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Review

Transgene expression systems in the *Triticeae* cereals

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SUMMARY

The control of transgene expression is vital both for the elucidation of gene function and for the engineering of transgenic crops. Given the dominance of the *Triticeae* cereals in the agricultural economy of the temperate world, the development of well-performing transgene expression systems of known functionality is of primary importance. Transgenes can be expressed either transiently or stably. Transient expression systems based on direct or virus-mediated gene transfer are particularly useful in situations where the need is to rapidly screen large numbers of genes. However, an unequivocal understanding of gene function generally requires that a transgene functions throughout the plant's life and is transmitted through the sexual cycle, since this alone allows its effect to be decoupled from the plant's response to the generally stressful gene transfer event. Temporal, spatial and quantitative control of a transgene's expression depends on its regulatory environment, which includes both its promoter and certain associated untranslated region sequences. While many transgenic approaches aim to manipulate plant phenotype via ectopic gene expression, a transgene sequence can be also configured to down-regulate the expression of its endogenous counterpart, a strategy which exploits the natural gene silencing machinery of plants. In this review, current technical opportunities for controlling transgene expression in the *Triticeae* species are described. Apart from protocols for transient and stable gene transfer, the choice of promoters and other untranslated regulatory elements, we also consider signal peptides, as they too govern the abundance and particularly the sub-cellular localization of transgene products.

Contents

Introduction

The *Triticeae* tribe within the *Pooideae* subfamiliy of the grass family *Poaceae* includes ca. 350 annual and perennial species assigned to ca. 30 genera out of which only about half are commonly accepted among taxonomists (Barkworth and von Bothmer, 2009). Thanks to their outstanding importance as food, feed and industrial raw material, the small grain cereals bread wheat (*Triticum aestivum*), durum wheat (*Triticum turgidum* ssp. *turgidum*), barley (*Hordeum vulgare*), rye (*Secale cereale*) and triticale (*x Triticosecale*) are the most prominent representatives of the *Triticeae*. Over the last decade, technological developments have moved the genetic transformation of the *Triticeae* cereals from being difficult

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to achieve to becoming routine. The pressure of continued human population growth and the probability of non-beneficial climate change only serve to increase the need to increase the supply of plant-based food and energy, and transformation technology will play a part in this effort—not just in the generation of superior cultivars, but also in the elucidation of gene function. In plant cells which are directly accessible to transformation, both transient over-expression (TOX) and transient-induced gene silencing (TIGS) approaches have been elaborated to provide rapid screening methods for large numbers of genes. As a contrast, some viral vectors can be exploited to ensure the systemic dispersal of a transiently expressed transgene beyond the site of primary infection. Promoter sequences are the key to the spatial, temporal and quantitative pattern of transgene expression, particularly in stable transformants, where the transgene is ubiquitously present.

A major limiting factor inhibiting advances in *Triticeae* transgene technology is that many of the best characterized plant promoters have been developed in dicotyledonous species; many of these have proven to be inefficient or ineffective in a monocotyledonous environment, for reasons which remain ill understood. Nevertheless, the number of functional transgene promoters in the *Triticeae* has been increasing with time. Transgene expression, and the subsequent site of transgene product accumulation can further be manipulated by the judicious choice of non-promoter regulatory elements such as introns and signal peptides, respectively. Transformation technology can take advantage of the complexity of gene regulation, by deliberately engineering these components of a given transgene's sequence.

Here we review the current state-of-the-art of *Triticeae* transgene expression systems. We cover both transient and stable gene transfer methods, as both are important for specific applications. A particular focus has been given to promoters and other regulatory sequences which can be exploited to control the localization in time and space and/or the abundance of transgene transcript and gene product in transgenic *Triticeae* species.

Methods of gene transfer

Transient expression systems

The value of transient transgene expression in plants has been only recently comprehensively reviewed (Jones et al., 2009; Shepherd, 2009). Briefly, "transient" implies expression over a period ranging from a few hours to several days. Transience, in part at least, reflects the expression of non-integrated recombinant DNA, which by definition is not replicated subsequent to its introduction. In some cases, genomic integration does occur, but the period of transgene expression is delimited by the life span of the recipient cell, which is unable to proliferate under the imposed experimental conditions. Many transient expression systems rely for their efficacy on supplying a large number of transgene copies per transformed cell, while in stable transgenics, a high transgene copy is frequently associated with silencing rather than amplification of transgene expression. Furthermore, transgene expression is not, in the case of transient systems, dependent on the genomic site of integration. The phenotypic consequences of transient transgenesis are often more consistently detectable, although post-transcriptional transgene silencing resulting from high transgene copy numbers can also affect transient expression systems.

A major feature of transient expression is that it avoids the time-consuming and laborious generation and maintenance of stable transgenic lines. This advantage is especially relevant in the context of the cereals which remain less readily amenable to stable transformation than are many dicotyledonous species. A range of physical, chemical and biological methods of DNA delivery into various cereal tissue types is available, making transient expression assays particularly attractive both for basic research and for some biotechnological applications.

The development of the micro-projectile (tungsten or gold) bombardment (or so called "biolistic" or "ballistic") technique of delivering DNA into a cereal cell represented an important breakthrough for the assessment of gene function via transient over-expression. As each transfer event necessarily affects only an individual cell, the expression of the transgene can be monitored at the single cell level. DNA vectors designed for transient over-expression need not include more than the target expression cassette. Since the biolistic approach allows for the inclusion of multiple vectors in a single bombardment, various transgenes and/or construct types such as scorable markers (*gus*, Jefferson, 1987 or *gfp*, Davis and Vierstra, 1998) can be simultaneously assayed in the same cell. Co-bombardment can also be used to genetically modify the target cell to provide the appropriate conditions for transgene expression; an example of this approach was the use of an *Mlo* over-expression construct to suppress the basal powdery mildew (*Blumeria graminis*) resistance of bombarded barley cells, and in so doing, produce a host–pathogen interaction environment which was more conductive for the expression of the transgenes under test (Shirasu et al., 1999; Elliott et al., 2002).

The biolistic method has been extensively applied in the *Triticeae* cereals to estimate the activity of promoter/*gus*-fusions. It has been repeatedly demonstrated that the number of detectable GUS-positive cells is correlated with promoter activity (Onate et al., 1999; Rubio-Somoza et al., 2006). More recently, a comparison of the strength of four different promoters in stably transgenic barley lines with their activities in transiently transformed leaves also revealed a strong correlation (Himmelbach et al., 2010). Consequently, transient tests can provide reliable information on the relative promoter activity in transgenic plants, yet absolute quantitative levels of expression unfortunately cannot be predicted in this way for stably transformed lines. Thus, stably transformed plants remain the gold standard for promoter studies, however, the biolistic approach can provide a viable alternative, especially where the aim is to pre-screen many candidate sequences or to rapidly acquire comparative gene expression information.

Although the biolistic method is not restricted to any particular plant cell type, the target cells clearly need to be physically accessible to bombardment, a requirement which does limit its applicability in the cereals. Most transient expression experiments in this group of plants have therefore been conducted on either the grain endosperm (Knudsen and Müller, 1991) or the leaf epidermis (Douchkov et al., 2005). Since only a small proportion of the cells is transformed, the accumulation of sufficient material for molecular biological and biochemical analysis can be problematical. This limitation has driven the development of enhanced tissue capture methods such as laser capture micro-dissection (Emmert-Buck et al., 1996) and microfluidic single-cell analysis (Stone et al., 2004; Marcus et al., 2006a,b). The ease and rapidity of the transient expression approach have been greatly improved by the recent combination of GATEWAY^{TM} cloning technology with robotics-based microscopic evaluation. It is now technically possible to perform functional analyses of thousands of genes within only a fraction of the time needed to generate sufficient populations of stably transgenic plants (Douchkov et al., 2005; Ihlow et al., 2008). The benefit of this high-throughput approach system has been well illustrated by the exploration of both the host–pathogen interaction in the barley leaf epidermis (Panstruga, 2004; Zimmermann et al., 2006; Shen et al., 2007) and by a phenomics-based study of gene expression in dehydration-stressed barley (Marzin et al., 2008).

