A Set of Modular Binary Vectors for Transformation of Cereals[1][W][OA]

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Genetic transformation of crop plants offers the possibility of testing hypotheses about the function of individual genes as well as the exploitation of transgenes for targeted trait improvement. However, in most cereals, this option has long been compromised by tedious and low-efficiency transformation protocols, as well as by the lack of versatile vector systems. After having adopted and further improved the protocols for *Agrobacterium*-mediated stable transformation of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*), we now present a versatile set of binary vectors for transgene overexpression, as well as for gene silencing by double-stranded RNA interference. The vector set is offered with a series of functionally validated promoters and allows for rapid integration of the desired genes or gene fragments by GATEWAY-based recombination. Additional in-built flexibility lies in the choice of plant selectable markers, cassette orientation, and simple integration of further promoters to drive specific expression of genes of interest. Functionality of the cereal vector set has been demonstrated by transient as well as stable transformation experiments for transgene overexpression, as well as for targeted gene silencing in barley.

Cereals represent crops of foremost economic importance worldwide (http://faostat.fao.org). Consequently, they are major targets in plant research, biotechnology, and commercial crop plant improvement, especially in the context of global climate changes and the rapidly growing demand for human nutrition.

A vast amount of different genetic resources has been generated and collected in databases worldwide (Alonso and Ecker, 2006; Stein, 2007). Assemblies of large EST datasets (approximately 855,000 ESTs for wheat [*Triticum aestivum*] and approximately 437,000 ESTs for barley [*Hordeum vulgare*]) provided important insight into the genome organization and led to in silico prediction of about 50,000 unique genes for wheat and barley, respectively (Zhang et al., 2004; Stein, 2007). Although bioinformatics, transcriptome analysis (Close et al., 2004; Druka et al., 2006; Zierold et al., 2005), transient overexpression, virus-induced gene silencing (VIGS; Lacomme et al., 2003), and transient-induced gene silencing (TIGS; Douchkov

et al., 2005) have greatly extended the information on putative roles of genes, we are left with the major challenge of elucidating gene functions by modulation of their expression in planta. The recent development of reliable and efficient *Agrobacterium*-mediated transformation technologies for cereals (for review, see Shrawat and Loerz, 2006; Goedeke et al., 2007) has stimulated a variety of strategies toward functional gene characterization, thereby paving the way for deeper understanding of crop plant biology in cereals.

Comprehensive analyses of gene function include stable transformation with sequences for overexpression or knock-down of plant genes. Binary vectors used for generation of transgenic cereal species are typically cumbersome due to their large size and the rather limited number of useful restriction sites. To bypass laborious preparation of constructs, GATEWAY technology (Invitrogen) is used especially for binary vectors generating knock-down lines. GATEWAY-derived cloning systems are based on the site-specific recombination system from bacteriophage λ (Landy, 1989) and circumvent traditional cloning methods involving restriction and ligation of DNA sequences. A number of GATEWAY-based binary vector sets for plant functional genomics have been developed, thereby allowing overexpression or knock-down of effector genes, expression of fusion proteins (Karimi et al., 2002; Curtis and Grossniklaus, 2003; Chung et al., 2005; for review, see Earley et al., 2006), and transformation of multiple genes (Chen et al., 2006).

Most overexpression studies employ a strong, constitutive promoter, such as the cauliflower mosaic virus *35S* promoter (Odell et al., 1985), followed by phenotypic analysis of the transgenic plant. In many cases, ectopic expression experiments gave

important insight into gene function (Jack et al., 1994). However, as possible consequence of ubiquitous overexpression and misdirection of gene products, undesirable pleiotropic effects on the plant may be caused. In addition, strong accumulation of unnecessary proteins leads to wasteful energy consumption, which could, in turn, generate phenotypes that are not directly correlated with the recombinant protein itself. To avoid such unwanted pleiotropic effects that occlude phenotypic analysis, transgene expression can be controlled temporally and spatially by the use of cell- and tissue-specific (Luo et al., 2006; Vickers et al., 2006) or chemically inducible (Holtorf et al., 1995; Zuo and Chua, 2000; Deveaux et al., 2003; Maizel and Weigel, 2004) promoters. Most promoters available to date are derived from dicotyledonous plants. Unfortunately, such promoters are typically dysfunctional in cereal species. Thus, expression of transgenes in cereals has been largely driven by ubiquitous promoters, such as those from the maize (*Zea mays*) ubiquitin 1 (*Ubi1*; Christensen et al., 1992; Oldach et al., 2001) or the rice (*Oryza sativa*) actin 1 gene (*Act1*; McElroy et al., 1990; Vickers et al., 2006). However, a few specific promoters derived from cereal species have been characterized and used to drive transgene expression. To confine transgene expression to the cereal seeds, several grain-specific promoters, such as the oat (*Avena sativa*) *AsGlo1* (Vickers et al., 2006), the barley hordein (*Hor2-4*, *Hor3-1*; Patel et al., 2000; Cho et al., 2002), and the rice glutelin B1 (*GluB1*; Patel et al., 2000) promoters, have been employed recently. Even though drought-inducible promoters were described for barley and rice (Xiao and Xue, 2001), stress-induced expression systems that are functionally verified in cereals or other monocotyledonous species are not yet available. As a consequence, there is growing demand for transformation technology that permits controllable expression of transgenes in cereals.

