Expanding the Repertoire of Selectable Markers for *Aspergillus* Transformation

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11.1 Introduction

Aspergilli are a unique group of saprophytic filamentous fungi that dwell in wide spectrum of habitat such as soil, decaying organic matter, and moist indoor environments. Enormous nutritional flexibility underlies their successful lifestyle. The genus *Aspergillus* is gifted with rich ability to secrete degradative enzymes, organic acids and secondary metabolites. These fungi are capable of growth in a wide range of pH (between pH 2.0 and pH 11.0), temperature (10–50 °C) and high osmolarity (Kis-Papo et al. 2003). These endowments have fascinated both academic and industrial researchers for over a century. While *A. nidulans* is an extensively used genetic model organism and an academic favorite, many other Aspergilli are industrial workhorses (Meyer 2008). For instance, *A. niger* is an avid citric acid producer (Karaffa et al. 2001); *A. oryzae* is used to brew sake and make soy sauce (Barbesgaard et al. 1992); *A. terreus* is employed for the production of itaconic acid (Okabe et al. 2009) and lovastatin (Bizukojc and Ledakowicz 2009). Some of them like *A. niger* and *A. oryzae* are choice platforms for heterologous protein production due to their exceptional secretion capacity (Su et al. 2012). In contrast, the genus also includes pathogens like *A. fumigatus*, *A. parasiticus*, and *A. flavus*.

Many whole genome sequences now available for Aspergilli are indicative of their acknowledged value. Genome sequences of several industrially and medically important *Aspergillus* species are freely accessible (Arnaud et al. 2012; Gibbons and Rokas 2013). This has enabled a better appreciation of gene expression, metabolism, and its regulation. Understanding the organization, regulation, and manipulation of fungal genes requires the development of various genetic tools. Due to the industrial importance of these fungi many of such tools may have remained trade secrets. Low transformation efficiency, limited choice of selection markers, and poor frequency of targeted gene insertions are largely responsible for the delay in development of genetic engineering toolkit for these organisms.

A. nidulans was the first *Aspergillus* to be successfully transformed and this involved the use of protoplasts (Tilburn et al. 1983). Subsequently other DNA delivery methods were described along with modifications to suit individual fungal strains (for details see other chapters of this book). Each of these methods has its own advantages and disadvantages (Prabha and Punekar 2004). Regardless of these variations, the transforming DNA either integrates into the host

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genome or replicates autonomously. With rare exceptions, integrative transformation is predominant in filamentous fungi including Aspergilli. Selectable markers are necessary and are used to score transformants (and DNA integration events) in a background of the recipient host. The repertoire of convenient selection markers available for *Aspergillus* transformation is limited. While some markers work well across Aspergilli others are species specific. Several earlier reviews have stressed on various transformation techniques and development of strains with different genetic backgrounds, to study filamentous fungal biology (Fleissner and Dersch 2010; Jiang et al. 2013; Kuck and Hoff 2010; Lubertozzi and Keasling 2009; Meyer 2008; Meyer et al. 2011; Ruiz-Diez 2002; Su et al. 2012; Prabha and Punekar 2004; Weld et al. 2006). However, the description of selectable markers for *Aspergillus* transformation has attracted limited attention. A comprehensive and up-to-date account on various selection markers and different strategies exploiting these markers in manipulating Aspergilli is given here.

11.2 Selectable Markers for *Aspergillus* Transformation

Selectable markers are one of the important molecular tools that distinguish transformed cells from the untransformed host. They generally fall into three categories: resistance markers, nutritional markers, and bidirectional markers. Besides, a few reporter genes also serve as convenient visual markers to select transformants. Selectable markers functionally tested in Aspergilli are described below. Three specific markers, namely *bar*, *agaA*, and *sC* as examples relating to *A. niger* transformation, are particularly emphasized.

11.2.1 Resistance Markers

Resistance markers allow the growth of an organism in the presence of inhibitors (antibiotic or antimetabolite). These are routinely used to transform wild type/natural isolates of fungal strains; they circumvent the need to create an auxotrophic host background. The only requirement is that the recipient organism is sensitive to the selection pressure applied. These markers are particularly useful for organisms with little genetic information available. Resistance markers by and large are dominant and provide a tool for positive selection—the gene imparting a survival phenotype to the recipient on the respective selection medium. Table 11.1 provides a list of such selectable markers reported till date for Aspergilli; of these, *hph*, *bar*, and *benA* are more commonly used. Interestingly, *hsv-1 tk* (coding for herpes simplex virus type 1 thymidine kinase) provides for a negative selection—its expression is lethal to the host organism when grown in the presence of ganciclovir. Lethality is due to the conversion of ganciclovir into a cytotoxic nucleoside analogue. In combination with *ble*, *hsv-1 tk* was used to provide both positive and negative selection in *A. fumigatus* (Krappmann et al. 2005).

Resistance to L-phosphinothricin (PPT) (through the *bar*/*pat* gene) was first used as selection marker in *Neurospora crassa* (Avalos et al. 1989). Inhibition of glutamine synthetase and subsequent accumulation of ammonia are thought to be the major mode of PPT action. Other cellular targets of PPT comprise membrane depolarization (Ullrich et al. 1990) and membrane transport process (Trogisch et al. 1989). The *bar* gene imparts resistance by acetylating PPT; it serves as a dominant selection marker as many fungi are sensitive to PPT (Ahuja and Punekar 2008). Selection of *A. niger* transformants using *bar* gene constructs was achieved with both a homologous promoter (*A. niger PcitA*, Dave and Punekar 2011) and a heterologous promoter (*A. nidulans PtrpC*, Ahuja and Punekar 2008). Besides *bar* transformants, spontaneously resistant *A. niger* colonies were obtained. Analysis of this spontaneous resistance, caused by mutations in the *A. niger* glutamate uptake system, was helpful in fine-tuning *bar* selection conditions. While host sensitivity to PPT may be an issue, the virtues of this marker are many. PPT is an active ingredient of several commercial herbicides (such as Basta, Bayer Crop-Science, India) and is readily accessible. Due to lower toxicity to