The expression of genes responsible for the accumulation of protein in the grain is of particular interest in the cereals, and has been led by the development of a polyethylene glycol (PEG)-mediated gene transfer method applied to barley and wheat

Table 1
Promoters shown to be functional in the *Triticeae* cereals. *Bgh* – *Blumeria graminis* f.sp. *hordei*; *Bgt* – *Blumeria graminis* f.sp. *tritici*; E – explants tested by transient expression assay; S – promoter tested in stably transgenic plants; T – promoter tested by transient expression assay.

Promoter	Specificity; inducer	Transgene	Target species	Reference
Ubiquitous				
Cauliflower Mosaic Virus 35S (*35S*)	Ubiquitous	*bar*	Barley^{S}	Wan and Lemaux (1994)
		gfp	Wheat^{S}	Furtado and Henry (2005)
Sugarcane Bacilliform Virus (*ScBV*)	Ubiquitous	*gus*	Barley^{S}, Wheat^{S}	Al-Saady et al. (2004)
Maize *ALCOHOL DEHYDROGENASE 1* (*ADH1*)	Ubiquitous	*gus*	Barley^{S}	Wan and Lemaux (1994)
			Wheat^{S}	Nehra et al. (1994)
Maize *UBIQUITIN-1* (*UBI-1*)	Ubiquitous	*gus, gfp*	Barley^{S}	Murray et al. (2004)
		gus	Wheat^{S}	Stoeger et al. (1999a)
		bar	Rye^{S}	Popelka et al. (2003)
		gfp	Triticale^{S}	Hensel et al. (2009)
Maize *HISTONE 2B* (*H2B*)	Ubiquitous	*gus*	Wheat^{S}	Rasco-Gaunt et al. (2003)
Maize *SUPPRESSOR MUTATOR* (*SPM*)	Ubiquitous	*phiC31 INTEGRASE*	Wheat^{S}	Rubtsova et al. (2008)
Recombinant *emu*	Mature embryo protoplasts^{E}, ubiquitous	*gus*	Wheat^{T}	Last et al. (1991)
	Ubiquitous	*pat, npt*	Wheat^{S}	Chamberlain et al. (1994)
Rice *ACTIN 1* (*ACT1*)	Ubiquitous	*gus*	Wheat^{S}	Becker et al. (1994)
		gfp		Primavesi et al. (2008)
		gus	Barley^{S}	Tingay et al. (1997)
Rice *Tungro Bacilliform Virus*	Phloem, ubiquitous	*gus*	Wheat^{S}	Mathur and Dasgupta (2007)
Wheat *BENZOXAZINONE 3* (*BX3*)	Mesophyll protoplasts^{E}, ubiquitous	*luc*	Wheat^{T}	Nomura et al. (2008)
Wheat *BENZOXAZINONE 4* (*BX4*)	Mesophyll protoplasts^{E}, ubiquitous	*luc*	Wheat^{T}	Nomura et al. (2008)
Grain				
Barley *HORDEIN B1* (*HOR-B1*)	Endosperm	*gus*	Barley^{T}	Knudsen and Müller (1991)
		gfp, gus	Barley^{S}, Wheat^{S}	Cho et al. (1999, 2002), Vickers et al. (2006) Piston et al. (2008)
Barley *HORDEIN C* (*HOR-C*)	Immature endosperm^{E}; Nitrogen-modulated	*gus*	Barley^{T}	Entwistle et al. (1991), Müller and Knudsen (1993)
Barley *HORDEIN D* (*HOR-D*)	Endosperm	*gfp, gus*	Barley^{S}, Wheat^{S}	Cho et al. (1999, 2002), Piston et al. (2008)
Barley *Bifunctional α-AMYLASE/SUBTILISIN INHIBITOR* (*ISA*)	Pericarp, embryo and aleurone	*gfp*	Barley^{S}	Furtado et al. (2009)
Barley *α-AMYLASE*	Aleurone protoplasts^{E}	*HvXYN1*	Barley^{T}	Caspers et al. (2001)
Barley *β-AMYLASE*	Sub-aleurone	*gus*	Barley^{S}	Okada et al. (2000)
Wheat *α-GLIADIN*	Endosperm	*gus*	Wheat^{S}	Van Herpen et al. (2008)
Wheat *γ-GLIADIN*	Endosperm	*gus*	Wheat^{S}	Piston et al. (2009)
Wheat *High-molecular-weight GLUTENIN 1-D1* (*HMWGLU-1 D1*)	Endosperm	*gus*	Wheat^{S}	Lamacchia et al. (2001)
	Endosperm, anther	*SnRK1*	Barley^{S}	Zhang et al. (2001)
Wheat *High-molecular-weight GLUTENIN 1Bx17* (*HMW 1Bx17*)	Endosperm^{E}	*gus*	Wheat^{T}	Oszvald et al. (2008)
Wheat *Low-molecular-weight GLUTENIN G1D1* (*LMWG1D1*)	Endosperm	*gus*	Barley^{S}	Stoeger et al. (1999a)
Wheat *PUROINDOLINE a* (*PINa*)	Endosperm	*gus*	Wheat^{S}	Wiley et al. (2007)
Wheat *PUROINDOLINE b* (*PINb*)	Endosperm	*gus*	Wheat^{S}	Wiley et al. (2007)
Oat *GLOBULIN 1* (*GLO1*)	Endosperm	*gfp*	Barley^{S}	Vickers et al. (2006)
Rice *GLUTENIN B1* (*GLUB-1*)	Endosperm	*XYNA D2*	Barley^{S}	Patel et al. (2000)
Barley *ISOAMYLASE* (*ISA1*)	Endosperm^{E}	*gfp*	Barley^{T}	Sun et al. (2003)
Barley *(1,3-1,4)-β-GLYCANASE ISOENZYME EII* (*EII*)	Endosperm	*E. coli EII*	Barley^{S}	Jensen et al. (1996)
Wheat *GRANULE-BOUND STARCH SYNTHASE 1* (*GBSS1*)	Central starchy endosperm	*gus*	Wheat^{S}	Kluth et al. (2002)
Wheat *ADP GLUCOSE PYROPHOSPHORYLASE* (*AGP2*)	Endosperm incl. aleurone	*gus*	Wheat^{S}	Thorneycroft et al. (2003)
Barley *LIPID TRANSFER PROTEIN W1* (*LTP W1*)	Aleurone	*gus*	Wheat^{S}	Simmonds et al. (2000)
Barley *JEKYLL*	Nucellar projection	*gfp*	Barley^{S}	Radchuk et al. (2006)
Maize *MYB-RELATED PROTEIN 1* (*MRP-1*)	Transfer cells	*gus*	Barley^{S}	Barrero et al. (2009)
Wheat *PATHOGENESIS-RELATED 60* (*PR60*)	Transfer cells	*gus*	Barley^{S}, Wheat^{S}	Kovalchuk et al. (2009)
Rice *PATHOGENESIS-RELATED 9* (*PR9*)	Transfer cells	*gus*	Barley^{S}	Li et al. (2008)
Rice *PATHOGENESIS-RELATED 602* (*PR602*)	Transfer cells	*gus*	Barley^{S}	Li et al. (2008)
Barley *EARLY-MATURING* (*EM*)	Pericarp, embryo, aleurone	*gfp*	Barley^{S}, Wheat^{S}	Furtado and Henry (2005), Furtado et al. (2009)