Knock-down approaches aim at perturbation of gene function due to the elimination of transcripts using antisense RNA, RNA interference (RNAi), or the generation of dominant-negative effects by interfering with protein complexes (Olive et al., 1996; Ramirez-Parra et al., 2003). Again, temporal and spatial control of effector-gene expression largely supports the interpretation of transgene-induced phenotypes.

At present, two major transformation strategies for monocotyledonous plants are established. Compared to biolistic techniques (Stoeger et al., 1999; Bhalla et al., 2006) *Agrobacterium*-mediated transformation offers several advantages (Tzfira and Citovsky, 2006), such as simpler integration patterns resulting in lower mutational consequences for the transgenic plant (Latham et al., 2006) and limited transgene silencing via cosuppression. In addition, the option for fine tuning the *Agrobacterium*-based transformation protocols renders more and more cereal species amenable for efficient genetic engineering (Shrawat and Loerz, 2006; Conner et al., 2007). These advantages prompted us to implement the GATEWAY cloning system and

expression cassettes into a vector set for *Agrobacterium*-mediated transformation, thereby providing the versatility to use the vector set for the transformation of a large panel of cereal species and genotypes.

Although GATEWAY-based binary vectors have been developed for dicotyledonous plants (e.g. Wesley et al., 2001; Curtis and Grossniklaus, 2003; Tzfira et al., 2005), these are typically not useful for monocotyledons, mainly because of the limited functionality of promoters that are used to drive either the gene of interest or the plant selection marker. But also, other specific vector elements, such as the plant-selectable marker and the origin of replication, may impede the amenability of a binary vector. The pVS1 origin of replication derived from *Pseudomonas aeruginosa* conferred high plasmid stability in *Agrobacterium* even under nonselective conditions (Itoh et al., 1984), thus ensuring the persistence of an effective population of transformation-competent bacteria during the entire period of cocultivation with target plant cells. This may be especially crucial in transformation systems for atypical *Agrobacterium* hosts, such as monocotyledonous plants. However, some binary GATEWAY destination vectors have been generated especially for use in monocotyledons (Miki and Shimamoto, 2004; www.bract.org). Unfortunately, these do not allow convenient and comprehensive modification with regard to the promoters and the plant selection marker to tailor derivatives for further specific approaches.

Here, we provide a set of generic binary vectors that is made available for phenotypic studies in stably transformed cereal species. Its modular configuration permits convenient insertion of promoter and effector sequences, as well as of plant selection marker cassettes of choice. The insertion of effector sequences into the binary overexpression and knock-down vector series is facilitated by the highly efficient GATEWAY recombination system. The spectrum of applications is further extended by the options to test constructs in transient expression assays (e.g. in barley) prior to starting the laborious stable transformation procedure and by the option to transform monocotyledonous and dicotyledonous plants using the same binary vector. Vector derivatives with strong, constitutive promoters, such as the maize ubiquitin promoter (*ZmUbi1*; Furtado and Henry, 2005), the double-enhanced CaMV *35S* promoter (*d35S*; Furtado and Henry, 2005), or the rice actin promoter (*OsAct1*; McElroy et al., 1990; Vickers et al., 2006), are provided. In addition, the wheat glutathione *S*-transferase promoter (*TaGstA1*; Altpeter et al., 2005) permits the expression of transgenes confined to leaf epidermis in a constitutive manner. With the availability of a combination of the highly efficient GATEWAY cloning system, a selection of cereal promoters controlling the expression of genes of interest, different plant selection markers, together with the option of further convenient vector modifications, the functional characterization of DNA sequences will be greatly facilitated in cereal species.