Table 11.1 Resistance (dominant) markers in Aspergilli

Selectable marker	Marker gene function (CDS length)	Metabolic target	Species transformed
bar	Phosphinothricin acetyltransferase (552 bp)	Glutamine synthetase	*A. niger*[a], *A. fumigatus*[b], *A. nidulans*[b]
benA	Benomyl resistant β-tubulin (1344 bp)	Mitosis	*A. flavus*, *A. parasiticus*, *A. nidulans*
ble	Phleomycin-binding protein (381 bp)	DNA scission	*A. nidulans*, *A. oryzae*
blmB	Bleomycin *N*-acetyltransferase (900 bp)	DNA scission	*A. oryzae*[c]
cbx	Carboxin-resistant succinic dehydrogenase mutant (567 bp)	TCA cycle	*A. oryzae*[d], *A. parasiticus*[d]
gliT	Gliotoxin sulfhydryl oxidase (1005 bp)	Not known	*A. fumigatus*[e]
hph	Hygromycin B phosphotransferase (1020 bp)	Translation	*A. niger*, *A. nidulans*, *A. sydowii*, *A. giganteus*, *A. terreus*, *A. ficcum*
hsv-1 tk	Thymidine kinase (1131 bp)	Nucleotide metabolism	*A. fumigatus*[f]
oliC	Oligomycin resistant mitochondrial ATP synthase subunit (432 bp)	ATP synthase	*A. nidulans*, *A. niger*
ptrA	Thiamine biosynthetic enzyme (984 bp)	Thiamine antagonist	*A. oryzae*, *A. nidulans*, *A. kawachii*, *A. terreus*, *A. fumigatus*

Table modified and updated from Prabha and Punekar (2004)
[a]Ahuja and Punekar (2008)
[b]Nayak et al. (2006)
[c]Suzuki et al. (2009)
[d]Shima et al. (2009)
[e]Carberry et al. (2012)
[f]Krappmann et al. (2005)

humans, affordable cost and PPT availability, *bar* selection is attractive. The compact size of *bar* (CDS around 0.5 kb) allows construction of expression vectors with larger inserts for genome engineering. The *bar* marker was successfully employed in *A. niger*: to select for EGFP expression (Dave and Punekar 2011), for deletion analysis of *A. niger citA* promoter (Dave and Punekar 2011), and to disrupt *agaA* from *A. niger* (see next section for details). It is also applied in introducing *ldh* (lactate dehydrogenase), *gbuA* (4-guanidinobutyrase), and *adc* (arginine decarboxylase) expression constructs in this fungus (unpublished work).

Several other resistance markers (not listed in Table 11.1) are available for filamentous fungi, but so far they have not been tested in Aspergilli. These include *bsd* (blasticidin S resistance in *Rhizopus niveus*; Yanai et al. 1991), *sur* (sulfonylurea resistance in *Penicillium chrysogenum* and *Magnaporthe grisea*, Sweigard et al. 1997) markers and more recently *nat* (nourseothricin resistance in *N. crassa*, *Cryphonectria parasitica* and *P. chrysogenum*; Smith and Smith 2007; Hoff et al. 2010; de Boer et al. 2013) and *ergA* (terbinafine resistance in *P. chrysogenum*; Sigl et al. 2010).

Some of the disadvantages of resistant markers which may limit their utility include poor selection with few markers due to background growth, random integration of the marker gene into the host genome, some of the markers like *oliC* are species specific, some agents are mutagenic to the fungus (i.e., phleomycin), dominant selection can be too harsh requiring a nonselective phase before cultivating under selective pressure, and restricted availability and toxicity of the antibiotic/antimetabolite required for selection. Most importantly, dominant marker genes are susceptible to horizontal transfer in the environment.

It is therefore highly desirable to eliminate the resistance gene from the genetically modified fungus before its large scale use.

11.2.2 Nutritional Markers

Nutritional markers are usually developed from metabolic pathways. The selection involves the complementation of an auxotrophic strain with the corresponding wild type allele. Development of auxotrophic recipient *Aspergillus* strains is often tricky due to the nutritional versatility of these organisms, poor understanding of their metabolism, and restricted range of available genetic tools. The nutritional markers described so far in Aspergilli are listed in Table 11.2. Markers like *argB* and *riboB* are commonly deployed in *Aspergillus* transformation. Disruption of *argB* (coding for ornithine carbamyltransferase of arginine biosynthesis pathway) leads to arginine auxotrophy and this is exploited as a transformation marker by complementing a matching *argB*$^{-}$ strain (Table 11.2). Either an *argB* mutant or a deliberately *argB* disrupted recipient is used (Lenouvel et al. 2002). Insertion of multiple copies of the bacterial *aspA* gene relieved the nutritional deficiency in NAD-glutamate dehydrogenase negative *A. nidulans* (Hunter et al. 1992). Thus, the *aspA* marker is unique in providing an efficient means of selecting multi-copy transformants.

Arginase (encoded by *agaA*) catabolizes L-arginine to L-ornithine and urea; this defines the only route for arginine utilization in most fungi. The *A. niger agaA* gene when disrupted using a *PtrpC-bar* cassette (by homologous recombination) results in an arginase negative phenotype. The D-42 strain (*agaA*::*bar*) is therefore unable to grow on arginine as sole nitrogen source (Dave et al. 2012). While *agaA* mutants are reported from *N. crassa* (Morgan 1970) and *A. nidulans* (Bartnik et al. 1977), the *A. niger* D-42 strain is the first example of targeted *agaA* disruption in filamentous fungi. Combination of D-42 strain and arginase expression construct (*PcitA-agaA*) defines *agaA* as a novel nutritional marker for *A. niger* transformation. Details of applying this selection marker may be found in an accompanying protocol (see Chap. 39), while the strategy to identify *agaA* transformants is shown in Fig. 11.1. Depending on the integration event, the transformants obtained on arginine plates may either be *agaA*$^{+}$*bar*$^{+}$ (ectopic) or *agaA*$^{+}$*bar*$^{-}$ (homologous). Such an *agaA*/*bar* combination describes a two-way selection with a potential for marker reuse.