Table 1 (*Continued*)

Promoter	Specificity; inducer	Transgene	Target species	Reference
Barley *LIPID TRANSFER PROTEIN 6* (*LTP6*)	Pericarp, epidermis^{E}	*gus* *gfp*	Barley^{S} Barley^{T}	Federico et al. (2005)
Barley *DEHYDRIN 12* (*DHN12*)	Embryo^{E}	*C1, Bperu*	Wheat^{T}	Doshi et al. (2006)
Barley *LIPID TRANSFER PROTEIN 1* (*LTP1*)	Embryo	*C1, Bperu*	Wheat^{S} Triticale^{S}	Doshi et al. (2007)
Barley *TRYPSIN INHIBITOR 1* (*LTR1*)	Embryo^{E}	*C1, Bperu*	Wheat^{T}	Doshi et al. (2006)
Leaf				
Wheat *GLUTATHIONE S TRANSFERASE A1* (*GSTA1*)	Leaf epidermis	*TaPERO*	Wheat^{S}	Altpeter et al. (2005)
Arabidopsis *SENESCENCE-ASSOCIATED GENE 12* (*SAG12*)	Senescent tissue	*LTP*	Wheat^{S}	Sykorova et al. (2008)
Bract				
Barley *LEMMA 1* (*LEM1*)	Bract, spike^{E}	*gfp*	Barley^{T}, Wheat^{S}	Skadsen et al. (2002), Somleva and Blechl (2006)
Barley *LEMMA 2* (*LEM2*)	Lemma, palea, coleoptile	*gfp*	Barley^{S}	Abebe et al. (2006)
Phloem				
Rice *SUCROSE SYNTHASE 1* (*SS1*)	Phloem	*GNA*	Wheat^{S}	Stoeger et al. (1999b)
Tapetum				
Rice *TAPETUM-SPECIFIC* (*OSG6B*)	Tapetum	*Split-BARNASE*	Wheat^{S}	Kempe et al. (2010)
Abiotic stress-induced				
Arabidopsis *RESPONSIVE TO DESICCATION STRESS* (*RD29*)	Stress induced	*AtDREB1A*	Wheat^{S}	Pellegrineschi et al. (2004)
Barley *COLD-REGULATED GENE* (*COR14b*)	Leaf^{E}, cold-induced	*gus*	Barley^{T}	Dal Bosco et al. (2003)
Barley *DEHYDRIN 4s* (*DHN4s*)	Germinated seedling^{E}; dehydration-, NaCl-, ABA-induced	*gus, gfp*	Barley^{T}	Xiao and Xue (2001)
Barley *FRUCTAN 6 FRUCTOSYLTRANSFERASE* (*6-SFT*)	Leaf^{E}; sucrose-induced	*gus*	Barley^{T}	Nagaraj et al. (2001)
Barley *LIPID TRANSFER PROTEIN* (*LTP4.2*)	Leaf^{E}; cold-induced	*gus*	Barley^{T}	Molina et al. (1996)
Barley *LIPID TRANSFER PROTEIN* (*LTP4.3*)	Leaf^{E}; cold-induced	*gus*	Barley^{T}	Molina et al. (1996)
Barley *LOW TEMPERATURE-RESPONSIVE GENE* (*BLT4.9*)	Shoot meristem^{E}; cold-induced	*gus*	Barley^{T}	Dunn et al. (1998)
Barley *LOW TEMPERATURE-RESPONSIVE GENE* (*BLT101.1*)	Leaf^{E}; cold-induced	*gus*	Barley^{T}	Brown et al. (2001)
Barley *PHOSPHATE TRANSPORTER GENE* (*PHT1;1*)	Low phosphate-induced	*gfp*	Barley^{S}	Schünmann et al. (2004)
Grape *STILBENE SYNTHASE* (*VST1*)	Wound-induced	*gus, VST1*	Barley^{T}	Leckband and Lörz (1998)
ABA element with rice *ACTIN 1* core promoter (*ACT*-100)	Stress-induced	*VaP5CS*	Wheat^{S}	Gruszka Vendruscolo et al. (2007)
Rice *LATE EMBRYOGENESIS ABUNDANT* (*HVA1s*)	Germinated seedling^{E}, root^{E};	*gus, gfp*	Barley^{T}	Xiao and Xue (2001)
	Dehydration-, NaCl-, ABA-induced	*XYLANASE (XYNA)*		Xue (2003)
Rice *RESPONSIVE TO ABA* (*RAB16Bj*)	Germinated seedling^{E}; Dehydration-, NaCl-, ABA-induced	*gus, gfp*	Barley^{T}	Xiao and Xue (2001)
Rice *WATER STRESS-INDUCED* (*WSI18j*)	Germinated seedling^{E}; Dehydration-, NaCl-, ABA-induced	*gus, gfp*	Barley^{T}	Xiao and Xue (2001)
Sweet potato *PEROXIDASE* (*SWPA2*)	Oxidative stress-induced	*AtNDPK2*	Barley^{S}	Um et al. (2007)

Table 1 (*Continued*)

Promoter	Specificity; inducer	Transgene	Target species	Reference
Wheat *ABIOTIC STRESS-INDUCED DEHYDRATATION RESPONSIVE ELEMENT-BINDING FACTOR* (*AIDFa*)	Callus^{E}; NaCl-, drought (PEG)-, cold-, ABA-induced	*gus*	Wheat^{T}	Xu et al. (2008)
Wheat *COLD-SPECIFIC* (*WCS120*)	Leaf^{E}; cold-induced	*luc*	Barley^{T}, Wheat^{T}, Rye^{T}	Ouellett et al. (1998)
Wheat *HIGH AFFINITY PHOSPHATE TRANSPORTER* (*PT2*)	Root; low phosphate-induced	*gus*	Wheat^{S}	Tittarelli et al. (2007)
Wheat *VERNALIZATION-RELATED GENE* (*VER2*)	Leaf^{E}; vernalization-induced	*gfp*	Wheat^{T}	Xu et al. (2004)
Barley *ABSCISIC ACID-INDUCED PROTEIN* (*HVA22*)	Grain^{E}; ABA-induced	*gus, gfp*	Barley^{T}	Zou et al. (2004, 2008)
Barley *DEHYDRIN* (*DHN1-2*)	Aleurone, Grain^{E}; ABA-induced	*gus*	Barley^{T}	Robertson (2003)
Wheat *EARLY METHIONINE-LABELED POLYPEPTIDE GENE* (*EMH5*)	Embryo^{E}; Mannitol-(osmotically), ABA-induced	*gus*	Barley^{T}, Wheat^{T}	Onde et al. (1994)
Wheat *EARLY METHIONINE-LABELED POLYPEPTIDE GENE* (*CS41*)	Aleurone protoplasts^{E}; ABA induced	*gus*	Barley^{T}	Onde et al. (1994)
Biotic stress-induced				
Agrobacterium tumefaciens MANNOPINE SYNTHASE (*MAS*)	*Bgh*-, *Fusarium graminearum*-induced	Metchni-kowin	Barley^{S}	Rahnamaeian et al. (2009)
Barley *GERMIN-LIKE PROTEIN* (*GER4a, GER4b, GER4d, GER4e, GER4f*)	Leaf^{E}; *Bgh*-induced	*gus*	Barley^{T}	Himmelbach et al. (2010)
Barley *GERMIN-LIKE PROTEIN* (*GER4c*)	Coleoptile, root (weak), epidermis; *Bgh*-, *Bgt*-, *Rhynchosporium secalis*-induced	*gus*	Barley$^{T,\ S}$, Wheat^{T}	Himmelbach et al. (2010)
Barley *LIPID TRANSFER PROTEIN* (*LTP4.3*)	Leaf^{E}; *Xanthomonas campestris pv. translucens*-induced	*gus*	Barley^{T}	Molina et al. (1996)
Barley *STEM RUST RESISTANCE GENE* (*RPG1*)	Epidermis, Leaf^{E}; *Puccinia graminis* induced	*HvRPG1*	Barley^{T}	Horvath et al. (2003)
Grape *STILBENE SYNTHASE* (*VST1*)	Immature embryo^{E}, Leaf; *Bgh*-, *Botrytis cinera*-induced; *Puccinia recondita* f.sp. *tritici*-, *Septoria nodorum* -induced	*gus, VST1*	Barley^{T} Barley^{S} Wheat^{S}	Leckband and Lörz (1998) Serazetdinova et al. (2005)

