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Temporal and spatial control of transgene expression using a heat-inducible promoter in transgenic wheat

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Summary

Constitutive promoters are widely used to functionally characterise plant genes in transgenic plants, but their lack of specificity and poor control over protein expression can be a major disadvantage. On the other hand, promoters that provide precise regulation of temporal or spatial transgene expression facilitate such studies by targeting over-expression or knockdown of target genes to specific tissues and/or at particular developmental stages. Here, we used the *uid*A (beta-glucuronidase, GUS) reporter gene to demonstrate that the barley *Hvhsp17* gene promoter can be induced by heat treatment of 38–40 °C for 1–2 h in transgenic wheat. The GUS enzyme was expressed only in those tissues directly exposed to heat and not in neighbouring leaf tissues. The induction of HSP::GUS was demonstrated in all organs and tissues tested, but expression in older tissues was lower. Generally, proximal root sections showed less GUS activity than in root tips. This heat-inducible promoter provides the ability to investigate the function of candidate genes by overexpression or by down-regulation of target gene expression (for example by RNAi) in selected tissues or developmental stages of a transgenic plant, limited only by the ability to apply a heat shock to the selected tissues. It also allows the investigation of genes that would be lethal or reduce fertility if expressed constitutively.

Introduction

Investigations of gene function in transgenic plants require methods to finely control the expression of candidate genes. Experimental control of transgene expression is usually achieved by constructing a gene cassette linking the coding sequence to a promoter that exhibits a predefined pattern and level of expression in the species to be transformed. Commonly used promoters that give relatively strong expression in most tissues throughout development include the 35S and 34S promoters from cauliflower mosaic virus and figwort mosaic virus, respectively, and promoters of constitutively expressed plant genes such as actin and ubiquitin (reviewed by Potenza *et al.*, 2004). Many promoters from genes that are normally regulated in a tissue-specific manner have also been utilised in transformation experiments. These promoters offer reproducible transgene expression patterns in predefined cell types but cannot usually be modulated subsequently. Examples of robust tissue-specific promoters that have been used to drive plant transgenes include those which are expressed specifically in vascular tissues (reviewed by Liu and Jia, 2003), anthers (reviewed by Kato *et al.*, 2010) or seed tissues (reviewed by Furtado *et al.*, 2008; Jones, 2005; Qu and Takaiwa, 2004). In addition, there is potential in the future to design customised, synthetic tissue-specific promoters (Venter, 2007).

The ability to induce *de novo* transgene expression at specific stages of plant development or in selected tissues in the same transgenic plant is a useful research tool. Inducible promoters are particularly important when constitutive up- or down-regulation of the target gene has deleterious effects on plant growth and development, when constitutive expression requires unacceptable inputs of energy or nutrients or when the effects of the transgene will be compared between different tissues or growth stages of the same plant. Several inducible promoters have been described in transgenic applications including those activated by application of specific chemicals or stresses such as heat, drought, mechanical damage and light (reviewed by Arguello-Astorga and Herrera-Estrella, 1998; Corrado and Karali, 2009; Moore *et al.*, 2006; Padidam, 2003). In transgenic wheat, only two promoters inducible by abiotic stresses have been described previously. An ABA-responsive element from the barley *HVA22* gene fused to a rice actin minimal promoter was induced by drought in transgenic wheat plants (Vendruscolo *et al.*, 2007), and the rd29A promoter from *Arabidopsis thaliana* (which acts through an ABA independent pathway) was also demonstrated to be induced by drought (Pellegrineschi *et al.*, 2004).

A cDNA clone encoding an 18-kDa, class I heat-shock protein (*Hvhsp18*) was cloned and used to identify a genomic clone encoding a 17-kDa, class 1 heat-shock protein (*Hvhsp17*) by Marmiroli *et al.* (1993). Upstream regulatory elements in the genomic clone, including two heat-shock elements (HSE) and a metal response element (MRE), were identified using bioinformatics, and the upstream region was demonstrated to possess heat-inducible promoter activity in protoplast transient expression studies (Raho *et al.*, 1995). However, in stable tobacco transgenic lines, expression of the Hvhsp17::GUS construct was strictly restricted to xylem tissues of the stem and petioles (Raho *et al.*, 1996). Here, we characterise the inducibility and resulting expression pattern of the *uid*A gene driven by the barley heat-shock promoter *Hvhsp17* in transgenic wheat.

Results

Transient testing of pHSPdGUS

Induction of GUS expression from pHSPdGUS (Figure 1) was initially tested using a transient expression assay. The scutellar

epithelium of immature wheat embryos was bombarded with micro-particles carrying either pHSPdGUS or a construct carrying the *uid*A gene under the control of the constitutive rice actin promoter (McElroy *et al.*, 1991) and cultured as described in the Experimental Procedures. As expected, blue foci were observed with and without heat shock with the constitutive promoter construct (pAct1::GUS) but only after heat-shock treatment when pHSPdGUS was used (Table 1).

Generation of stable transgenic wheat lines

A total of 21 independent transgenic lines in which the HSP::GUS gene cassette was detected by PCR (Experimental Procedures) were produced. Clearly visible heat-inducible GUS expression, with no GUS expression in the absence of heat shock, was demonstrated in T1 seeds from 14 of the 20 lines which were tested. For T1 seeds from the other six lines tested, staining was either absent or too weak to be unambiguous. Seven lines with heat-inducible GUS expression in T1 seeds were selected at random for T2 seed production. Four of these lines, in which the heat-induced GUS expression in T2 seed either did not segregate indicating homozygosity (B2295-R8P2) or segregated in ratios of approximately 3 : 1 indicating that these lines are likely to have single transgene insertion sites (B2295-R2P1, B2295-R5P1 and B2301-R9P8a), were selected, and T2 plants from these lines were used for all subsequent experiments.