The inability of *A. niger* D-42 strain (*agaA*::*bar*) to grow on arginine is consistent with a single pathway for arginine utilization. Both biochemical and bioinformatics approaches support the absence of a functional arginine decarboxylase in *A. niger*. But a pathway to catabolize agmatine was shown to exist in this fungus (Kumar 2013). Introducing a functional arginine decarboxylase is expected to confer the ability to utilize L-arginine to the D-42 mutant. Therefore, arginine decarboxylase could become another novel marker based on fungal arginine catabolism. Initial attempts to express bacterial arginine decarboxylase in *A. niger* D-42 strain for this purpose, have been unsuccessful (unpublished). However, as in *S. cerevisiae*, an alternative route for polyamine biosynthesis was reconstituted by introducing *E. coli* arginine decarboxylase (Klein et al. 1999); we still hope to develop this gene as a successful marker for *A. niger*.

11.2.3 Bidirectional Markers

At times it is advantageous if the same marker can be used for both gain and loss of function. All such two-way selection markers reported to date happen to be nutritional markers (Table 11.3). Such markers are called bidirectional markers as they provide a two-way selection. *pyrG* and *amdS* are frequently used bidirectional markers. As is typical for bidirectional markers, *pyrG* allows both positive and negative selection—*pyrG* mutants are auxotrophic for uridine/uracil but are resistant to 5-fluoroorotic acid (5-FOA). The cloned *amdS* gene was first used as homologous transformation marker in *A. nidulans* (Tilburn et al. 1983). The selection is based on utilization of acetamide by *amdS* expressing transformants. For many Aspergilli that cannot utilize acetamide,

Table 11.2 Nutritional markers in Aspergilli

Selection marker	Marker gene function (CDS length)	Selection	Species transformed
acuD	Isocitrate lyase (1617 bp)	Acetate utilization	*A. nidulans*
adeA	Phosphoribosylaminoimidazolesuccinocarboxamide synthase (2184 bp)	Adenine prototrophy	*A. oryzae*
adeB	Phosphoribosylaminoimidazolecarboxylase (3000 bp)	Adenine prototrophy	*A. oryzae*
agaA	Arginase (975 bp)	Arginine utilization	*A. niger*[a]
argB	Ornithine carbamyltransferase (1194 bp)	Arginine prototrophy	*A. nidulans*, *A. niger*, *A. oryzae*, *A. terreus*
aspA	Aspartase (1437 bp)	Aspartate utilization	*A. nidulans*[b]
bioDA	DAPA[f] synthase and dethiobiotin synthetase (2364 bp)	Biotin prototrophy	*A. nidulans*[c]
hemA	5-Aminolevulinate synthase (1911 bp)	5-Aminolevulinate prototrophy	*A. oryzae*
hoa	Homoserine *O*-acetyltransferase (1572 bp)	Methionine prototrophy	*A. oryzae*[d]
pkiA	Pyruvate kinase (1581 bp)	Fermentable carbon utilization	*A. nidulans*
prn	Proline catabolism (2457 bp)	Proline utilization	*A. nidulans*
pyroA	Not known (915 bp)	Pyridoxine prototrophy	*A. nidulans*[e], *A. fumigatus*[e]
qutE	Catabolic quinate dehydrogenase (462 bp)	Quinate utilization	*A. nidulans*
riboB	Putative GTP cyclohydrolase (1230 bp)	Riboflavin prototrophy	*A. nidulans*, *A. fumigatus*[e]
trpC	Trifunctional enzyme of tryptophan biosynthesis (1995 bp)	Tryptophan prototrophy	*A. nidulans*, *A. niger*

Table modified and updated from Prabha and Punekar (2004)
[a]Dave et al. (2012)
[b]Hunter et al. (1992)
[c]Magliano et al. (2011)
[d]Iimura et al. (1987)
[e]Nayak et al. (2006)
[f]DAPA 7,8-Diaminopelargonic acid

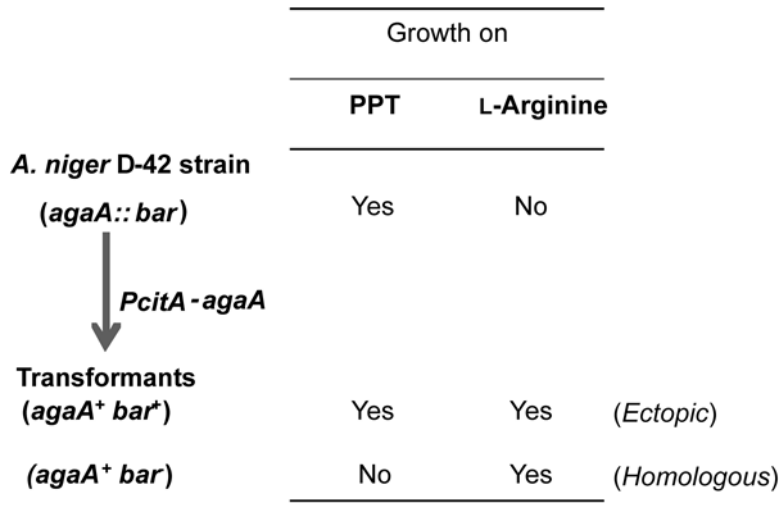

Fig. 11.1 Strategy for *agaA* based selection of transformants in *A. niger*. The *PcitA-agaA* gene construct complements and confers growth phenotype on arginine to D-42 strain (*agaA*::*bar*). Two types of transformants are expected depending upon the nature of integration event

amdS based selection works directly in the wild type background. In this sense, *amdS* represents a nutritional marker which is dominant. Since *amdS* transformants become sensitive to fluoroacetamide, *amdS* was also exploited as a bidirectional marker (Michielse et al. 2005). Bidirectional markers are convenient tools as they can be repeatedly exploited in the same host. This feature is particularly useful when the range of available markers is restricted. The list of bidirectional markers available for fungi, and for Aspergilli in particular, however is limited (Table 11.3).