Table 1 (*Continued*)

Promoter	Specificity; inducer	Transgene	Target species	Reference
Maize *PR PROTEIN* (*PRms*)	Aleurone protoplasts^{E}; *Fusarium moniliforme* (elicitor)-induced	*cat*	Barley^{T}	Raventos et al. (1995)

endosperm-derived protoplasts (Lee et al., 1989, 1991; Diaz and Carbonero, 1992). A major output of this line of research has been an understanding of the activity of a number of grain-specific gene promoters (Table 1). The same gene transfer method has been applied to protoplasts derived both from embryonic wheat cell suspensions (Ahmed et al., 1997) and from barley leaves (Junker et al., 1987). Protoplasts derived from roots, leaves, developing endosperm or aleurone of mature endosperm retained their tissue specificity to some extent, thus permitting an analysis of regulatory elements in promoters (Diaz et al., 1995). More recently, protoplasts from wheat leaf mesophyll were also used to analyse the role of proteins in photo-oxidative stress tolerance. Transformed protoplasts over-expressing superoxide dismutase revealed a lower level of oxidative damage, whereas the over-expression of glutathione reductase showed no effect. Both enzymes were targeted to the chloroplasts using a signal peptide, and their expression was documented using activity measurements (Melchiorre et al., 2009). As an alternative to PEG-mediated DNA uptake, electroporation was employed to transiently transform cereal protoplasts. Depending on the starting material both methods showed different efficacies. While electroporation was more efficient using protoplasts derived from leaf and root material, protoplasts from endosperm were solely amenable to PEG-mediated gene transfer (Diaz, 1994). In all cases the conditions for the isolation of the protoplasts from different tissues and the technical parameters of gene transfer required careful optimisation. Although the establishment of transient gene expression in protoplasts is technically challenging, this approach is particularly useful to rapidly assess gene function and offers a viable alternative to the time-consuming stable transformation of *Triticeae* cereals.

A more recent development has flowed from the discovery of cell-penetrating peptides (CPPs), which *in vivo* are thought to be involved in the translocation of bioactive macromolecules across the plasmamembrane (Jarver and Langel, 2006). These have been exploited to deliver to permeabilized wheat zygotic immature embryo cells not only large proteins such as the 270 kDa GUS (β-glucuronidase), but also a 7.2 kb reporter plasmid (Chugh and Eudes, 2008). In the same study, the expression of the *gus* reporter gene was further enhanced by the addition of Lipofectamine™ 2000. CPPs did not appear to have any cytotoxic effect, as embryo germination was not compromised. They represent therefore a promising avenue for improving the efficacy of gene transfer and expression analysis in the cereals.

Transiently induced gene silencing (TIGS) in single epidermal cells achieved via biolistic transformation is now a well-established means of studying cell-autonomous phenomena such as the interaction between host and pathogen (Schweizer et al., 1999; Panstruga, 2004) and the drought response (Marzin et al., 2008). Its principle is based on the RNA-interference (RNAi) phenomenon, in which the transgene is composed of two inverted repeats of the target gene sequence (Schweizer et al., 1999; Taylor and Fauquet, 2002; Altpeter et al., 2005). A side effect of the bombardment process is that the target cells suffer from a relatively high level of stress, and this can have a major influence on the interpretation of the resulting data. For example, wheat expresses a strong stress-induced resistance, which is a particular problem for TIGS experiments where a period of 48–96 h post-bombardment is typically required for an effective depletion of the cellular pool of the targeted protein. This problem has been partially resolved using constructs designed to reduce the basal stress resistance response (Shirasu et al., 1999; Elliott et al., 2002).

Virus-induced transgene expression profits from two major advantages. First, transgene expression is not confined to the sites of viral infection, because a gene silencing signal spreads systemically across the plant, and second, a comparatively high transgene dosage associated with high expression can be achieved thanks to virus proliferation in infected cells (Schwach et al., 2005; Ding and Voinnet, 2007; Garcia-Ruiz et al., 2010). Several modified viruses are available to over-express foreign genes in dicotyledonous plants. However, only few positive-strand RNA viruses are capable to systemically infect cereals. In a previous study, French et al. (1986) showed that Brome mosaic virus (BMV) can be used to transiently express recombinant genes in barley protoplasts. It has a comparatively broad host range including monocots and dicots (De Jong and Ahlquist, 1995). Later, Choi et al. (2000) described a modified version of the Wheat streak mosaic virus (WSMV), permitting the insertion of foreign sequences into the viral polyprotein open reading frame. In the same study, the foreign polypeptide was shown to be released from the polyprotein by proteolytic cleavage and found to be substantially over-expressed in systemically infected leaves of wheat and barley. Similarly, Barley stripe mosaic virus (BSMV) has been developed as an alternative vector for systemic transient over-expression in wheat (Tai and Bragg, 2007). However, there have been no further reports on the application of virus-induced over-expression in the *Triticeae*, which is likely due to the problem that systemic spread of the virus takes some days and thus may also result in virus-induced gene silencing. Nevertheless, this method is still considered to be a potential alternative approach to transient gene expression especially for those tissues that are not well accessible for biolistics.