Preliminary investigation of GUS expression

Preliminary investigations of the induction of GUS expression were carried out using the four independent transgenic wheat lines described earlier. From each line, 24 T2 seeds were germinated and, after 7 days, the whole seedlings were subjected to a 38 °C heat shock for 2 h and then tested for GUS activity. The observations of GUS staining for all 24 seedlings for each line are summarised below and representative examples are shown in Figure 2 for illustration. Blue staining in lines B2295-R8P2 and B2301-R9P8a was stronger and more extensive in shoots and roots than in lines B2295-R2P1 and B2295-R5P1 (Figure 2). In line B2295-R2P1, staining in the roots was generally confined to the distal region of the roots but in other lines staining extended further up the roots with strong staining visible in the vascular tissue of some roots. In some roots, particularly of lines B2295-R8P2 and B2301-R9P8a, darkly stained spots were visible which appeared to correspond with the positions of lateral root primordia (Figure 2c, B2295-R8P2, inset). In shoot tissue, staining was generally stronger in the coleoptile than in the first leaf (the first leaf of line B2295-R2P1 was particularly weak) (Figure 2). At the base of the coleoptiles, no staining was visible in many seedlings despite strong staining further up the coleoptiles (for example, Figure 2c, B2295-R2P1). For all seedlings in which no GUS activity was detected (15), the presence/absence of the transgene was confirmed by PCR and only one of these 15 contained the transgene.

Table 1 Transient GUS expression in immature wheat scutella bombarded with pHSPdGUS or pAct1::GUS with or without a 40 °C heat-shock treatment

Construct	Experiment	Heat shock	% Embryos with blue foci*
pAct1::GUS	1	–	81.0 ± 7.62
		+	68.3 ± 1.67
	2	–	35.8 ± 8.18
		+	ND
pHSPdGUS	1	–	0
		+	65.0 ± 8.66
	2	–	0
		+	52.6 ± 3.91

*Results are the mean of three plates from each experiment ±SEM. ND, not determined.

Optimisation of heat treatment conditions

The effect of varying the temperature on the induction of gene expression driven by the heat-shock promoter was investigated. Duplicate 4–6-cm pieces of shoot from germinated seedlings (approximately 14 days old) of the four independent transgenic lines containing the HSP::GUS cassette, and null segregants, were incubated in a water-bath at ten temperatures ranging from 4 to 44 °C for 2 h. Small (0.5 cm long) samples were used for histochemical (GUS) assays, and the remaining tissue was used for fluorometric (MUG—4-methylumbelliferyl β-D-glucuronide) assays of GUS activity. The results of MUG assays indicated that the optimum temperature for induction of expression driven by the heat-shock promoter was between 38 and 40 °C (Figure 3a). These results were reflected in the blue colour observed in the corresponding samples used for histochemical GUS assays (data not shown). A similar experiment was conducted to determine the effect of duration of heat treatment on GUS expression in the same transgenic lines. The experiment was conducted as described earlier except that all samples were incubated at 38 °C but for times ranging from 0 to 180 min. GUS expression was observed after 15 min and generally increased in intensity until 120 min after which no obvious further increase in intensity or extent of blue staining was observed (Figure 3b). Results of fluorometric MUG assays were consistent with the histochemical GUS assay and confirmed that incubation beyond 120 min did not generally result in further increase in expression (data not shown). No GUS activity was observed in null lines using GUS or MUG assays (Figure 3a, b). These experiments were repeated using duplicate

Figure 1 Diagrammatic representation of pHSPdGUS. HSP indicates the 3′ end (656 bp) of the *Hvhsp17* gene promoter containing the two heat-shock elements and the metal response element identified by Marmiroli *et al.* (1993). GUS indicates the β-glucuronidase reporter gene. NOS indicates the nopaline synthetase terminator sequence. Target sites for restriction enzymes used in cloning are indicated (note that there are two additional *Apo* I sites in the vector sequence downstream of the NOS terminator). The plasmid vector is pGEM7zf(-) (Promega UK Ltd., Southampton, Hampshire, UK).