RESULTS AND DISCUSSION

GATEWAY Compatibility of Binary Destination Vectors

Traditional cloning of DNA sequences for overexpression or RNAi knock-down experiments into binary plant transformation vectors is laborious and time consuming. To facilitate generation of binary vectors for cereal species, we used the GATEWAY system for recombinational cloning (Fig. 1A). GATEWAY technology takes advantage of a modified bacteriophage λ recombination system, thereby allowing a highly efficient, site-specific, and reliable exchange of DNA fragments between plasmids. The recombination reaction requires an entry vector containing a gene of interest flanked by appropriate recombination sites (e.g. attL1 and attL2), a recombination enzyme (Clonase), and a binary destination vector. The binary destination vector contains compatible recombination sites (e.g. attR1 and attR2) integrated downstream of the plant promoter of choice. For generation of RNAi constructs, an inverted repeat of such GATEWAY insertion cassettes is required. To enable the formation of an RNA hairpin structure, the inverted repeat of insertion cassettes is recommended to be separated by a spacer or intron sequence. To this end, we used a wheat *RGA2* intron in this study (Douchkov et al., 2005).

To allow for efficient introduction of gene sequences of interest into the entry vector, plasmid pIPKTA38 was used (Douchkov et al., 2005), which lacks the negative bacterial selection marker *ccdB* but contains the multiple cloning site (MCS) instead. The *Swa*I restriction site present in the MCS permits the highly
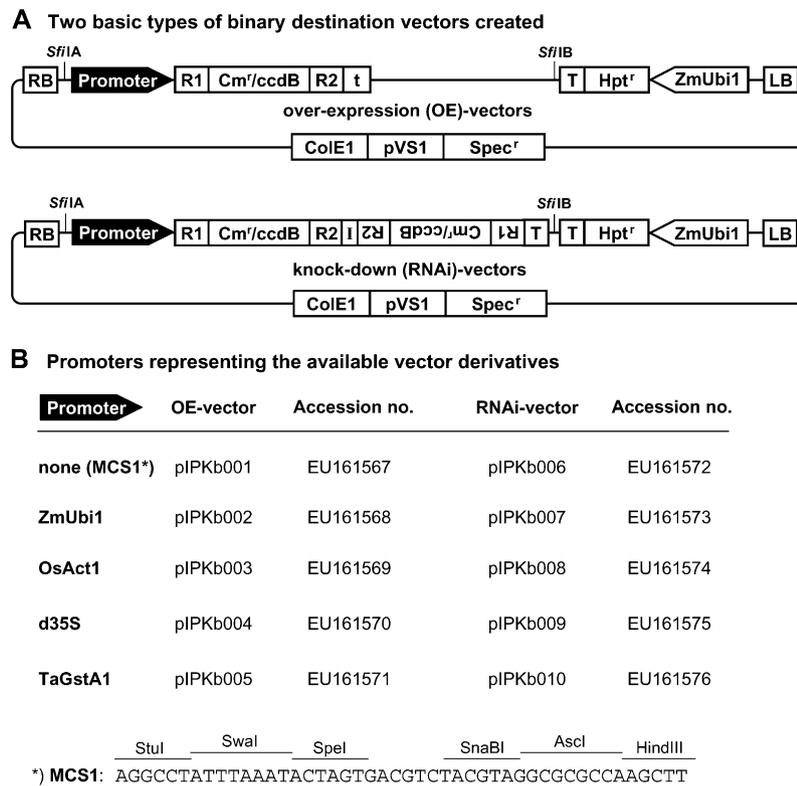
Figure 1. Schematic representation of the modular binary destination vectors generated. A, There are two basic vector types designed for overexpression (OE) and for RNAi-mediated knock-down (RNAi). The GATEWAY destination cassettes of the OE vectors consist of R1 (attR1 recombination attachment site), *Cm*^{r} (chloramphenicol acetyltransferase gene), *ccdB* (negative selection marker), and R2 (attR2 recombination attachment site) sequences. The RNAi vectors further contain the wheat *RGA2* intron (I) separating the inverted repeat of the GATEWAY destination cassettes. Transcription is terminated either by the *A. tumefaciens nos* (t) or the CaMV *35S* termination signal (T). All vectors can be digested using endonuclease *Sfi*I to obtain two fragments, one containing the GATEWAY expression cassette and the other comprising all other components of the vector; i.e. RB (right border), ColE1 (origin of replication for *E. coli*), pVS1 (origin of replication for *A. tumefaciens*), *Spec*^{r} (streptomycin/ spectinomycin bacterial resistance), LB (left border), and the plant selection marker *Hpt*^{r} (hygromycin phosphotransferase) controlled by the maize *ZmUbi1* promoter. The latter *Sfi*I fragment can be readily exchanged by respective fragments of compatible binary vectors (e.g. available from DNA Cloning Service) that carry other plant selectable marker expression cassettes. B, Expression of the GATEWAY cassettes of the binary vectors pIPKb001 to pIPKb010 is driven by the promoters specified below (i.e. the constitutive *ZmUbi1*, *OsAct1*, and *d35S* promoters, and the epidermis-specific *TaGstA1* promoter). To enable convenient integration of further promoters, vectors pIPKb001 and pIPKb006 contain a MCS1 upstream of the GATEWAY destination cassettes. Sequences of all binary destination vectors presented are available under the GenBank accession numbers given.