As a bidirectional marker *pyrG* is convenient and is frequently used. The availability and cost of 5-FOA is sometimes a concern. *sC* is superior as a marker in this regard. ATP sulfurylase (encoded by *sC*) activates inorganic sulfate to form adenosine 5-phosphosulfate (APS) (Fig. 11.2a). The *sC* gene sequence is highly conserved in Aspergilli (Varadarajalu and Punekar 2005) making it easy to use across species. Since ATP sulfurylase is the first committed step in sulfate assimilation, *sC* mutants are incapable of using sulfate as source of sulfur. At the same time these mutants display selenate (a toxic analogue of sulfate) resistance. Both sC^- (selenate resistant) and sC^+ (sulfate utilization) phenotypes can thus be selected for. Homologous transformation with the *sC* marker was first reported in *A. nidulans* (Buxton et al. 1989) and later in *A. fumigatus* (De Lucas et al. 2001). A heterologous *sC* gene was used to transform *A. niger* (Buxton et al. 1989) and *A. oryzae* (Yamada et al. 1997). A mutant (sC^-) background is a prerequisite to use *sC* marker. Spontaneous *sC* mutants are easily isolated on selenate media as they are resistant to selenate. Mutations in the *sB* gene (encoding sulfate permease) could also result in selenate resistance. However, only *sC* mutants are chromate sensitive and can be distinguished from *sB* mutants (Fig. 11.2b, c). Although *sC* has a long CDS, the high degree of sequence conservation (across Aspergilli) at this genomic locus (Varadarajalu and Punekar 2005) could in principle be used for marker assisted homologous integration events.

11.2.4 Visual Marker Systems

Some reporter genes may also serve as selection markers since they allow the transformants to be visually distinguished. Examples of well-known

Table 11.3 Bidirectional markers from Aspergilli

Selection marker	Marker gene function (CDS length)	Selection	Species transformed
acuA	Acetyl CoA synthase (2013 bp)	– Acetate utilization – Fluoroacetate resistance	*A. nidulans*
amdS	Acetamidase (1647 bp)	– Acetamide utilization – Fluoroacetamide resistance	*A. nidulans*, *A. niger*, *A. oryzae*, *A. ficcum*, *A. terreus*
niaD	Nitrate reductase (2622 bp)	– Nitrate utilization – Chlorate resistance	*A. nidulans*, *A. parasiticus*, *A. oryzae*, *A. niger*, *A. alliaceus*, *A. flavus*
pyr4/*pyrG*	OMP decarboxylase (843 bp)	– Uridine/uracil prototrophy – 5-FOA resistance	*A. niger*, *A. nidulans*, *A. oryzae*, *A. parasiticus*, *A. aculeatus*, *A. sojae*, *A. fumigatus*, *A. awamori*
sC	ATP sulfurylase (1725 bp)	– Sulfate utilization – Selenate resistance	*A. nidulans*, *A. oryzae*[a], *A. fumigatus*[b], *A. niger*[c]

Table modified and updated from Prabha and Punekar (2004)
[a]Yamada et al. (1997)
[b]De Lucas et al. (2001)
[c]Varadarajalu and Punekar (2005)

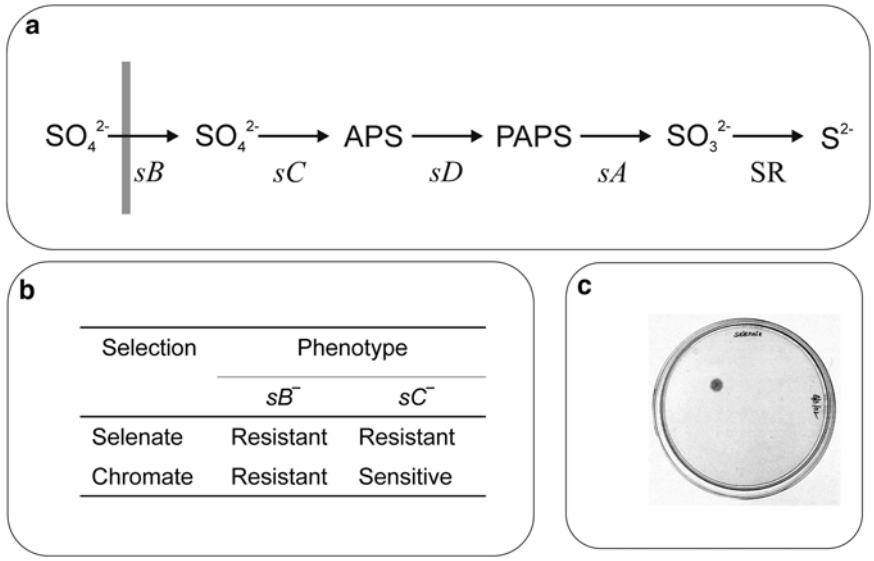

Fig. 11.2 Selection of *sC* mutants. (**a**) Sulfate assimilation pathway in *Aspergillus*, (**b**) strategy to distinguish sC^- and sB^- mutants, and (**c**) spontaneous *A. niger* sC^- mutant (one out of 10^6 spores spread per plate)

reporters include *lacZ* (β-D-galactosidase acting on X-Gal), *gusA* (β-glucuronidase acting on X-Gluc), and laccase genes. They were used as reporters in *A. nidulans* (Kanemori et al. 1999) and *A. parasiticus* (Miller et al. 2005). Heterologous laccases were used as reporters in *A. nidulans* and *A. niger* (Mander et al. 2006). Since then various laccase genes from *A. niger* genome have been cloned and functionally annotated (Ramos et al. 2011). Laccases oxidize artificial substrates like ABTS (2,2-azino-di-(3-ethylbenzthiazoline sulfonate)), ADBP/DMA (4-amino-2,6-dibromophenol/3,5-dimethylaniline), and DMPPDA (*N,N-dimethyl-p*-phenylenediamine sulfate) thereby help locate the transformants on agar plates containing these substrates. The green fluorescence

protein (GFP) and its variants have been extensively used as reporters in Aspergilli (Jiang et al. 2013; Nitsche et al. 2013). However, utility of GFP as a visual marker is constrained because scoring transformants requires their exposure to UV light. In a rare example, the *bar-egfp* protein fusion gene was created to combine selectable and visible markers for *Beauveria bassiana* (Jin et al. 2008).

11.3 Selection Markers in Genome Manipulation Strategies

Transformation with the help of different selectable markers enables genetic manipulation of fungi to study various cellular metabolic processes. Gene knock-in (gene insertion, replacement, or over-expression) and knock-out (gene deletion) approaches have led to the elucidation of many metabolic/gene functions. Industrial strain development may require introduction of one or more steps (genes) of a metabolic pathway. Multiple selectable markers or multiple use of the same marker often becomes a necessity. For instance, a quadruple auxotrophic host (*niaD*$^{-}$*sC*$^{-}$ Δ*argBadeA*$^{-}$/*adeB*$^{-}$) was developed to construct more auxotrophic strains of *A. oryzae* (as also other deuteromycetes wherein sexual crossing is impossible) (Jin et al. 2004). Transformation in *Aspergillus* is almost always an integrative event. Therefore one is quickly restricted by the availability of fresh markers. This limitation may be overcome by devising suitable strategies and judicious use of available markers. Aspects of selectable marker exploitation are highlighted below and include locus specific (homologous) DNA integration events for gene disruption, deletion, or insertion as well as marker rescue.