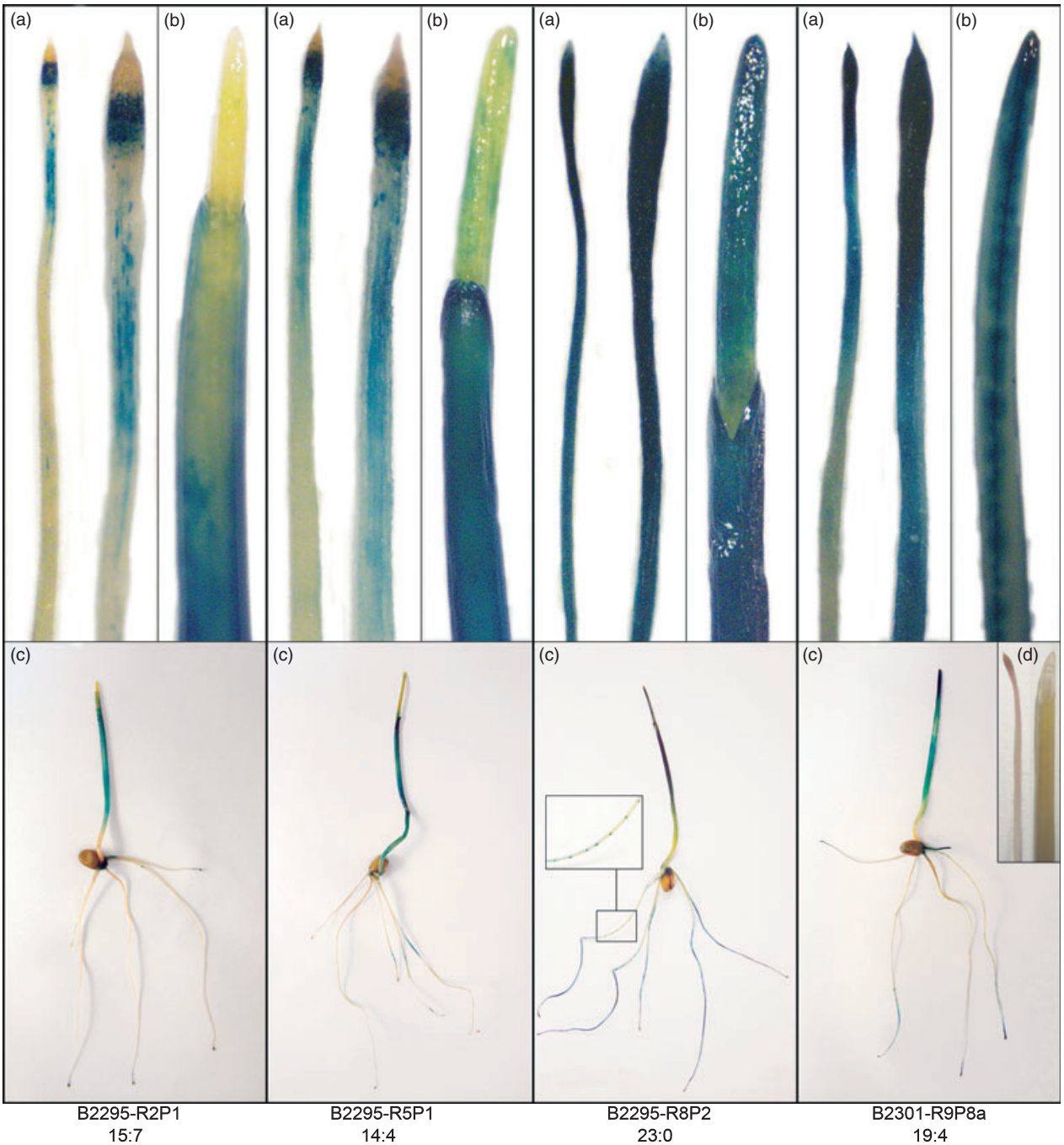

Figure 2 Induction of GUS expression after 38 °C heat shock for 2 h in 7-day-old T2 generation seedlings from four independent transgenic lines containing HSP::GUS. Line identifiers and ratio of seedlings in which GUS activity was detected to those in which none was detected are shown below each panel. (a) Root (two images of the same root at different magnifications are shown for each transgenic line), (b) coleoptile, (c) whole seedling, (d) null segregant.

1.5 cm leaf pieces excised from 7-week-old plants with similar results (data not shown).

Developmental age and range of tissue-types that displayed inducible GUS expression

Glasshouse-grown transgenic and null segregant plants (confirmed by PCR) from each of the four independent transgenic lines were used to investigate the range of tissues that displayed inducible GUS expression. Two transgenic plants and one null segregant from each of the four lines were used for each experiment. Whole plants at 4, 8, 12, 16 and 20 weeks after germination were heat-shocked as described in the Experimental Procedures. Appropriate tissue samples were taken from the developmental series for histochemical GUS assays: leaf and root pieces from all plants, stem (node and internode) for all except 4-week-old plants, immature inflorescences for 8-week-old plants only and floral parts (glumes, seeds and anthers) for 12-, 16- and 20-week-old plants. For 4- and 8-week-old plants, 1.5-cm leaf segments were sampled from approximately the midpoint of both youngest and oldest leaves. For 12-, 16- and

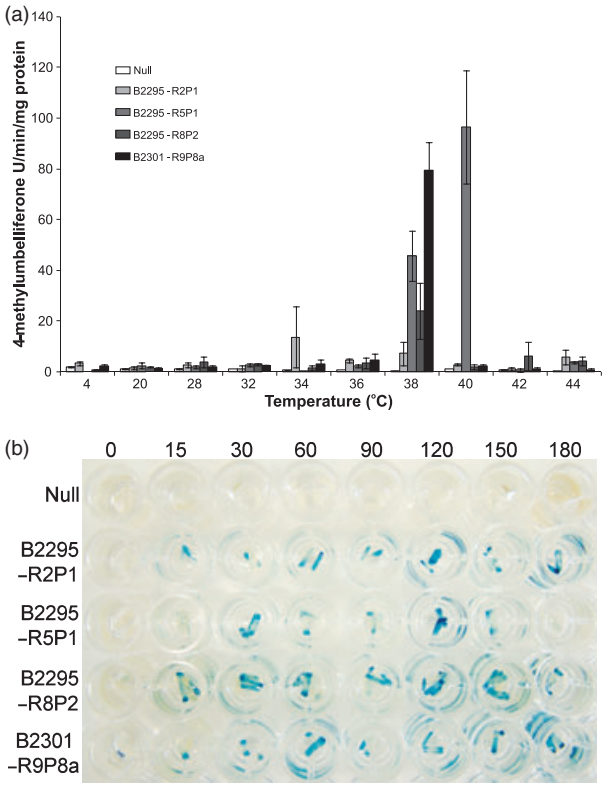

Figure 3 Induction of expression driven by the HSP::GUS expression cassette in four independent transgenic lines and null segregants. Duplicate 4–6-cm samples of shoot tissue were excised from 14-day-old seedlings and incubated at a range of temperatures for 2 h (a) and for various times (indicated in min) at 38 °C (b) and induction of expression measured by fluorometric 4-methylumbelliferyl β-D-glucuronide assay (a) and histochemical GUS assay (b). Error bars in (a) indicate SEM. 1 U 4 MU/min/mg protein = 8.3 pmol/min/mg protein.