efficient introduction of sequences by using a cyclic-cut-ligation reaction involving the concurrent use of the *Swa*I restriction endonuclease and T4 DNA ligase (Douchkov et al., 2005). To obtain the successfully recombined destination vector with high efficiency, two bacterial selection schemes were imposed. Entry vector (kanamycin resistance) and destination vector (spectinomycin and chloramphenicol resistance) contain different antibiotic selection markers. In addition, upon recombination, the gene of interest replaces the *ccdB* negative selection marker that poisons most *Escherichia coli* strains (Bernard and Couturier, 1992). In addition to pIPK38, any appropriate GATEWAY-compatible entry vector containing a DNA sequence of interest can be used to generate a respective binary vector for overexpression or knock-down approaches.

Interchangeability of Promoter Sequences

In the vectors presented, transgene expression is driven either by several strong, constitutive promoters (*ZmUbi1*, *d35S*, and *OsAct1*) or the epidermis-specific wheat glutathione *S*-transferase promoter (*TaGstA1*). To permit future extensions of the range of promoters controlling the gene of interest, MCS1 was introduced to create the generic destination vectors pIPKb001 and pIPKb006 (Fig. 1B). Additional promoter sequences can thus be incorporated directly into these plasmids prior to or following a GATEWAY recombination reaction. Thus, versatility is provided that is required to employ the vectors to functionally test new promoters or other regulatory elements or to integrate known promoter sequences that possess particularly useful properties.

Interchangeability of Plant Selection Markers

Although the hygromycin phosphotransferase (*hpt*) selection marker of the binary plasmid 6U (DNA Cloning Service) is widely employed for barley and wheat (Goedeke et al., 2007; Hensel et al., 2008), different plant selection markers, such as phosphinothricin-*N*-acetyl transferase (*pat*), may be preferred for some target species or required for iterative gene-stacking approaches (Halpin, 2005). Compatible binary vectors, such as 7U (DNA Cloning Service), containing further expression cassettes of plant selection markers have been conveniently swapped to some of the binary vectors by using the rare-cutting enzyme *Sfi*I (see Fig. 1A). Likewise, binary plasmids available from the DNA Cloning Service with further plant selection gene cassettes, such as neomycin phosphotransferase II (*nptII*; plasmid 9U) or dihydrofolate reductase (*dhfr*; plasmid 5U), can be readily combined with any of the binary vectors presented here. In addition, any of the binary vectors available can be used to create a respective vector devoid of a plant selectable marker expression cassette. Those vectors might be useful in exceedingly efficient transformation systems that do not necessarily require the application of selective conditions. The benefit of such an approach would be to obtain transgenic plants instantly free of a selectable marker gene. To this end, the *Sfi*I fragment containing the plant selection marker has to be exchanged with the compatible markerless fragment of the binary vector B-BA (DNA Cloning Service). Furthermore, the vector series provides the potential to introduce alternative plant selection markers, such as the phospho-mannose-isomerase gene (Reed et al., 2001; Goldstein et al., 2005) or sequences for site-specific recombination-mediated marker deletion strategies (*Cre/loxP*; Darbani et al., 2007) through the generation of respective *Sfi*I-compatible plasmids.

The vector set also allows the expression units for the plant resistance marker as well as the overexpression/knock-down cassettes to be juxtaposed in two orientations. By using the binary plasmid 6U, both transcription units are oriented convergently, whereas the plasmid 65U (DNA Cloning Service) permits the cassettes to be positioned in tandem.