Homologous recombination is necessary for targeted integration of the DNA along with the selectable marker. Homologous recombination occurs by a single crossover event (type I integration) resulting in the insertion of foreign DNA (and the marker) into the target locus or by a double crossover event (type III integration) leading to replacement of target gene by selection marker. Both these strategies are extensively employed with minor modifications. However, homologous recombination events are rare while ectopic integrations (illegitimate recombination, type II integration) are more common in Aspergilli. Frequency of homologous recombination increases when longer homologous flanking sequences, corresponding to the target locus, are used. While 30–50 bp flanking sequences serve well in *S. cerevisiae* (Hua et al. 1997), much longer flanking sequences (0.5–2.0 kb) are required in Aspergilli (Meyer 2008). The frequency of homologous integration was significantly improved when the components of the nonhomologous end-joining (NHEJ) pathway (of DNA recombination) were deleted in *N. crassa* (Ninomiya et al. 2004). This was extended to Aspergilli by marker-assisted knock out in *A. nidulans* (*nkuA*::*argB*; Nayak et al. 2006), *A. niger* (*kusA*::*amdS* and *kueA*::*pyrG*; Honda et al. 2011; Meyer et al. 2007), *A. oryzae* (*ku70*/*80*::*ptrA*; Takahashi et al. 2006), *A. fumigatus* (*ku80*::*pyrG*; Krappmann et al. 2006), and *A. sojae* (*ku70*/*80*::*ptrA*;Takahashi et al. 2006). This approach has also enabled the genome-wide deletion project in *A. nidulans* (Meyer et al. 2011).

11.3.1 Split Marker

The split marker technique, initially developed in *S. cerevisiae* (Fairhead et al. 1996), has found application in many filamentous fungi (Kuck and Hoff 2010). In this strategy, instead of a complete marker sequence, two partially overlapping fragments of the marker are used. The fragments are so designed that the functional marker is generated only after a recombination event (Fig. 11.3a). This technique requires two PCR products (generated either by two step PCR or by ligation fusion PCR), each comprising a fusion of flanking sequence of the target gene and an appropriate partial–inactive fragment of the marker DNA. The two PCR products have internal sequence overlaps such that a complete marker CDS is generated after recombination. On transformation, three crossover events are required for homologous integration of the split marker replacing the

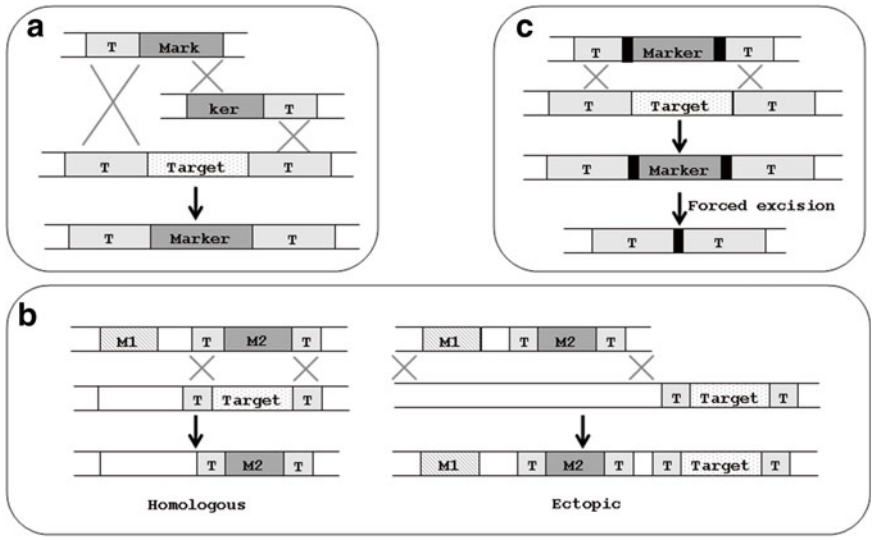

Fig. 11.3 Use of selection markers in fungal genome manipulation. Strategies for split marker (**a**), dual selection (**b**), and marker rescue (**c**). [*T* flanking homologous regions of the target gene, *M1* positive selectable marker, *M2* negative selectable marker, *black bars* direct DNA repeats]

target gene (Kuck and Hoff 2010). The probability of this event increases when the fragments are in close proximity and when they are at the desired locus. Ectopic integration of either of the fragments of such a split marker will be nonfunctional and hence those transformants are not selected in the screen. The split marker approach significantly improves the chances of homologous over ectopic integrations; but the transformation frequency decreases notably as three recombination events are required. An *hph* split marker was used to disrupt *pyrG* and *acuB* genes of *A. niger* (Nielsen et al. 2007); interestingly, *pyrG* was later rescued (also see Sect. 3.3 below) by eliminating the *hph* marker. Disruption of *A. fumigatus* genes involved in trehalose biosynthetic pathway (*tpsA* and *tpsB*, Al-Bader et al. 2010) and also of photoreceptors (*lreA* and *fphA*, Fuller et al. 2013) was achieved through the split marker strategy.

11.3.2 Dual Selection

Dual selection strategy makes use of two markers—one for positive and another for negative selection. This allows one to distinguish between homologous (positive selection) and ectopic (negative selection) integrations of a gene-cassette. The positive selection marker (often resistance or prototrophy) is designed to be locus specific while the other marker (often lethality or auxotrophic) provides for negative selection. The ectopic transformants express both the markers whereas a targeted gene replacement results in the loss of negative marker and is amenable to positive selection (Fig. 11.3b). Although tested in many fungi (such as Gardiner and Howlett 2004; Khang et al. 2005), dual selection is reported in only two species of Aspergilli. The *niaD*, *areA*, and the tannase genes of *A. sojae* were individually disrupted by dual selection using *pyrG* and *oliC* markers (Takahashi et al. 2004). Homologous integration at *pyrG* locus of *A. awamori* was achieved through the *hph* and *amdS* twin marker system (Michielse et al. 2005).