Figure 4 Histochemical staining in a range of tissues from transgenic lines containing HSP::GUS. Equivalent plant tissues were either not heat-treated (left side) or heat-shocked at 38 °C for 2 h (right side). Top to bottom: leaf segment, roots from B2301-R9P8a 8-week-old plants; stem (internode), stem (node) from B2295-R5P1 8-week-old plants; glume, palea/lemma, anther from B2295-R8P2 12-week-old plants; immature inflorescence from B2301-R9P8a 8-week-old plants; seed coat, endosperm from B2301-R9P8a 12-week-old plants.

20-week-old plants, 2–3-cm leaf segments were taken from the flag and third leaves. For all plants, root samples (approximately 2–5 cm in length) were taken from growing root tips and from older root segments closer to the stem base. Stem samples were 2–3 cm long and were from the first node and internode below the immature inflorescence or peduncle. Floral parts were sampled by removing one spikelet from the base and one from the mid-section of the oldest and youngest ears of each plant (from opposite sides of the ear pre- and postheat-shock) and removing and opening the florets to allow X-Gluc. penetration. Seeds were bisected both longitudinally and transversely facilitating access of X-Gluc. solution to the endosperm. Plants were sampled before and after heat shock. GUS activity was not seen in any tissues prior to heat shock (Figure 4) nor in null segregant plants regardless of heat treatment (data not shown). The results of these experiments are summarised in Table 2 and Figure 4. Heat-inducible GUS expression was found in all tissues tested, although some variation between lines was evident, and the ability to induce GUS expression was markedly lower in plants older than 16 weeks.

In 4-week-old plants, GUS activity was induced by heat shock in leaves and roots of both plants of all four lines except the roots of one plant of line B2296-R8P2. In 8-week-old plants, GUS activity was induced in leaf, stem (internode and node) and root tissue of three or four lines, but not always in both of the duplicate plants and in immature inflorescences of at least one plant of three of the four lines. At 12 weeks old, GUS activity was induced in all tissue types tested in at least one plant of all lines except for the node in line BB2301-R9P8a, the roots and anther/pollen in line B2295-R2P1 and the endosperm in line B2295-R5P1. At 16 weeks old, all leaves, glumes, endosperm and seed coats and most stem segments were positive, but GUS activity was only induced in pollen in line B2295-R8P2 and the roots of a single plant in one or two of the four lines. At 20 weeks old, the plants were senescing, and GUS activity

Table 2 Histochemical localisation of GUS activity in different tissues of heat-shocked transgenic wheat plants expressing GUS under the control of a barley heat-shock promoter. The tissues were sampled from plants at 4-, 8-, 12-, 16- and 20-weeks-old both before and after heat shock at 38 °C for 2 h. Two transgenic plants and one null segregant plant were tested for each line and developmental stage. No GUS staining was observed in nonheat-shocked tissue or in null segregant plants (data not shown). np—tissue not present in plants of that specific age

			Stem							
		Leaf	Internode	Node	Root	Glumes	Seed coat	Endosperm	Anther/pollen*	Immature inflorescence
4 weeks	B2295-R2P1	+ +	np	np	+ +	np	np	np	np	np
	B2295-R5P1	+ +	np	np	+ +	np	np	np	np	np
	B2295-R8P2	+ +	np	np	+ –	np	np	np	np	np
	B2301-R9P8a	+ +	np	np	+ +	np	np	np	np	np
8 weeks	B2295-R2P1	+ +	+ –	+ +	+ +	np	np	np	np	– –
	B2295-R5P1	+ +	+ +	+ +	+ +	np	np	np	np	+ ?
	B2295-R8P2	+ +	+ +	– ?	? –	np	np	np	np	+ –
	B2301-R9P8a	+ +	+ +	+ +	+ +	np	np	np	np	+ +
12 weeks	B2295-R2P1	+ +	+ ?	+ –	– –	+ +	+ +	+ +	? –	np
	B2295-R5P1	+ +	+ +	+ –	+ +	+ +	+ +	? ?	+ ?	np
	B2295-R8P2	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	np
	B2301-R9P8a	+ +	+ –	– –	+ –	+ +	+ +	+ –	+ –	np
16 weeks	B2295-R2P1	+ +	? ?	+ +	? –	+ +	+ +	+ +	– –	np
	B2295-R5P1	+ +	– –	+ ?	– –	+ +	+ +	+ +	– –	np
	B2295-R8P2	+ +	+ +	+ +	– –	+ +	+ +	+ +	+ +	np
	B2301-R9P8a	+ +	+ –	+ +	+ –	+ +	+ +	+ +	– –	np
20 weeks	B2295-R2P1	+ –	– –	– –	– –	– –	+ +	– –	– –	np
	B2295-R5P1	+ –	– –	– –	– –	– –	+ +	+ –	– –	np
	B2295-R8P2	– –	– –	– –	– –	+ –	+ –	– –	– –	np
	B2301-R9P8a	– –	– –	– –	– –	? –	+ ?	– –	– –	np

Presence/absence of GUS activity in a particular tissue is indicated by +/– for each of the two transgenic plants tested. Ambiguous results are indicated by ?.
*No GUS staining was observed in the anther wall.

was only induced in the few tissues that were still green on some plants.

Inducible GUS expression in developing seeds

Heat induction of GUS expression in developing seeds of four transgenic wheat lines containing HSP::GUS was assayed. Seeds were harvested at various times postanthesis, halved and one half subjected to heat shock and the other half stored at ambient temperature, prior to histochemical localisation of GUS activity (Figure 5). In early stages of development, the endosperm is surrounded by the embryo sac and further thick layers of maternal tissue which are not fused to the endosperm. During seed development, these maternal layers become progressively compacted, with fusion of some layers, and also become more strongly adhered to the endosperm tissue. (For more information on wheat seed morphology and changes in cell layers during seed development see http://www.wheatbp.net). Strong GUS staining of the maternal layers was observed until 28 days postanthesis (dpa) with staining in some regions persisting until 34 dpa (the latest developmental stage tested). Strong GUS activity was observed throughout the endosperm of heat-shocked seeds until approximately 12 dpa. From 12 to 18 dpa, GUS activity was induced throughout the endosperm, but less activity was observed in nonperipheral cells. By 21 dpa, little or no induction of GUS expression was observed in central starchy endosperm and prismatic cells and induction declined in all cells except those in the transfer region from 21 dpa onwards. By approximately 28 dpa, little or no induction of GUS expression occurred in any cells except those in the transfer region where induction persisted until 34 dpa. No induction of GUS expression was observed in nonheat-shocked seeds (data not shown).