Virus-induced gene silencing (VIGS) transiently down-regulates targeted host genes and provides an additional powerful tool for plant reverse genetics (Baulcombe, 1996; Ruiz et al., 1998). It is particularly useful when genes that cause lethality are to be knocked out. The approach exploits the fact that RNA viruses produce double-stranded RNA (dsRNA) molecules during replication that in turn trigger the plant RNAi defense mechanism. This conserved, natural response prevents the plants from accumulating potentially dangerous viral RNAs. Therefore, upon the integration of an appropriate plant target sequence into the viral genome, almost all host genes can be silenced along with the invading viral RNA. In the *Triticeae*, only two vectors based on BSMV and BMV have thus far been shown to be functional in VIGS approaches (Holzberg et al., 2002; Ding et al., 2006). The characteristics of these two vector systems have been reviewed recently in detail (Hein et al., 2009; Cakir et al., 2010). BSMV is a positive-sense RNA virus and represents the type member of the *Hordeiviridae*. Its genome is composed of three single-stranded RNAs designated α, β and γ RNA. The full-length γ RNA-derived cDNA was cloned and modified to allow for the expression of foreign DNA inserts in sense, antisense or inverted repeat orientation (Petty et al., 1989). In the monocotyledons, VIGS was pioneered by the use of BSMV to down-regulate the *PHYTOENE DESATURASE* (*PDS*) gene in barley (Holzberg et al., 2002). PDS is required for the synthesis of carotenoids, which protect chlorophyll from photo-bleaching. Successfully silenced tissues showed reduced *PDS* mRNA levels and displayed white, photo-bleached areas, which appeared as early as 5 days after inoculation (Bruun-Rasmussen et al., 2007). In BSMV, the effective plant gene insert size was typically between about 120 and 500 bp (Scofield et al., 2005; Bruun-Rasmussen et al., 2007). Long inserts interfered with virus accumulation, thereby imposing a selection pressure for the loss of the plant gene insert. This inherent instability could also explain the transient nature of VIGS mediated by the BSMV (Bruun-Rasmussen et al., 2007). However, Bruun-Rasmussen et al. (2007) also showed that BSMV-induced gene silencing can be seed-transmissible. BSMV-VIGS was documented in numerous studies as a powerful tool for functional genomics of disease resistance in wheat and barley (Scofield et al., 2005; Hein et al., 2005; Cloutier et al., 2007; Zhou et al., 2007; Brueggeman et al., 2008; Meng et al., 2009; Hu et al., 2009). This system also facilitates the simultaneous knock-down of two plant genes represented in a single BSMV-VIGS construct, albeit with a lower efficiency as compared to single-gene approaches (Cakir and Tör, 2010). As a particular advantage, the simultaneous silencing of two genes offers the possibility to readily indicate the knock-down of a gene that itself lacks any phenotype when silenced individually. Furthermore, simultaneous silencing of genes may help to manipulate more complex regulatory pathways or networks. Until recently, all studies using BSMV-VIGS involved *in vitro* transcription for the generation of infectious RNA for plant inoculations. The development of BSMV-VIGS DNA vectors and their transfer to plant material using particle bombardment eventually rendered VIGS amenable for high-throughput applications (Meng et al., 2009). BMV was also reported to be a suitable vector for VIGS in barley (Ding et al., 2006). Despite its establishment and some useful modifications towards reduced viral pathogenicity and improved vector accumulation in recipient cells (Hema and Kao, 2004; Ding et al., 2006), BMV-based VIGS has not yet been applied for the functional analysis of novel gene candidates in the *Triticeae*.

Taken together, several well-established methods of transient expression tailored for different requirements are available for the *Triticeae* cereals. Especially in combination with high-throughput analysis approaches these can greatly facilitate the rapid functional validation of candidate genes.

Stable transgenesis

The generation of stably transgenic plants was long considered as difficult in monocotyledonous species, both because they are less easily infected by *Agrobacterium* spp. and because it is less straightforward to regenerate shoots *in vitro* from somatic tissue. However, most of these perceived limitations have been removed by the judicious choice of explant and technical advances in cell culture and gene transfer methods. The development of technology to stably transform temperate cereals has been comprehensively reviewed elsewhere (Kumlehn and Hensel, 2009).

In brief, the biolistic transformation of immature embryos emerged as the early method of choice. Protocols established for wheat (Becker et al., 1994; Nehra et al., 1994; Rasco-Gaunt et al., 2001) and barley (Wan and Lemaux, 1994) were rapidly adapted for cereal rye (Castillo et al., 1994; Popelka et al., 2003), triticale (Zimny et al., 1995) and macaroni wheat (Bommineni et al., 1997). Eventually, *Agrobacterium*-based protocols were also established for wheat and barley (Cheng et al., 1997; Tingay et al., 1997). Their superiority lay both in better transgene integration patterns and a higher transformation efficiency – in barley, for example, more than 10 independent transgenic regenerants can be routinely obtained from 100 inoculated embryos (Matthews et al., 2001; Coronado et al., 2005; Bartlett et al., 2008; Hensel et al., 2008). However, in wheat (Wu et al., 2003; Hensel et al., 2009), cereal rye (Popelka and Altpeter, 2003) and triticale (Hensel et al., 2009), the transformation efficiency remains on par with what is achievable with the biolistic method. When Holme et al. (2006) agro-infected isolated barley ovules at the zygote or few-celled embryo stage, the outcome was not merely the efficient generation of transgenic progeny, but a demonstration that the method was effective (albeit less efficient) even without the use of selective conditions. An additional feature of isolated ovules is that *Agrobacterium*-mediated transformation appears to be less dependent on the host genotype than is the case when other recipient explants from cereals are used (Holme et al., 2008). Kumlehn et al. (2006) were able to combine haploid technology and *Agrobacterium*-mediated gene transfer to instantly generate homozygous barley transgenic lines. This method, based on embryogenic pollen cultures, has been employed in studies featuring both gene function and promoter characterization (Stein et al., 2005; Radchuk et al., 2006).

More recently, hairpin RNAi technology has been successfully established and applied for the stable down-regulation of native genes in transgenic *Triticeae* crops. An advantage of RNAi over knock-out mutants is that silencing of the target gene can be defined according to the choice of promoter. Moreover, RNAi allows for several members of a gene family to be targeted using a conserved DNA sequence, an approach that is particularly relevant in wheat that carries homologous genomes. Pursuing a biolistic approach, Travella et al. (2006) were able to manipulate the expression of both a *PHYTOENE DESATURASE* (*PDS*) and an *ETHYLENE INSENSITIVE 2* (*EIN2*) gene in wheat and demonstrated that the reduction of transcript was correlated with the phenotype severity within the phenotypic series generated. Each of the two genes targeted in this study is represented by three alleles derived from the A, B and D genomes of wheat. The relative silencing of the individual alleles of each gene and their total transcript levels were evaluated by qRT-PCR, indicating that all copies were equally affected. Further examples of quantification of individual allele transcripts of wheat genes targeted by RNAi via biolistics were demonstrated by Uauy et al. (2006) and Yue et al. (2008). The complex transgene integration pattern often obtained through biolistics is not necessarily a disadvantage in RNAi approaches which themselves aim at gene silencing. On the other hand, *Agrobacterium*-mediated transformation of *Triticeae* species using hairpin constructs has also been successfully applied to validate the function of candidate genes (e.g. Radchuk et al., 2006; Eichmann et al., 2010) or to generate lines with valuable characteristics (Li et al., 2005; Regina et al., 2006; Gil-Humanes et al., 2008; Zalewski et al., 2010; Nemeth et al., 2010). Table 2 provides a comprehensive overview on RNAi approaches using stably transgenic *Triticeae* species.

Regulation of transgene expression

The strength, timing, location and responsiveness to environmental signals of gene expression are determined by the gene's regulatory context. While transcription is largely controlled by the promoter, enhancement by untranslated mRNA-sequences (such as introns) can also play a role. The abundance of mRNA at a given time and place is, in addition, dependent on its stability, a property which can be influenced by the identity of its 5′ and 3′ UTRs (Baek and Skinner, 2006; Bhat et al., 2004). For an overview of promoters that have been functionally validated in the *Triticeae*, see Table 1.

Ubiquitous transgene expression

Promoters conferring ubiquitous expression (also referred to as "constitutive promoters") have been heavily used during the devel-

Table 2
Stable expression of RNAi contructs in transgenic *Triticeae* cereals.

