisions followed by polarized development with both physical and functional separation of antipodals from egg and synergid cells. We would like to know exactly which postmeiotic stage(s) of both male and female gametophyte development are most sensitive to *Spcdc25* expression. However, as *Spcdc25* was not transmitted to the T_1 generation, this would require establishing new transformants.

Overexpression of *Spcdc25* induces cells to divide at a smaller threshold size than wild type, both in fission yeast and in dicot plant cells (Russell & Nurse, 1987; Bell *et al.*, 1993). Therefore one possibility is that *Spcdc25* expression in wheat makes the gametophyte nonviable by perturbing the threshold mitotic cell size in that first postmeiotic mitotic division. Equally valid is the second hypothesis, that the expression of *Spcdc25* affects the second mitotic division, i.e. sperm-cell production from the generative nucleus. The lethality of *Spcdc25* expression on pollen development in AGP2.1 and AGP2.3 plants prevented any tests of these hypotheses.

The finding that expression of *Spcdc25* perturbs gametophytic development is surprising, given the relatively mild effects of *Spcdc25* on the sporophytic phase in tobacco (Bell *et al.*, 1993; McKibbin *et al.*, 1998; Wyrzykowska *et al.*, 2002). However, the 35S promoter (used to regulate *Spcdc25* expression in tobacco and *Arabidopsis*) is known to be inactive in pollen (Wilkinson *et al.*, 1997). Our results therefore suggest that the regulation of mitotic divisions in the gametophyte is more vulnerable to perturbation by cell-cycle regulatory genes. However, surprisingly little is known about cell-cycle gene expression during the gametophytic phase or, indeed, during embryogenesis of the sporophytic phase. Sauter *et al.* (1998) approached the problem by studying cell-cycle gene expression in sperm cells, egg cells, synergids and antipodal cells of maize, and in the zygotes that formed following *in vitro* gamete fusion. Whereas two maize homologues to *cdc2* (*Zmcdc2*A and B) were expressed in all cell types, the expression of two cyclin homologues (*ZmCycB*1;1 and *ZmCycB*2;1) could not be detected in the gametophyte cells, and these genes began to be transcribed only between 12 and 24 h after *in vitro* gamete fusion (Sauter *et al.*, 1998). Hence the differential expression of these cyclin genes did mark a clear difference between the gametophytic and sporophytic generations. *ZmCycB* transcripts appear in late G2 (Sauter *et al.*, 1998), a time comparable to when SpWee1 and SpCdc25 phosphoregulate Cdc2 in fission yeast (Nurse, 1990). In the latter, cyclin binding to Cdc2 is important, leading to a conformational change in the Cdc2 protein that, in turn, results in exposure of an ATP-binding domain on Cdc2. This change in conformation allows Cdc25 phosphatase access to the ATP-binding domain (Nurse, 1990). The

absence of zygotic *ZmCycB* transcripts in gametophytic cells could possibly alter the conformation of Cdc2 protein so that it might be vulnerable to attack from the *Spcdc25* phosphatase, leading to unscheduled or aberrant division. Hence these data do indeed suggest that gametophytic mitotic divisions and cell-cycle gene expression are regulated differently from sporophytic ones.

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