Vector Elements Facilitating Transgenic Plant Analysis

Phenotypic characterization of transgenic plants often includes analysis of integration patterns of T-DNA within the plant genome. This involves determination of the integration events of the T-DNA sequences as well as verification of complete T-DNA transfer to the plant, especially when sequences are used that cause negative selection pressure. To simplify screening for plants harboring the complete T-DNA sequence, primer pairs spanning the overexpression cassette were generated. To verify the integration of entire hairpin constructs (derivatives of pIPKb007–pIPKb010), the sense and antisense repeats of the hairpin cassette can be detected independently by specific primer pairs, the first spanning the region between the promoter and the *RGA2* intron, and the second the region between the *RGA2* intron and the terminator, respectively. This feature proved to be highly beneficial because, in our experience, not all generated transgenic plants surely contain both inverted sequence repeats of a given hairpin construct. All of the available primers can be employed regardless of the sequence of interest introduced to the destination vector because they are designed to anneal with sequences flanking the GATEWAY cassettes. Primer sequences and information on their target templates are available (see Supplemental Table S1; Fig. 1). By using these primers for PCR followed by DNA sequence analysis, the integrity of the T-DNA was conveniently verified in a large number of transgenic lines (data not shown).

Functional Analysis of Binary Overexpression Vectors

Functionality of plasmids with respect to integrity of the destination cassette, promoter strength, and general transformation efficiency was tested by introduction of the *gus* reporter in the overexpression vector

series (pIPKb002–pIPKb005) followed by transformation of barley and subsequent expression analysis. Transgenic barley lines carrying overexpression sequences were generated using methods based on the cocultivation of immature barley embryos with *Agrobacterium* followed by regeneration under antibiotic (hygromycin) selection. This procedure yielded transformants with an efficiency ranging between about 30% and 60% (related to the number of barley embryos used), as was previously observed for plasmid 6U derivatives without GATEWAY cassettes (Hensel et al., 2008). Following transformation using the overexpression vectors presented here, the *gus* reporter was verified by PCR in >90% of the T_0 plants tested and was inherited to the T_1 generation according to Mendelian rules (data not shown). Expression of the *gus* reporter under the control of the *ZmUbi1* promoter in barley leaf segments of a segregating T_1 population obtained by gene transfer using pIPKb002_GUS is shown in Figure 2A.

For quantification of promoter strength in transgenic barley lines, the specific GUS activity generated under the control of the *ZmUbi1* promoter (pIPKb002_GUS), the *OsAct1* promoter (pIPKb003_GUS), the CaMV *d35S* promoter (pIPKb004_GUS), and the *TaGstA1* promoter (pIPKb005_GUSI) was measured (Fig. 2B). Analysis of T_1 seedling pools from independently derived primary transgenic lines revealed the strongest average specific GUS activity (97 ± 76 fluorescence units [FU] h^{-1} μg^{-1}) obtained by the *ZmUbi1* promoter, followed by the *OsAct1* promoter (40 ± 30 FU h^{-1} μg^{-1}), the *TaGstA1* promoter (26 ± 19 FU h^{-1} μg^{-1}), and the CaMV *d35S* promoter (15 ± 9 FU h^{-1} μg^{-1}). In contrast to the overexpression lines, wild-type plants only showed background GUS activity (5 ± 2 FU h^{-1} μg^{-1}). The moderate average expression obtained by the *TaGstA1* promoter has to be assigned to its specificity for the epidermis that represents only a minor proportion of the leaf. In an additional experiment using the same transgenic plants carrying the