Promoter	Specificity; inducer	Transgene	Target species	Reference
Ubiquitous				
Cauliflower Mosaic Virus 35S + maize *ADH1* intron	Ubiquitous	*TmVRN1*	Wheat	Loukoianov et al. (2005)
		TmVRN2	Wheat	Yan et al. (2004)
		TtNAM-A1	Wheat	Uauy et al. (2006)
Maize *UBIQUITIN-1* (*UBI-1*)	Ubiquitous	*BYDV-PAV orf1/orf2*	Barley	Wang et al. (2000)
		HvABA8' OH1	Barley	Gubler et al. (2008)
	Ubiquitous; *Bgh*	*HvBI*	Barley	Eichmann et al. (2010)
		HvCKX1	Barley	Zalewski et al. (2010)
	Ubiquitous; *Bgh*	*HvRBOHF2*	Barley	Proels et al. (2010)
		HvWHY1	Barley	Melonek et al. (2010)
		TaCKX1	Wheat Triticale	Zalewski et al. (2010)
		TaHMWGLU-1D1	Wheat	Yue et al. (2008)
		TaMSH7	Barley	Lloyd et al. (2007)
		TaEIN2 TaPDS	Wheat	Travella et al. (2006)
		WSMV NLA	Wheat	Fahim et al. (2010)
Grain				
Barley *HORDEIN D* (*HOR-D*)	Endosperm	*Taγ-GLIADIN*	Wheat	Gil-Humanes et al. (2008)
Wheat *High-molecular-weight GLUTENIN 1-D1* (*HMWGLU-1 D1*)	Endosperm	*TaCSLF6*	Wheat	Nemeth et al. (2010)
		TaSBEIIa and b	Wheat	Regina et al. (2006)
			Barley	Regina et al. (2010)
Barley *JEKYLL*	Nucellar projection	*HvJEKYLL*	Barley	Radchuk et al. (2006)

opmental stages of the transformation technology itself, as well as to drive the expression of the first generation of transgenic crops. However, the level of expression is dependent on the cell type, the developmental stage and on the perception of environmental triggers. In the *Triticeae*, the *Cauliflower mosaic virus 35S* promoter (*CaMV35S*) confers comparatively weak expression, but historically has been much employed. In an improved version, its enhancer region is present as a tandem repeat (Himmelbach et al., 2007), but this enhancer can interfere *in trans* with the specificity and strength of other promoters used in the same transformation vector (Yoo et al., 2005). The *CaMV35S* promoter is not active during gametophytic development or early embryogenesis in dicotyledonous species (Custers et al., 1999), which we recently reconfirmed for immature barley pollen (G. Hensel, unpublished). Popular alternatives to *CaMV35S* are maize *UBI-1* and rice *ACT1*, both of which have been well characterized in transgenic barley and wheat (Stoeger et al., 1999a; Vickers et al., 2006; Primavesi et al., 2008).

In RNAi approaches, the expression of inverted repeat constructs has mostly been driven by the ubiquitous maize *UBI-1* or *CaMV35S* promoters. For example, Yan et al. (2004) and Loukoianov et al. (2005) created *VRN2* and *VRN1* knock-down lines in winter wheat that showed accelerated and delayed flowering time, respectively. Further knock-down phenotypes resulting from ubiquitous expression of RNAi constructs were generated in wheat by Li et al. (2005), in barley by Eichmann et al. (2010) and Proels et al. (2010), as well as by Zalewski et al. (2010) in barley, wheat and triticale.

Cell-type-specific transgene expression

Ubiquitous expression of a transgene may be undesirable, especially if it is associated with deleterious pleiotropic effects. As the grain is the focus of economic yield in *Triticeae* crops, some effort has been given to identifying and characterizing grain-specific promoters (Table 1). The largest group of these are the endosperm-specific promoters derived from genes responsible for the synthesis of barley hordein and wheat gliadin and glutenin. Surprisingly, the highest level of endosperm-specific transgene expression in the *Triticeae* has been achieved using the oat *GLO-1* promoter (Vickers et al., 2006). The activity of most endosperm-specific promoters is specific to a particular window of grain development and/or the sub-region of the endosperm (Table 1). Grain-specific promoters specific for the embryo or the pericarp have also been identified. Aside from the set of grain-specific promoters, only a small number of functionally proven *Triticeae* promoters has been shown to be consistently active in a particular tissue; specificities include the epidermis, the bracts, the phloem and the tapetum (Table 1). To an extent, these tissue specificities reflect the concentration of research activities on enhancing resistance against pathogens, the manipulation of nutrient transport and the elaboration of F_1 hybrid technology.

By judicious choice of promoter used in conjunction with hairpin RNAi constructs, expression of endogenous genes can be down-regulated within a particular tissue or developmental stage, so reducing the risk of off-target effects and allowing for a greater level of precision in the identification of the target gene's effect on phenotype. Using the endosperm-specific HMW *1Dx5* subunit promoter of wheat, Regina et al. (2006, 2010) down-regulated the two isoforms of *STARCH BRANCHING ENZYME* (*SBE-IIa* and *SBE-IIb*) genes, which caused reduced amylopectin content in the grains of barley and wheat, respectively. Similarly, the (1,3;1,4)-β-D glucan content of wheat grains was reduced by endosperm-specific RNAi targeting the *CSLF6* gene (Nemeth et al., 2010). Radchuk et al. (2006) used the target's own promoter driving a hairpin construct to knock down the barley *JEKYLL* gene, leading to reduced transcript levels associated with impaired development of the reproductive organs and a delay in flowering time. Using the endosperm-specific barley *HOR-D1* promoter, RNAi technology has also been used to knock down the wheat γ-*GLIADIN* gene family (Gil-Humanes et al., 2008).

Pathogen-controlled transgene expression

Plants perceive attacking pathogens from their associated molecular pattern, and this recognition triggers the expression of an immune response, which includes a re-programming of gene expression, the localized lignification of cell walls, the synthesis of the anti-microbial phytoalexins and the accumulation of pathogenesis-related (PR) proteins (Jones and Dangl, 2006). Changes in the cereal transcriptome induced by pathogen attack have been studied by a range of molecular platforms (cDNA-AFLP: Zhang et al., 2003; suppression subtractive hybridization: Bogacki et al., 2008; array hybridization: Zellerhoff et al., 2010). Although these analyses have identified large numbers of differentially regulated genes, few of the relevant promoters have been functionally validated by transiently or stably expressed promoter/reporter gene fusions. Transcript profiling has revealed that the most abundant PR transcript in the epidermis of powdery mildew-infected barley encodes GER4, a germin-like protein associated with superoxide dismutase activity. Eight GER4-encoding genes form a single locus cluster on chromosome 4H (Druka et al., 2002; Himmelbach et al., 2010), and the promoter regions (of ∼3 kb in length) have been isolated from various *GER4* genes and fused to the *gus* reporter gene (Himmelbach et al., 2010). Reporter gene activity in transiently transformed barley leaves induced by infection with powdery mildew has been recorded for six *GER4* gene promoters, although the level of their expression strength varied. The *GER4c* (the most strongly induced gene) promoter has also been tested in stable transgenic barley in response to both powdery mildew and scald (*Rhynchosporium secalis*) infection. In both cases, inoculation with the pathogen induced the localized activation of the promoter (Himmelbach et al., 2010). This highly localized, epidermis-specific expression of the *GER4c* promoter provides a useful tool for the engineering of disease resistance in the cereals.

The promoter of *VST1* (*STILBENE SYNTHASE*), a gene isolated from *Vitis vinifera*, was used by Leckband and Lörz (1998) to produce pathogen-induced expression in barley and wheat. Stilbene synthase catalyses the formation of the phenolic phytoalexin trans-resveratrol, which has anti-fungal activity (Hart, 1981). The same phytoalexin is also produced in response to elicitor (Melchior and Kindl, 1990), UV light (Schöppner and Kindl, 1979) and wounding (Langcake, 1981). The native *Vst1* promoter, as well as a modified version in which four copies of the *CaMV35S* enhancer were included, was used to drive the expression of the *VST1* transgene or *gus*. When the *gus* fusion was stably transformed into barley suspension cells, the level of GUS accumulation was increased by some ten fold, whether driven by the native or the enhanced version of the *VST1* promoter. In transformed barley plants expressing *VST1* driven by the enhanced promoter, a wounding treatment led to the accumulation of *VST1* transcript. Expression was also induced by infection with powdery mildew. In transgenic wheat, both promoters were responsible for the generation of phytoalexin in response to infection by the fungal pathogens causing both brown (leaf) rust (*Puccinia recondita* f.sp. *tritici*) and Septoria blotch (*Septoria nodorum*) (Serazetdinova et al., 2005).