11.3.3 Marker Rescue

The list of markers at our disposal is not extensive and therefore recycling/reusing a marker is of essence. The issue is particularly acute when the same recipient has to be transformed repeatedly.

The problem was cleverly addressed by the advent of a "blaster cassette" tool for sequential deletion of multiple genes in yeast. This method allows the rescue and reuse of a single selectable marker, mostly a bidirectional marker. The yeast *URA*-blaster cassettes are tripartite sequences consisting of *URA3* gene flanked on both sides by direct DNA repeats (Alani et al. 1987; Fonzi and Irwin 1993). Transformants become prototrophic for uracil upon *URA3* integration. Forced excision of *URA3*, through recombination between the two direct repeats, is achieved in the presence of 5-FOA. The desired locus continues to remain disrupted as one copy of the direct repeat is left behind (Fig. 11.3c). This rescues the *URA* marker and the organism is rendered auxotrophic for uracil. The *URA*-blaster cassette is again available for the next round of gene integration/disruption.

A *URA3* homolog was used to develop the corresponding blaster cassette (*pyrG*-blaster) for Aspergilli. The *pyrG*-blaster was successfully used to disrupt *rodA* gene of *A. fumigatus* (d'Enfert 1996) and *lacA* and *glaA* genes of *A. niger* (Storms et al. 2005). Deletion of *A. nidulans aroC* was achieved with a *pyrG*-blaster module that combines ET cloning (RecE and RecT mediated recombinogenic engineering approach) to rescue the marker (Krappmann and Braus 2003). Marker rescue based on the *cre/loxP* recombination system is also available for Aspergilli. Here, a *loxP* direct DNA repeat is placed on both sides of *pyrG* marker. The Cre recombinase recognizes and catalyzes reciprocal recombination between the pair of *lox* repeats, thereby rescuing the marker. Marker rescue using *cre/loxP* system was used to disrupt *pabaA* and *veA* loci of *A. fumigatus* (Krappmann et al. 2005) and *ligD* gene of *A. oryzae* (Mizutani et al. 2012). Successive disruption of *yA* and *wA* genes was achieved in *A. nidulans* using *cre/loxP* blaster cassettes (Forment et al. 2006). As an improvement, a self-excising marker cassette that employs the prokaryotic β-rec/*six* site-specific recombination system was adopted to recover the *ptrA* marker after disrupting *abr2* and *pksP* genes of *A. fumigatus* (Hartmann et al. 2010).

Marker rescue strategy may also be used to transiently disrupt a gene. As noted above, NHEJ disruption favors homologous recombination. However, such NHEJ disrupted strains display increased sensitivity towards DNA damaging conditions such as γ irradiation (Meyer et al. 2007). Restoring the NHEJ function after the required genetic manipulation is therefore desirable. Transient disruption and subsequent recovery of NHEJ function was demonstrated in *A. nidulans*. The *pyrG*-blaster module served to disrupt *nkuA*; required genetic manipulation was done and finally *nkuA* function was regained by recombining the flanking direct repeats of *nkuA* itself (Nielsen et al. 2008).

11.4 Conclusions

The importance of Aspergilli as an industrially and medically important group of fungi is well established. A growing interest in both basic and applied research on Aspergilli acknowledges this fact. Many whole genome sequences are now available and await exploitation. Both genome annotation and strain development require genetic manipulations. Selectable markers are central to these objectives. A range of selection markers are now available to engineer *Aspergillus* genomes. More are being developed based on the knowledge of the fungal genomes. Selectable markers find direct applications in strain improvement programs for industrially important Aspergilli. Strategies for safe and judicious use of available markers through locus specific integration, marker rescue, and self-excision subsequent to site-specific recombination continue to emerge.

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References

Ahuja M, Punekar NS (2008) Phosphinothricin resistance in *Aspergillus niger* and its utility as a selectable transformation marker. Fungal Genet Biol 45:1103–1110

Alani E, Cao L, Kleckner N (1987) A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics 116:541–545

Avalos J, Geever RF, Case ME (1989) Bialaphos resistance as a dominant selectable marker in *Neurospora crassa*. Curr Genet 16:369–372

Al-Bader N, Vanier G, Liu L, Gravelat FN, Urb M, Hoareau CM, Campoli P, Chabot J, Filler SG, Sheppard DC (2010) Role of trehalose biosynthesis in *Aspergillus fumigatus* development, stress response, and virulence. Infect Immun 78:3007–3018

Arnaud MB, Cerqueira GC, Inglis DO, Skrzypek MS, Binkley J, Chibucos MC, Crabtree J, Howarth C, Orvis J, Shah P, Wymore F, Binkley G, Miyasato SR, Simison M, Sherlock G, Wortman JR (2012) The *Aspergillus* Genome Database (AspGD): recent developments in comprehensive multispecies curation, comparative genomics and community resources. Nucleic Acids Res 40:653–659

Barbesgaard P, Heldt-Hansen HP, Diderichsen B (1992) On the safety of *Aspergillus oryzae*: a review. Appl Microbiol Biotechnol 36:569–572

Bartnik E, Guzewska J, Klimczuk J, Piotrowska M, Weglenski P (1977) Regulation of arginine catabolism in *Aspergillus nidulans*. In: Smith JE, Pateman JA (eds) Genetics and physiology of *Aspergillus*. Academic, London, pp 243–254

Bizukojc M, Ledakowicz S (2009) Physiological, morphological and kinetic aspects of lovastatin biosynthesis by *Aspergillus terreus*. Biotechnol J 4:647–664

Buxton FP, Gwynne DI, Davies RW (1989) Cloning of a new bidirectionally selectable marker for *Aspergillus* strains. Gene 84:329–334

Carberry S, Molloy E, Hammel S, O'Keeffe G, Jones GW, Kavanagh K, Doyle S (2012) Gliotoxin effects on fungal growth: mechanisms and exploitation. Fungal Genet Biol 49:302–312

d'Enfert C (1996) Selection of multiple disruption events in Aspergillus fumigatus using the orotidine-5′-decarboxylase gene, pyrG, as a unique transformation marker. Curr Genet 30:76–82