Figure 2. Overexpression of GUS in transgenic barley. The amount of GUS protein is dependent on the promoter controlling the overexpression cassette. A, Barley transformants (T_1 generation; 10 d old) expressing the *gus* reporter under the control of the *ZmUbi1* promoter (pIPKb002_GUS) were analyzed for GUS activity. The photograph represents leaf segments as typical examples of constitutive *gus* reporter activity in a segregating population. B, Ten-day-old seedlings from independently generated transgenic barley lines expressing the *gus* reporter under the control of the *ZmUbi1* (pIPKb002_GUS), *OsAct1* (pIPKb003_GUS), CaMV *d35S* (pIPKb004_GUS), and *TaGstA1* (pIPKb005_GUSI) promoters, respectively, were pooled (15 T_0 plants each) for quantitative fluorimetric GUS measurements. Wild-type barley plants (white bars) served as controls. Specific GUS enzyme activity is shown. Quantification was reproduced twice with very similar results.
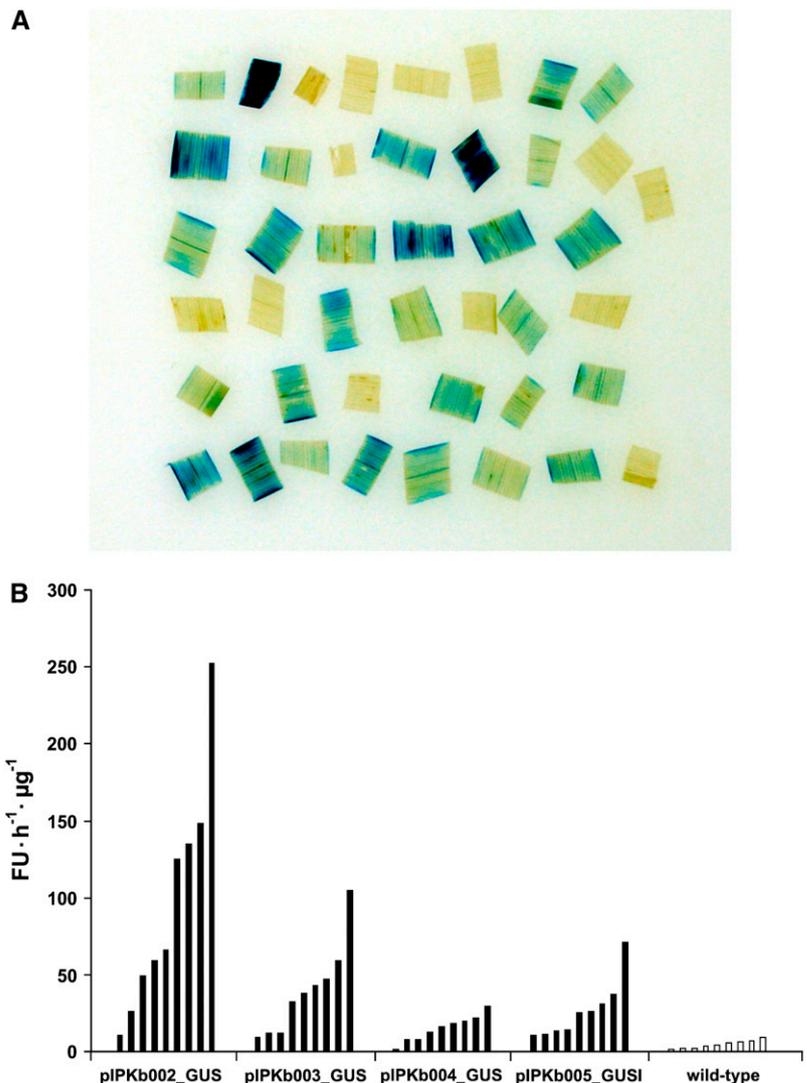
gus reporter under the control of the *TaGstA1* promoter, reporter expression was compared in epidermis pealed off from the abaxial side of leaves to that of the corresponding leaf remnants with the upper epidermis still attached to their adaxial surface (because upper epidermis cannot be removed appropriately). Fluorescence spectroscopy revealed that GUS activity in isolated epidermis was, on average, 10 times as strong as in the corresponding leaf remnants. This result not only confirmed our earlier finding that the *TaGstA1* promoter drives specific expression in barley leaf epidermis (Altpeter et al., 2005), but also that this particular promoter specificity is retained in the context of pIPKb005. Furthermore, the epidermis represents an important tissue for plant defense against pathogens that enter the plant via a direct mode of penetration through the epidermis (e.g. powdery mildew fungus [*Blumeria graminis*] and Fusarium head blight [*Fusarium culmorum*]). The epidermis-specific wheat *TaGstA1* promoter could therefore be used to control the expression of antifungal effector genes, thereby interfering with pathogen infection.

Derivatives of pIPKb002 to pIPKb005 with various genes of interest integrated in the GATEWAY destination site were successfully used to produce stable transgenic barley and wheat plants. The molecular and phenotypic characterization of these plants will be published elsewhere. Moreover, the vectors pIPKb002_GUS and pIPKb004_GUS carrying the *gus* reporter under the control of the *ZmUbi1* and the *d35S* promoter, respectively, were used to stably transform tobacco (*Nicotiana tabacum*). Expectedly, these plants showed ubiquitous expression of the *gus* reporter as revealed by fluorescence spectroscopy (data not shown). This result indicates that the vector set presented here provides the opportunity to transform both mono- and dicotyledonous plant species with the same binary vector.

Functional Analysis of Binary RNAi Vectors

The discovery of RNAi triggered by double-stranded RNA paved the way for the high-throughput production of loss-of-function mutants for functional genomics in plants, including cereals (Waterhouse et al., 1998). The set of binary destination vectors for cereals described here allows the constitutive expression of RNAi sequences under the control of the *d35S* promoter, as well as of the *ZmUbi1* and the *OsAct1* promoter. Furthermore, we provide a destination vector containing the wheat *TaGstA1* promoter, which permits the epidermis-specific knock-down of gene expression.