Expression did not spread to nonheat-treated tissue

To investigate the spatial boundaries of the heat-induced expression of GUS, we exposed defined parts of leaves from two independent transgenic lines to heat shock and looked for spread of expression into nonheat-shocked tissue. Lines B2295-R8P2 and B2305-R9P8a were selected for these experiments because, in most previous experiments, heat-induction of GUS expression had been strongest in these lines. Distal and proximal sections of leaves, excised immediately prior to heat shock from two transgenic plants and one null plant for each line, were heat-shocked in a water bath for 2 h at 38 °C as described in the Experimental Procedures. GUS expression was strongly induced in heat-shocked tissue with pronounced delineation between heat-treated and untreated tissue. No GUS staining was observed in null plants irrespective of heat treatment (Figure 6). The same clear-cut delineation of GUS expression between heat-treated and non heat-treated parts of the leaf was observed when parts of the leaves were heat-shocked while still attached to the plant (data not shown). In this case, the treated leaves were maintained at ambient temperature for 24 h before excision, and histochemical GUS assay was performed to determine whether heat-induced GUS expression spread to adjacent tissues after heat shock.

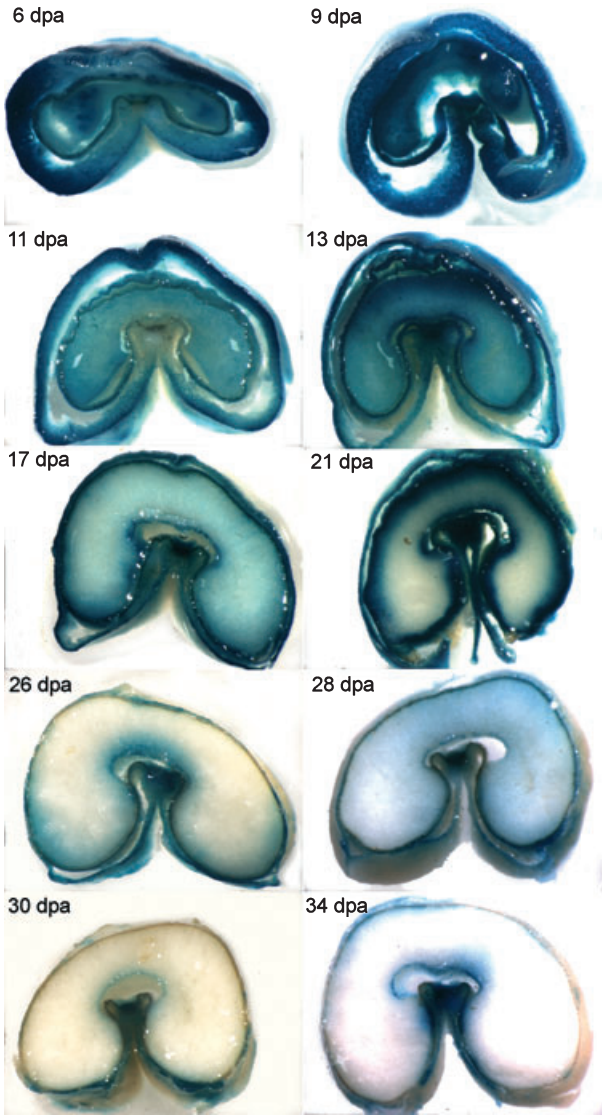

Figure 5 Histochemical localisation of GUS expression in developing seeds from transgenic wheat plants containing HSP::GUS. The experiment was repeated using different transgenic lines and with slightly different time points. Left side: line B2295-R2P1; right side: line B2301-R9P8a. dpa, days postanthesis.

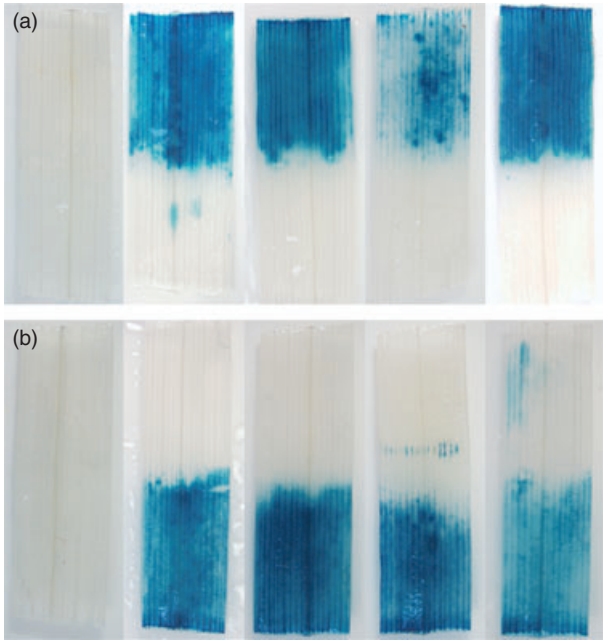

Figure 6 Investigation of transmission of heat-induced GUS expression to noninduced leaf tissue in excised leaves (20–25 cm long) from two independent transgenic wheat lines containing HSP::GUS. All leaf pieces are orientated distal end uppermost and are 2 cm sections spanning the junction of heat-shock/nonheat-shock. Panel (a): distal half heat-shocked; panel (b): proximal half heat-shocked. Both panels, left to right: null; B2295-R8P2 plant 1; B2295-R8P2 plant 2; B2301-R9P8a plant 1; B2301-R9P8a plant 2.