Dave K, Ahuja M, Jayashri TN, Sirola RB, Punekar NS (2012) A novel selectable marker based on *Aspergillus niger* arginase expression. Enzyme Microb Technol 51:53–58

Dave K, Punekar NS (2011) Utility of *Aspergillus niger* citrate synthase promoter for heterologous expression. J Biotechnol 155:173–177

de Boer P, Bronkhof J, Dukić K, Kerkman R, Touw H, van den Berg M, Offringa R (2013) Efficient gene targeting in *Penicillium chrysogenum* using novel Agrobacterium-mediated transformation approaches. Fungal Genet Biol 61:9–14

De Lucas JR, Dominguez AI, Higuero Y, Martinez O, Romero B, Mendoza A, Garcia-Bustos JF, Laborda F (2001) Development of a homologous transformation system for the opportunistic human pathogen *Aspergillus fumigatus* based on the *sC* gene encoding ATP sulfurylase. Arch Microbiol 176:106–113

Gibbons JG, Rokas A (2013) The function and evolution of the *Aspergillus* genome. Trends Microbiol 21: 14–22

Fairhead C, Llorente B, Denis F, Soler M, Dujon B (1996) New vectors for combinatorial deletions in yeast chromosomes and for gap-repair cloning using 'split-marker' recombination. Yeast 12:1439–1457

Fleissner A, Dersch P (2010) Expression and export: recombinant protein production systems for *Aspergillus*. Appl Microbiol Biotechnol 87:1255–1270

Fonzi WA, Irwin MY (1993) Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 134:717–728

Forment JV, Ramon D, MacCabe AP (2006) Consecutive gene deletions in *Aspergillus nidulans*: application of the *Cre/loxP* system. Curr Genet 50:217–224

Fuller KK, Ringelberg CS, Loros JJ, Dunlap JC (2013) The fungal pathogen *Aspergillus fumigatus* regulates growth, metabolism, and stress resistance in response to light. mBio 4:e00142-13

Gardiner DM, Howlett BJ (2004) Negative selection using thymidine kinase increases the efficiency of recovery of transformants with targeted genes in the filamentous fungus *Leptosphaeria maculans*. Curr Genet 45: 249–255

Hartmann T, Dumig M, Jaber BM, Szewczyk E, Olbermann P, Morschhauser J, Krappmann S (2010) Validation of a self-excising marker in the human pathogen *Aspergillus fumigatus* by employing the beta-rec/*six* site-specific recombination system. Appl Environ Microbiol 76:6313–6317

Hoff B, Kamerewerd J, Sigl C, Zadra I, Kück U (2010) Homologous recombination in the antibiotic producer *Penicillium chrysogenum*: strain *ΔPcku70* shows up-regulation of genes from the HOG pathway. Appl Microbiol Biotechnol 85(4):1081–1094

Honda Y, Kobayashi K, Kirimura K (2011) Increases in gene-targeting frequencies due to disruption of *kueA* as a *ku80* homolog in citric acid-producing *Aspergillus niger*. Biosci Biotechnol Biochem 75:1594–1596

Hua SB, Qiu M, Chan E, Zhu L, Luo Y (1997) Minimum length of sequence homology required for in vivo cloning by homologous recombination in yeast. Plasmid 38:91–96

Hunter GD, Bailey CR, Arst HN (1992) Expression of a bacterial aspartase gene in *Aspergillus nidulans*: an efficient system for selecting multicopy transformants. Curr Genet 22:377–383

Iimura Y, Gomi K, Uzu H, Hara S (1987) Transformation of *Aspergillus oryzae* through plasmid-mediated complementation of the methionine-auxotrophic mutation. Agric Biol Chem 51:323–328

Jiang D, Zhu W, Wang Y, Sun C, Zhang KQ, Yang J (2013) Molecular tools for functional genomics in filamentous

fungi: recent advances and new strategies. Biotechnol Adv 31:1562–1574

Jin FJ, Maruyama J, Juvvadi PR, Arioka M, Kitamoto K (2004) Development of a novel quadruple auxotrophic host transformation system by *argB* gene disruption using *adeA* gene and exploiting adenine auxotrophy in *Aspergillus oryzae*. FEMS Microbiol Lett 239:79–85

Jin K, Zhang Y, Luo Z, Xiao Y, Fan Y, Wu D, Pei Y (2008) An improved method for *Beauveria bassiana* transformation using phosphinothricin acetlytransferase and green fluorescent protein fusion gene as a selectable and visible marker. Biotechnol Lett 30:1379–1383

Kanemori Y, Gomi K, Kitamoto K, Kumagai C, Tamura G (1999) Insertion analysis of putative functional elements in the promoter region of the *Aspergillus oryzae* Taka-amylase A gene (*amyB*) using a heterologous *Aspergillus nidulans amdS-lacZ* fusion gene system. Biosci Biotechnol Biochem 63:180–183

Karaffa L, Sandor E, Fekete E, Szentirmai A (2001) The biochemistry of citric acid accumulation by *Aspergillus niger*. Acta Microbiol Immunol Hung 48:429–440

Khang CH, Park SY, Lee YH, Kang S (2005) A dual selection based, targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium oxysporum*. Fungal Genet Biol 42:483–492

Kis-Papo T, Oren A, Wasser SP, Nevo E (2003) Survival of filamentous fungi in hypersaline Dead Sea water. Microb Ecol 45:183–190

Klein RD, Geary TG, Gibson AS, Favreau MA, Winterrowd CA, Upton SJ, Keithly JS, Zhu G, Malmberg RL, Martinez MP, Yarlett N (1999) Reconstitution of a bacterial/plant polyamine biosynthesis pathway in *Saccharomyces cerevisiae*. Microbiology 145:301–307

Krappmann S, Bayram O, Braus GH (2005) Deletion and allelic exchange of the *Aspergillus fumigatus veA* locus via a novel recyclable marker module. Eukaryot Cell 4:1298–1307

Krappmann S, Braus GH (2003) Deletion of *Aspergillus nidulans aroC* using a novel blaster module that combines ET cloning and marker rescue. Mol Genet Genomics 268:675–683

Krappmann S, Sasse C, Braus GH (2006) Gene targeting in *Aspergillus fumigatus* by homologous recombination is facilitated in a nonhomologous end- joining-deficient genetic background. Eukaryot Cell 5:212–215