To test binary RNAi vectors for their performance in the TIGS system (Nielsen et al., 1999; Schweizer et al., 1999, 2000), we transiently knocked down the barley mildew resistance locus o (*Mlo* gene), which encodes a negative regulator of resistance to the powdery mildew fungus. Barley leaf segments were challenge inoculated with powdery mildew fungus (*B. graminis*

f. sp. *hordei*) and scored for their resistance phenotype at the single-cell level in successfully transformed epidermis cells, marked by expression of the *gus* reporter. Introduction of the empty binary vectors revealed an expected susceptibility to powdery mildew ranging from 18% to 38%. However, delivery of the binary RNAi constructs directed against the *Mlo* gene greatly reduced susceptibility (<5%) in all promoters tested (Fig. 3). Similar results were obtained with control vector (pIPKTA36; Douchkov et al., 2005) particularly designed for TIGS experiments targeting the *Mlo* gene. The results suggest that the function of the negative regulator of resistance (*Mlo*) has been eliminated or at least largely reduced by the RNAi constructs, thereby leading to a phenocopy of the loss-of-function *mlo* resistance allele.

Derivatives of pIPKb007 to pIPKb010 with fragments from various genes of interest integrated in the GATEWAY destination sites were successfully used to produce stable transgenic barley and wheat plants. Molecular and phenotypic characterization of these plants will be published elsewhere.

CONCLUSION

A series of modular binary plasmids for stable *Agrobacterium*-mediated transformation of cereals
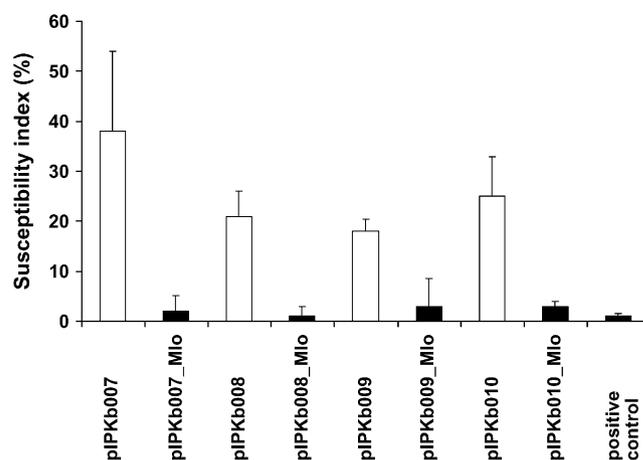
Figure 3. TIGS of the *Mlo* gene caused increased resistance to powdery mildew (*B. graminis* f. sp. *hordei*) infection. Binary RNAi constructs targeting the *Mlo* gene were cobombarded together with a *gus* expression plasmid (*ZmUbi1*-promoter-*gus* fusion) followed by challenge inoculation with powdery mildew (*B. graminis* f. sp. *hordei*) 3 d postbombardment. Expression of the RNAi sequence was controlled by the *ZmUbi1* (pIPKb007_Mlo), *OsAct1* (pIPKb008_Mlo), CaMV *d35S* (pIPKb009_Mlo), and *TaGstA1* (pIPKb010_Mlo) promoters, respectively. Cells transformed with the corresponding empty vectors (pIPKb007, pIPKb008, pIPKb009, and pIPKb010) served as controls. Plasmid pIPKTA36 was utilized as a positive control for RNAi-mediated gene silencing of the *Mlo* gene. Haustorium formation of powdery mildew was scored 48 h postinoculation. The susceptibility index represents the number of GUS-positive cells harboring at least one haustorium divided by the total number of GUS-stained cells. The mean SD of three independent experiments is shown.

such as barley and wheat is made freely available for noncommercial use. Vector derivatives are provided for overexpression studies or knock-down analyses. Modular configuration of the presented vectors allows for convenient introduction of coding sequences to be overexpressed or knocked down, any promoter sequence to drive the gene of interest, as well as any preferred plant selectable marker cassette. This provides the opportunity to generate vector derivatives tailored for the particular requirements of various plant transformation systems and for the ultimate elucidation of the function of any particular candidate DNA sequence. The introduction of genes of interest in these generic vectors is greatly facilitated by the implementation of the GATEWAY recombinational cloning system in both the overexpression and the knock-down vectors presented. Beside the highly beneficial simplification of cloning RNAi constructs, a major advance derives from the opportunity to easily generate overexpression and knock-down binary vectors using entire GATEWAY-compatible cDNA libraries. High versatility of the vector set is further provided through construction of derivatives with promoters functional in cereal species, which drive ubiquitous or epidermis-specific expression of transgenes.