Discussion

We wished to develop a method for inducing expression of candidate genes at particular developmental stages and to target transgene expression to particular tissues or parts of a transgenic wheat plant while leaving other parts unaffected. We also wished to ensure that the induction stimulus was relatively benign and able to be applied precisely. We therefore selected the promoter of the barley *Hvhsp17* gene as capable of fulfilling all of these criteria.

We fused 656 bp of the barley *Hvhsp17* gene promoter, including two heat-shock elements (HSE) and a MRE, to the *uid*A reporter gene. A similar construct had previously been demonstrated to drive heat-inducible expression in maize, barley, durum wheat and hexaploid wheat leaf mesophyll protoplasts (Raho *et al.*, 1995). We showed in transgenic wheat plants that this promoter was induced by heat treatment of 38–40 °C for 1–2 h. Histochemical staining of the heat-shocked tissues gave a deep blue colouration comparable to that of transgenic wheat plants expressing GUS driven by the rice *Act1* or maize *Ubi1* promoters (Jones and Sparks, 2009). Quantitative MUG assays were used to optimise the heat-shock conditions and to estimate the GUS enzyme activity. Replicate treatments from four independent, heat-shocked transgenic lines and controls showed a clear induction of GUS activity with an optimum at 38–40 °C. The maximum leaf GUS activity observed in the temperature range experiments was 97 U 4 MU/min/mg protein (equivalent to 800 pmol 4 MU/min/mg protein). This is within the ranges reported by several authors such as: 83–1250 pmol 4 MU/min/mg protein for Act 1::GUS in transgenic rice (Su *et al.*, 1998), 99–6000 pmol 4 MU/min/mg protein for CaMV 35S in transgenic rice (Terada and Shimamoto, 1990), but lower than the values given for heat-induced *Hvhsp17*::GUS expression in tobacco leaves (range from 1000 to 5000 pmol 4 MU/min/mg protein) (Raho *et al.*, 1996).

In the time course experiments, GUS activity was observed after only 15 min exposure to heat shock and increased until 120 min after which no further increase in GUS activity was observed. This is consistent with the induction of expression of the endogenous gene in barley seedlings (Gulli *et al.*, 2005). These authors isolated total RNA from barley seedlings which had been exposed to heat shock at 40 °C for various time periods and reported no detection of transcript at 0, 5 and 10 min, an increase in transcript from 15 to 120 min, and no further increase beyond 120 min but with the transcript still detectable 22 h postheat shock.

The native *Hvhsp17* gene is known to be induced by heat and the heavy metal cadmium in whole barley seedlings as indicated by northern blots of total seedling RNA (Gulli *et al.*, 2005). However, it was not established whether expression was ubiquitous or restricted to only certain seedling tissues. We have confirmed in transgenic wheat, at a range of stages throughout development, that pHSPdGUS was heat-inducible. GUS expression was observed in many organs and tissues tested; however, in some tissues (such as the immature inflorescence), GUS staining was weak which may be because it is difficult to heat-shock tissues insulated by the stem. The absence of expression in the anther wall is consistent with expression from constitutive promoters maize Ubi1 and rice actin (C.A. Sparks and H.D. Jones, unpublished data). The relatively wide-spread nature of heat-induced GUS expression seen here contrasts with the results of Raho *et al.* (1996) who reported that 1.7 Kb of this promoter induced expression only in vascular bundles of transgenic tobacco and who, because of the similarity between monocot and dicot xylem structure, hypothesised that 'a similar regulation could also operate in cereals'. We have demonstrated, at least in wheat using 656 bp of this promoter, that this is not the case and that the machinery for heat-induction of this promoter is present in many other tissues throughout the plant. Although GUS staining of some heat-shocked tissues (leaves, glumes and palea/lemma) of our transgenic wheat lines was more intense in the vascular bundles, expression was not confined to the vascular bundles in these tissues and expression was observed in other tissues. Both 1.7 Kb and 600 bp (but not 170 bp) of this promoter were sufficient to drive heat-inducible GUS expression in transient assays using protoplasts of cereals but not in tobacco protoplasts (Raho *et al.*, 1995).

We have also demonstrated that expression of the GUS enzyme was tightly confined to only those tissues directly exposed to heat: no GUS staining was observed in neighbouring leaf tissue even 24 h after heat shock of leaves of intact plants, and there was a very clear delineation between heat-shocked and nonheat-shocked tissue. Induction of HSP::GUS was demonstrated in all tissues tested, but there was some line-to-line variation and reduced expression in metabolically less-active and older tissues. In particular, proximal root sections showed less GUS activity than root tips, and there was a gradual decline in expression in endosperm tissue during grain development. In addition, it is known that biolistic transformation tends to generate transgenic plants with higher copy number and more complex integration patterns than methods using *Agrobacterium* often resulting in multiple integration sites and/or multiple copies at a particular locus. The segregation ratios in the T2 generation for the four lines selected for more detailed study indicated that one line may be homozygous and that the other three lines are likely to have single insertion sites (see Results). We did not attempt a molecular characterisation of the transgene copy number or the number of independently segregating insert loci in our lines. Therefore, as transgene insertion sites frequently vary in copy number, it is likely that copy number differs between lines. This factor, together with possible differences in transgene expression levels resulting from effects of integration site (which can result in no expression in some cases), may explain the variation in GUS expression levels observed between lines. To address these sources of variation, it is important that studies of transgene expression are conducted using multiple transgenic lines and more than one plant for each line. In this study, 21 transgenic lines were generated from which seven were selected at random for T2 seed production and four of these selected for further study. Twenty-four T2 seedlings from the four independent transgenic lines were used for preliminary experiments and at least two plants per line used in subsequent experiments. Variation in GUS staining of the same tissues from different plants of a single transgenic line was also observed. This could result from slight differences in the developmental stage of tissues tested or differences in the penetration of the heat shock to tissues that were insulated from the external heatsource (for example, floral tissues enclosed in the floret or immature inflorescences enclosed by the stem) in the experiments for which whole plants were subjected to heat shock in controlled environment chambers.