Another example of a heterologous pathogen-regulated promoter is provided by the *MAS* (*MANNOPINE SYNTHASE*) gene originating from the *Agrobacterium tumefaciens* Ti plasmid (Langridge et al., 1989). This promoter was successfully used to express the anti-microbial insect peptide metchnikowin (Mtk) in stably transformed barley plants (Rahnamaeian et al., 2009). Time course experiments revealed that *MAS* promoter activity responded to the presence of auxin, as well as of fungi causing powdery mildew (*Blumeria graminis*) and Fusarium head blight (*Fusarium graminearum*). However, the beneficial root-colonizing Basidiomycete *Piriformospora indica* remained unaffected by the level of Mtk peptide present in the host.

Regulation of transgene expression by environmental signals

Plants are exposed to ever-changing environmental cues, and therefore have developed regulatory systems to control the expression of the genes required to ensure their survival under adverse conditions. The initial cues are perceived by a number of sensors, which leads to the production of stress hormones such as abscisic acid (ABA) and a range of secondary messengers (Ca^{2+}, reactive oxygen species and phospholipids). Signal transduction cascades then transcriptionally activate the various stress-responsive genes. Different stresses have been found to share the same secondary signal(s), leading to a substantial overlap in the spectrum of genes induced by various abiotic stresses. The stress response is also mediated by various ABA-independent pathways (Xiong et al., 2002). Several promoters responsive to osmotic stress, drought or exogenously applied ABA have been successfully validated in both transient and stably transformed cereals. The *Arabidopsis thaliana RD29A* promoter is activated via an ABA-independent pathway (Chak et al., 2000) and is targeted by the transcription factor DREB1 (dehydration responsive element binding) (Agarwal et al., 2006). When an *RD29A*/*DREB1* fusion was expressed in wheat plants subjected to 2 days of water depletion, it was demonstrated that the promoter was as efficiently induced in wheat as it is in *A. thaliana*, and that the increased accumulation of the DREB1 protein delayed the appearance of drought symptoms (Pellegrineschi et al., 2004). A prominent class of *Triticeae* ABA-induced promoters is associated with the *LEA* (*LATE EMBRYOGENESIS*) gene family. The barley *A1* (Straub et al., 1994; Xue, 2003) and *DHN4* (Choi et al., 1999), as well as the rice *WSI18j* (Joshee et al., 1998) and *RAB16B* (Xiao and Xue, 2001) promoters have all been demonstrated through a transient transformation assay to be activated in response to drought, cold stress and exogenously applied ABA.

Since the constitutive over-expression of genes (such as the transcriptional activator *CBF3*) conferring cold resistance can affect plant development under both normal and high temperatures (Gilmour et al., 2000), the engineering of cold-inducible expression systems may provide a means of improving freezing tolerance. The promoters of several members of the barley *LTP* (*LIPID TRANSFER PROTEIN*) gene family and of two *LOW TEMPERATURE-RESPONSIVE* genes have proven to be cold-inducible (Table 1). *LTP4.2* and *LTP4.3* were active in both spring and winter barleys (Molina et al., 1996), suggesting that their inducibility is independent of genetic background. They may therefore be functional in other cereals as well. An example of a cold-inducible promoter functionally validated by transient expression assays across a broad range of plant species is represented by the wheat *WCS120* promoter, which was shown to be functional in barley, rye and rice, as well as in certain dicotyledonous species (Ouellett et al., 1998).

In contrast to the many genes modulated by a short exposure to cold, longer episodes of low temperature are required for vernalization, in which a stepwise process of metabolic changes eventually results in the triggering of flowering. When the *VER2* promoter identified in winter wheat was fused to *gfp*, reporter activity was only present in transiently transformed seedling wheat leaves, which have been subjected to vernalization before gene transfer (Xu et al., 2004).

The supply of available phosphate is a limiting factor for plant growth in many areas. Numerous morphological, physiological and molecular responses (the latter including the induction of genes encoding phosphate transporters) have evolved to allow plants to cope with phosphate starvation (Rausch and Bucher, 2002). Typically, the expression of phosphate transporters is induced exclusively in the roots during an episode of phosphate deprivation, although some are expressed independently of the level of available phosphate. The expression profiles of the promoters associated with the phosphate transporters barley *PHT1;1* (Schünmann et al., 2004) and wheat *PT2* (Tittarelli et al., 2007) have been analysed using reporter fusions in transgenic barley and wheat. In both cases, reporter gene expression was elevated in response to low phosphate concentrations in roots, whereas only low activity was observed in aerial tissues.

The nutrient status of the barley plant affects the synthesis of hordein, which represents ∼50% of the total grain protein (Shewry et al., 1978). The expression of *HOR-C* is influenced by the supply of nitrogen to the developing endosperm, and has been characterized in a transient expression system using a *HOR-C* promoter/reporter construct in the barley endosperm. Full promoter activity was, as expected, expressed only under conditions of optimal nitrogen supply. The strength of a hordein promoter derivative was modulated by the ratio of certain *cis* regulatory elements (namely GN4 and endosperm boxes), which were attached in different combinations to the distal end of the promoter (Müller and Knudsen, 1993).

Fructans, the major storage carbohydrate present in many cereals, are synthesized from sucrose. Nagaraj et al. (2001) isolated a *6-SFT* (*FRUCTAN 6-FRUCTOSYL TRANSFERASE*) promoter and fused it to the *gus* reporter gene to enable transient expression experiments in detached barley leaves. Light and sucrose are known to be inducers of fructan biosynthesis (Roth et al., 1997), and the response of the *6-SFT* promoter to both stimuli was positive. Unlike leaf mesophyll cells, epidermal cells (including guard cells) are not a storage tissue for fructans, but nevertheless showed inducible *6-SFT* promoter activity. Light-inducibility in transient assays using the *gus* reporter gene could also be demonstrated for the rice *GOS5* promoter in green tissues of several monocotyledonous species, including barley (Hensgens et al., 1993). The *6-SFT* and *GOS5* promoters may therefore be useful for transgene expression in whole leaves when light-regulated expression is relevant.

Intron-mediated enhancement of transgene expression

The functionality of plant introns and their effects on gene expression have been investigated in numerous studies, yet little is known about the underlying mechanisms of intron-mediated enhancement (IME), and different introns are anticipated to affect expression by different means (for reviews see: Koziel et al., 1996; Simpson and Filipowicz, 1996). It is however common sense, that plant introns act post-transcriptionally to increase the accumulation and translational activity of mRNA rather than enhancing the transcription rate. Unlike transcriptional enhancers, they must be contained within transcribed sequences and in the proper orientation to increase gene expression (Callis et al., 1987; Mascarenhas et al., 1990). Introns are of particular value for biotechnological approaches, since they do not appear to affect the specificity of the promoters used (Schünmann et al., 2004). Using mutated derivatives of the Arabidopsis *PAT1* first intron, Rose and Beliakoff (2000) surprisingly provided evidence that neither unique intron sequences nor splicing be generally required to increase mRNA accumulation. On the other hand, the composition of flanking exon sequences remarkably influences intron functionality (Maas et al., 1991; Luehrsen and Walbot, 1991). While not all introns result in elevated expression (Sinibaldi and Mettler, 1992), the magnitude of IME is commonly in the range of 2–10-fold, but over 100-fold enhancements have also been reported (Maas et al., 1991). The degree of enhancement is usually stronger for relatively weak promoters (Luehrsen et al., 1994), and the range of IME typically observed in dicots is less than increases seen in monocots (Simpson and Filipowicz, 1996). Whereas monocot introns were thus far not shown to stimulate transgene expression in dicots (Leon et al., 1991; Maas et al., 1991), dicot introns are typically functional in monocots. Consequently, any intron derived from angiosperms is potentially useful for transgenic approaches in *Triticeae* cereals. Vain et al. (1996) evaluated seven different constructs using maize, rice and petunia introns that were inserted along with small adjacent exon sequences into the 5′-leader sequence between the *CaMV35S* promoter and the *gus* coding sequence. They showed that most introns enhanced transient reporter gene expression in cell suspensions of both maize and bluegrass. In this study, the maize *UBI-1* first intron consistently resulted in the highest level of *gus*-expression. However, the stimulation was markedly stronger in maize as compared to bluegrass, indicating that the effect of a given intron varies within different grass species.