Although data providing direct functional proof of the newly developed binary vectors are presented here only for barley, it can be anticipated that the vector set will also be useful for any further cereal or monocotyledonous species. Moreover, some of the vectors generated have been shown to be amenable to the genetic transformation of both mono- and dicotyledonous plants.

Eventually, the presented vector set provides a potential basis for the implementation of further useful features, such as the integration of affinity or screenable tags that can be N- or C-translationally attached to the coding sequence, or for the development of systems that permit conditional gene expression or directed T-DNA insertion mutagenesis in cereal species.

MATERIALS AND METHODS

Plasmid Construction

All molecular biological manipulations were performed according to standard protocols (Sambrook and Russel, 2001). The constructs that involved PCR and synthetic oligonucleotides were verified by sequencing. Details of plasmid constructs are available in Supplemental Materials and Methods S1.

Plants and Powdery Mildew Fungus

Barley (*Hordeum vulgare* 'Ingrid' and 'Golden Promise') and powdery mildew (*Blumeria graminis* DC Speer f. sp. *hordei*) were cultivated as described elsewhere (Zimmermann et al., 2006). Leaf segments of plants were challenge inoculated with powdery mildew at a density of 150 to 200 conidia mm^{-2}.

Generation of Transgenic Plants

Immature barley 'Golden Promise' embryos were transformed with the GUS overexpression vector series using the *Agrobacterium tumefaciens* strain AGL1 as described elsewhere (Hensel et al., 2008). The resulting plantlets were selected on medium containing hygromycin (50 mg L^{-1}).

Transient Expression and TIGS

Binary plasmids were transiently expressed in bombarded barley leaf epidermal cells of 'Ingrid' by using a PDS-1000/He System (Bio-Rad) essentially as described previously (Douchkov et al., 2005). To monitor the transformation of epidermal cells, a plasmid expressing the *gus* reporter under the control of the maize (*Zea mays*) *Ubi1* promoter (pUbiGUS) was cotransformed together with the binary vector DNA. For TIGS, bombarded leaf segments were challenge inoculated with powdery mildew (*B. graminis* DC Speer f. sp. *hordei*) 48 h postbombardment. The interaction phenotype, represented by the fraction of GUS-specifically stained epidermis cells harboring at least one haustorium (susceptibility index in percent), was examined by light microscopy (Douchkov et al., 2005).

Protein and GUS Measurements

Leaf or peeled epidermis was ground in liquid nitrogen and 10 mg of material was resuspended in incubation buffer (50 mM sodium phosphate, pH 7.2, 1 mM EDTA, 0.1% [w/v] Triton X-100, 10 mM β-mercaptoethanol). GUS enzyme activity was measured in the soluble protein fraction by using 4-methylumbelliferyl-β-D-glucoside (2 mM) as a substrate. Fluorescence was recorded at 365-nm excitation and 456-nm emission wavelength using a luminescence spectrometer (GIBCO TEC Synergy HT). Protein concentration was determined employing standard methods (Bradford, 1976). Leaf segments were stained histochemically for GUS activity as described elsewhere (Jefferson et al., 1987; Schweizer et al., 1999).

Generation of Entry Vectors (Cyclic-Cut-Ligation) and Clonase Reaction

DNA fragments were ligated into the *Swa*I site of plasmid pIPKTA38 as previously described (Douchkov et al., 2005). Briefly, the ligation reaction containing T4 DNA ligase and the restriction endonuclease *Swa*I was incubated at 25°C for 1 h. Enzymes were then inactivated by heating to 65°C for 15 min. Additional *Swa*I enzyme was added for 1 h at 25°C to quantitatively eliminate religated plasmid. The resulting recombinant pIPKTA38 clones were transformed into *Escherichia coli* (DH10B) cells and verified by restriction analysis. Positive pIPKTA38 clones were used as entry vector in the LR reaction of the GATEWAY system with the binary destination plasmids pIPKb001 to pIPKb010. The Clonase reaction was essentially performed as described elsewhere (Douchkov et al., 2005).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU161567 to EU161576 (pIPKb001 to pIPKb010) and EU161577 (pSB156; supplemental data).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. PCR-based detection of overexpression or knock-down GATEWAY cassettes in transgenic plants.

Supplemental Table S1. Primer sequences for the PCR-based detection of overexpression or knock-down cassettes integrated in the plant genome.

Supplemental Materials and Methods S1. Further information on materials and methods.

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