While several tissue/stage-specific promoters have been identified and characterised (see Introduction), it is not currently possible to target transgene expression to many tissues/developmental stages. The promoter characterised here should therefore prove to be a valuable tool for future studies as it allows transgene expression to be specifically induced in transgenic wheat plants, limited only by the technical ability to apply a heat shock to the desired tissue and developmental stage.

Experimental procedures

Construction of pHSPdGUS

A genomic clone carrying a gene encoding a barley 17.03-kDa heat-shock protein (pHvhsp17) was kindly provided by Prof. N. Marmiroli (University of Parma, Italy) (Marmiroli *et al.*, 1993). This clone was subjected to three rounds of site-directed mutagenesis using the QUIKCHANGE site-directed mutagenesis kit (Agilent Technologies UK Limited, Stockport, Cheshire SK8 3GR, UK). First, a *Nco* I site in the promoter sequence was disrupted using the primer 5′-AAA TTT TAA GAT GGT GCA TGG ACC ACC CGG ACC ACC-3′ (and its reverse compliment) to change C to G at position -643 relative to the start codon. A *Nco* I site was introduced, incorporating the start codon, using the primer 5′-GCA AGC AAC ACC GAC CAT GGC GAT CGT GAG GAG G-3′ (and its reverse compliment) to change A to C and T to G at positions −1 and +4, respectively, relative to the start codon. The primer 5′-CTC CGG C**TG A**GT CCA TGG GAC TGC AGG CCC AAT TCG CCC-3′ (and its reverse compliment) was used to introduce a second *Nco* I site followed by a *Pst* I site closely following the stop codon (shown in bold above). The Hvhsp17 coding sequence was removed by *Nco* I digestion and self-ligation to give pHvhsp-P. pHvhsp-P was digested with *Eco* RI and *Pst* I and with *Apo* I and *Pst* I, and the gel-purified *Eco* RI-*Pst* I 2965 bp vector fragment and an *Apo* I-*Pst* I fragment containing 668 bp of promoter sequence and including the start codon were ligated to give pHvhsp-P670. The resulting plasmid contains an *Nco* I site (incorporating the start codon) followed by a *Pst* I site at the 3′ end of the 656 bp promoter sequence which includes both HSE and the MRE identified by Marmiroli *et al.*, (1993). A *Nco* I-*Pst* I fragment carrying the β-glucuronidase (*uid*A) reporter gene followed by the nopaline synthetase terminator sequence was ligated into *Nco* I-*Pst* I-digested pHvhsp-P670 to give pHSPdGUS (Figure 1).

Wheat transformation and growth conditions

For transient expression assays, immature embryos (0.5–2.5 mm) were isolated and bombarded according to Sparks and

Jones (2004), except that the embryo axis was not removed, and preculture (20 embryos per 9 cm Petri-dish) was on MS media containing 3% sucrose and 0.5% AGARGEL (Sigma-Aldrich, Gillingham, Dorset, UK) overnight at 26 °C in the dark before bombardment at 650 psi. Plates were then cultured as above for a further 48 h with incubation at 40 °C for 60 min after 24 h for heat-shock treatment.

Transgenic wheat (*Triticum aestivum* L. var. Cadenza) lines expressing the *uid*A reporter gene under the control of the heat-shock promoter were generated according to Sparks and Jones (2004). Plasmid pAHC20 (Christensen and Quail, 1996), which contains the *bar* gene, was cobombarded with pHSPd-GUS into immature scutella to allow selection of transformed plantlets in the regeneration phase. Integration of *bar* and *uid*A genes was confirmed by PCR, and T0 plants in which the transgenes were detected by PCR (and null controls) were grown for T1 seed production according to Sparks and Jones (2004). A total of 21 transgenic lines were generated from two separate bombardment experiments of 250 immature embryos each.

Seedlings were produced from seeds which had been sterilised as described by Sparks and Jones (2004). For time course and temperature range experiments, seeds were germinated on sterile Whatman 3MM chromatography paper, prewetted with sterile water in propagator trays, and for whole seedling experiments on prewetted sterile Whatman No.1 9-cm discs in Petri dishes, and then germinated in the dark in a growth room at 22 °C. For production of older plants, seedlings were potted in soil and grown in the glasshouse with 18–20 °C day and 14–16 °C night temperatures with a 16-h photoperiod provided by natural light supplemented with banks of Son-T 400 W sodium lamps (Osram Limited, Langley, Berkshire, UK) giving 400–1000 μmol/m^2/s PAR.

Heat-shock experiments

For temperature range and time course experiments, duplicate pieces of shoot/leaf were submerged in water (equilibrated to the desired temperature) in 15 mL sterile tubes and incubated in a water bath. Water was removed, and some plant tissue was used for histochemical GUS assay immediately and the remainder frozen in liquid nitrogen and stored at −80 °C for fluorometric GUS assay. Explants (including leaf pieces for identification of transgenic plants and null segregants, seeds and embryos) were heat-shocked in sealed 96-well micro-titre plates, with a drop of sterile water in each well to prevent desiccation, at 38 °C for 2 h in an incubator with air circulation.