Taking advantage of what is mainly known from other monocot species, quite a number of introns have been used to genetically transform *Triticeae* crops. For example, the *UBI-1* and *ACT1* promoters from maize and rice, respectively, are commonly used in conjunction with the first intron contained in the adjacent 5′-untranslated region so as to achieve strong ubiquitous transgene expression. However, very few studies have thus far been devoted to evaluate the specific effect of introns on expression in *Triticeae* species. Bourdon et al. (2004) showed that the insertion of the maize *RpoT* intron 4 within the firefly *luciferase* coding region in addition to the maize *UBI-1* 5′ untranslated leader intron results in a 1.5–2-fold expression enhancement in transgenic wheat. Whereas a further intron (*RpoT* i18) caused a slight decrease in expression, both intron combinations led to a stabilization of luciferase expression in the T1 and T2 progenies. In a more recent study, the integration of either the Arabidopsis *UBQ10* first intron or the maize *RpoT* intron 4 into the *luciferase* reporter coding region caused an elevation of both the reporter gene mRNA level and the accumulation of reporter protein in transgenic barley (Bartlett et al., 2009). While insertion of these two different introns in the same position resulted in different enhancement, their effect on transgene expression was consistent across the T1 and T2 generations. The highest expression was obtained using the *UBQ10* intron, which increased the average luciferase activity in primary transgenics almost 3-fold, with a more than 4-fold increase being observed in the single-copy lines.

In plant genetic transformation, introns have not only been used to enhance expression, but also to prevent reporter and selectable marker genes from being expressed by accidentally present microbes or *Agrobacterium* persisting in putatively transgenic tissue or plants (Vancanneyt et al., 1990; Wang et al., 1998). This approach has been especially important in the cereals, which are comparatively difficult to transform and where the generation of false-positive lines has sometimes led to misleading conclusions concerning the transformation methodology used (Langridge et al., 1992). The use of intron-containing reporter or selectable marker coding sequences has facilitated the establishment of reliable methods of *Agrobacterium*-mediated stable transformation in barley (Murray et al., 2004; Kumlehn et al., 2006), bread wheat (Wu et al., 2003) and durum wheat (Wu et al., 2008).

Introns are also thought to render hairpin RNAi constructs more effective when being inserted between the two inverted repeats of the RNAi-expression cassette (Smith et al., 2000). For example, the wheat *RGA2* intron was used by Douchkov et al. (2005) to establish a highly efficient transient gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells, as well as by Himmelbach et al. (2007) to create a set of generic binary vectors for transient and stable knock-down approaches in barley and other monocots.

Sub-cellular localization of transgene products

Although signal peptide-encoding sequences integrated within the open reading frame of a transgene do not modulate expression *sensu strictu*, they are nevertheless considered here because of their importance as a tool for controlling the accumulation of a gene product in the context of genetic transformation. Transit peptides govern the sub-cellular localization of many proteins. If *Triticeae* cereals are to be exploited as a production system for recombinant proteins, then maximizing the yield of transgene product is a critical aim. Ideally, over-expressed recombinant proteins should be directed to a sub-cellular environment where their stability is maintained, and where their high concentration does not impinge on processes essential for plant growth and development. The endoplasmic reticulum (ER) has been identified as the optimal destination in this context (Table 3, and references therein). The organismal origin of transit peptides appears to be non-critical for their functionality in heterologous systems, suggesting that the mechanisms underlying intracellular protein movement are highly conserved. Plastids represent a second choice of appropriate cellular compartment. Primavesi et al. (2008) achieved plastid-specific reporter gene expression in various stably transformed wheat tis-

Table 3
Signal peptides functionally validated in stably transgenic *Triticeae* cereals.

	Specificity	Transgene	Target species	Reference
Arabidopsis Basic chitinase	ER	*rCla1*	Barley	Ritala et al. (2008), Eskelin et al. (2009)
Barley α-Amylase	ER	*Aspergillus niger phyA*	Wheat	Brinch-Pedersen et al. (2000)
Barley α-Amylase/KDEL	ER/ ER-retention	CD4/CD28 scFv	Wheat	Brereton et al. (2007)
Barley High pI α-Amylase	ER	*(1,3-1,4)-β-GLUCANASE*	Barley	Jensen et al. (1996)
Barley Hordein D (Hor D)	ER	*(1,3-1,4)-β-GLUCANASE*	Barley	Horvath et al. (2000)
Maize Ferredoxin III	Plastids	*gfp*	Wheat	Primavesi et al. (2008)
Mouse Leader petide (LP)	ER	Anti-fungal peptide + scFv	Wheat	Li et al. (2008)
Pea Legumin/KDEL	ER/ ER-retention	HIV Diagnostic Reagent	Barley	Schünmann et al. (2002)
Rice FtsZ	Plastids	*gfp*	Wheat	Primavesi et al. (2008)
Wheat Small-subunit of RUBISCO	Plastids	*gfp*	Wheat	Primavesi et al. (2008)

sues, by assembling chimeric reporter gene constructs carrying particular transit peptides (from rice, maize and wheat) translationally fused to the *gfp* coding sequence. Further examples of transit peptides which have been functionally validated in *Triticeae* species are given in Table 3. Of other signal peptides which can cause elevated levels of recombinant protein, the most prominent is the C-terminal KDEL ER-retention signal. This counteracts protein excretion from the ER to the extracellular space, as exemplified both in barley and in wheat (Brereton et al., 2007; Schünmann et al., 2002).

Outlook

Recent improvements in transformation methodology for *Triticeae* cereals have spawned novel approaches to isolate and functionally characterize promoters and other regulatory elements controlling gene expression and protein accumulation. Compared to the dicotyledonous species, however, cereal transformation technology still faces significant technical hurdles. Techniques yet to be transferred from dicotyledonous systems include transient expression based on *Agrobacterium*-mediated gene transfer, gene knock-down using artificial micro-RNAs, plastid transformation, and chemically inducible expression. For the latter, the need is for a minimal promoter which can provide zero basal, but a strong level of expression when combined with appropriate *cis* elements. Similarly, there is a requirement for the large-scale functional analysis of promoter *cis* elements and the development of synthetic promoters which are functional in the *Triticeae*. Even the commonly anticipated effect of transcriptional enhancers has yet to be experimentally verified in *Triticeae* species. While stable transformation is relatively efficient in barley, the other *Triticeae* crops are lagging far behind, and in particular, the *Agrobacterium*-mediated transformation of wheat urgently requires improvement. Moreover, the strong genotype dependence of transformation methods still remains a barrier in some situations. The widespread genetic engineering of the *Triticeae* cereals still awaits the assembly of a well-characterized set of promoters. Finally, what is still limiting the establishment of powerful production platforms for valuable plant-made molecules is the means to engineer very high transgene expression and recombinant protein accumulation, especially in the *Triticeae* endosperm.

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