Whole seedlings were heat-shocked for 2 h, sealed in 9 cm Petri dishes, in a controlled environment (CE) cabinet at 38 °C, 80% relative humidity. Whole plants at 4, 8, 12, 16 and 20 weeks old were removed from pots and soil washed from the root system in tap water. After taking preheat-shock samples, the roots were briefly prewarmed to 34–35 °C while preheat-shock samples were taken from the aerial parts of the plants (approximately 5 min) before placing the plants in the CE cabinet with the root system submerged in water which had been equilibrated to 38 °C in the cabinet overnight. After 2 h, plants were removed from the cabinet and post heat-shock samples taken immediately.

For distal effects experiments, 15 mL Falcon tubes with slots cut in the lids were completely filled with water, submerged to approximately 2 mm from the top of the lids in a 38 °C water bath and allowed to equilibrate. Leaves (20–25 cm long) were excised from 6- to 7-week-old plants and either the distal half or the proximal half fed through the slots in the lids of the Falcon tubes and incubated for 2 h. Leaves were then removed and the heat-shocked half cut into approximately 2 cm lengths, with a further 2 cm section spanning the junction of heat shock/nonheat-shock and a final 2 cm section of nonheat-shocked leaf adjacent to this. Leaf pieces were transferred to 2 mL microtubes for histochemical GUS assay. The distal halves of leaves from intact plants were treated in the same way except that after heat shock, the plants were left at room temperature for 24 h before cutting sections for GUS assays.

Histochemical (GUS) and fluorometric (MUG) assays of GUS activity

Histochemical localisation of GUS activity *in situ* was assayed according to Jefferson *et al.* (1987) by incubation of plant tissue in X-Gluc. solution (0.5 mg/mL 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc.; dissolved at 50 mg/mL in methyl cellusolve), 0.1 M sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide at 37 °C for 16 h. Chlorophyll was then removed from green tissue by immersion in several changes of 70% ethanol, and all samples were stored in 70% ethanol.

GUS activity in leaf extracts was compared quantitatively by monitoring cleavage of the β-glucuronidase substrate 4-methyl-umbelliferyl β-D-glucuronide (MUG) (Gallagher, 1992). The assay was adapted so that large numbers of samples could be assayed and measured in a 96-well plate format. For leaves, 3×0.5 cm^2 sections were placed in an individual 1.5 mL microfuge tube and ground in 1 mL ice-cold extraction buffer (50 mM $NaHPO_4$ (pH7.0), 10 mM β-Mercaptoethanol, 10 mM Na_2EDTA, 0.1% Sarcosyl, 0.1% Triton X-100). Following extraction, tubes were vortexed and placed on ice for 10 min, centrifuged in a microfuge at 11 300 ***g*** for 10 min at 4 °C and the supernatant was transferred to a clean tube on ice. Assay reactions were prepared on ice by adding 500 μL sample extract to 500 μL substrate buffer (2 mM 4-methyl umbelliferyl β-D-glucuronide hydrate (MUG) (Sigma-Aldrich), mixing, then incubating at 37 °C, taking 100 μL samples at time 0, 5, 10 min and subsequently every 10 up to 90 min. Samples at each time point were added to individual wells of a microtitre plate containing 200 μL stop buffer (0.2 M Na_2CO_3). Once all time points had been collected, the plate was read in a Fluoroskan II fluorimeter plate reader (Flow Laboratories Ltd), set at 37 °C with excitation 365 nm and emission 455 nm. A negative control (500 μL extraction buffer + 500 μL substrate buffer) and positive control reaction [480 μL extraction buffer + 500 μL substrate buffer + 20 μL GUS enzyme (Sigma-Aldrich)] was set up for each assay. Protein content of each extract was determined using a Pierce BCA (Bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific, Cramlington, Northumberland, UK), with Bovine serum albumin (BSA) standard samples 0, 0.025, 0.125, 0.25, 0.5, 0.75, 1.0 mg/mL. Protein was measured using a spectrophotometer with plate reader at wavelength 562 nm. Fluorescence units were plotted against 4-MU concentration to create a standard curve. To calculate final fluorometric values, the negative control value was subtracted from all positive controls and test samples, drawing a graph of fluorometric (Fl) units against time (min). The linear part of the trace was established to obtain a trend line and the slope of this line determined and

expressed as Fl units per min. Using results from the protein determination assay, U/min/mg protein was determined. (1 U 4 MU/min/mg protein = 8.3 pmol/min/mg protein).

DNA preparation and PCR assays

Genomic DNA was prepared using the Promega Wizard™ Genomic DNA Purification Kit according to the Manufacturer's instructions. For identification of transgenic plants and null segregants, PCRs contained 1× REDDYMIX (Thermo Fisher Scientific), 0.3 μM each primer and I μL genomic DNA in a final volume of 20 μL. For PCRs of DNA prepared from leaf tissue after histochemical GUS assay reactions were as described earlier except that 15–100 ng DNA was used and reactions were in a final volume of 12.5 μL. PCR primers HSP3′fwd (5′-AGC AAA AGC GAA CAA CAT CCT ACC-3′) and GUS5′rev (5′-CGC TGA TCA ATT CCA CAG TTT TCG-3′) were used to test for integration of the heat-shock promoter-*uid*A fusion and amplify a 182 bp product. Cycling conditions were 95 °C, 2 min; 30 cycles of 95 °C, 30 s; 55 °C, 45 s; 72 °C, 60 s followed by a final extension of 72 °C for 10 min. PCR primers Bar1 (5′-GTC TGC ACC ATC GTC AAC C-3′) and Bar2 (5′-GAA GTC CAG CTG CCA GAA AC-3′) were used to test for integration of the selectable marker and amplify a 444 bp product. Cycling conditions were 95 °C, 5 min; 30 cycles of 95 °C, 60 s; 57 °C, 30 s; 72 °C 2 min followed by a final extension of 72 °C for 10 min. PCR products were visualised on 1% agarose gels containing ethidium bromide.

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