Kuck U, Hoff B (2010) New tools for the genetic manipulation of filamentous fungi. Appl Microbiol Biotechnol 86:51–62

Lenouvel F, van de Vondervoort P, Visser J (2002) Disruption of the *Aspergillus niger argB* gene: a tool for transformation. Curr Genet 41:425–432

Lubertozzi D, Keasling JD (2009) Developing *Aspergillus* as a host for heterologous expression. Biotechnol Adv 27:53–75

Magliano P, Flipphi M, Sanglard D, Poirier Y (2011) Characterization of the *Aspergillus nidulans* biotin biosynthetic gene cluster and use of the *bioDA* gene as a new transformation marker. Fungal Genet Biol 48:208–215

Mander GJ, Wang H, Bodie E, Wagner J, Vienken K, Vinuesa C, Foster C, Leeder AC, Allen G, Hamill V, Janssen GG, Dunn-Coleman N, Karos M, Lemaire HG, Subkowski T, Bollschweiler C, Turner G, Nusslein B, Fischer R (2006) Use of laccase as a novel, versatile reporter system in filamentous fungi. Appl Environ Microbiol 72:5020–5026

Meyer V, Arentshorst M, El-Ghezal A, Drews AC, Kooistra R, van den Hondel CA, Ram AF (2007) Highly efficient gene targeting in the *Aspergillus niger kusA* mutant. J Biotechnol 128:770–775

Meyer V (2008) Genetic engineering of filamentous fungi—progress, obstacles and future trends. Biotechnol Adv 26:177–185

Meyer V, Wu B, Ram AF (2011) *Aspergillus* as a multi-purpose cell factory: current status and perspectives. Biotechnol Lett 33:469–476

Michielse CB, Arentshorst M, Ram AF, van den Hondel CA (2005) Agrobacterium-mediated transformation leads to improved gene replacement efficiency in *Aspergillus awamori*. Fungal Genet Biol 42:9–19

Miller MJ, Roze LV, Trail F, Linz JE (2005) Role of cis-acting sites *NorL*, a TATA box, and *AflR1* in *nor*-1 transcriptional activation in *Aspergillus parasiticus*. Appl Environ Microbiol 71:1539–1545

Mizutani O, Masaki K, Gomi K, Iefuji H (2012) Modified *Cre-loxP* recombination in *Aspergillus oryzae* by direct introduction of *Cre* recombinase for marker gene rescue. Appl Environ Microbiol 78:4126–4133

Morgan DH (1970) Selection and characterisation of mutants lacking arginase in *Neurospora crassa*. Mol Gen Genet 108:291–302

Nayak T, Szewczyk E, Oakley CE, Osmani A, Ukil L, Murray SL, Hynes MJ, Osmani SA, Oakley BR (2006) A versatile and efficient gene-targeting system for *Aspergillus nidulans*. Genetics 172:1557–1566

Nielsen ML, de Jongh WA, Meijer SL, Nielsen J, Mortensen UH (2007) Transient marker system for iterative gene targeting of a prototrophic fungus. Appl Environ Microbiol 73:7240–7245

Nielsen JB, Nielsen ML, Mortensen UH (2008) Transient disruption of non-homologous end-joining facilitates targeted genome manipulations in the filamentous fungus *Aspergillus nidulans*. Fungal Genet Biol 45:165–170

Ninomiya Y, Suzuki K, Ishii C, Inoue H (2004) Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc Natl Acad Sci U S A 101:12248–12253

Nitsche BM, Burggraaf-van Welzen A-M, Lamers G, Meyer V, Ram AF (2013) Autophagy promotes survival in aging submerged cultures of the filamentous fungus *Aspergillus niger*. Appl Microbiol Biotechnol 97:8205–8218

Okabe M, Lies D, Kanamasa S, Park EY (2009) Biotechnological production of itaconic acid and its biosynthesis in *Aspergillus terreus*. Appl Microbiol Biotechnol 84:597–606

Prabha VL, Punekar NS (2004) Genetic transformation in Aspergilli: tools of trade. Indian J Biochem Biophys 41:205–215

Ramos JA, Barends S, Verhaert RM, de Graaff LH (2011) The *Aspergillus niger* multicopper oxidase family: analysis and overexpression of laccase-like encoding genes. Microb Cell Fact 10:78–89

Ruiz-Diez B (2002) Strategies for the transformation of filamentous fungi. J Appl Microbiol 92:189–195

Shima Y, Ito Y, Kaneko S, Hatabayashi H, Watanabe Y, Adachi Y, Yabe K (2009) Identification of three mutant loci conferring carboxin-resistance and development of a novel transformation system in *Aspergillus oryzae*. Fungal Genet Biol 46:67–76

Sigl C, Handler M, Sprenger G, Kurnsteiner H, Zadra I (2010) A novel homologous dominant selection marker for genetic transformation of *Penicillium chrysogenum*: overexpression of squalene epoxidase-encoding *ergA*. J Biotechnol 151:307–311

Smith RP, Smith ML (2007) Two yeast plasmids that confer nourseothricin-dihydrogen sulfate and hygromycin B resistance in *Neurospora crassa* and *Cryphonectria parasitica*. Fungal Genet Newsl 54:12–13

Storms R, Zheng Y, Li H, Sillaots S, Martinez-Perez A, Tsang A (2005) Plasmid vectors for protein production, gene expression and molecular manipulations in *Aspergillus niger*. Plasmid 53:191–204

Su X, Schmitz G, Zhang M, Mackie RI, Cann IK (2012) Heterologous gene expression in filamentous fungi. Adv Appl Microbiol 81:1–61

Kumar S (2013) Metabolism of guanidinium compounds in *Aspergillus niger*: role of ureohydrolases. Ph.D. thesis, Indian Institute of Technology Bombay, India

Suzuki S, Tada S, Fukuoka M, Taketani H, Tsukakoshi Y, Matsushita M, Oda K, Kusumoto K, Kashiwagi Y, Sugiyama M (2009) A novel transformation system using a bleomycin resistance marker with chemosensitizers for *Aspergillus oryzae*. Biochem Biophys Res Commun 383:42–47

Sweigard JA, Carroll AM, Farrall L, Valent B (1997) A series of vectors for fungal transformation. Fungal Genet Newsl 44:52–53

Takahashi T, Hatamoto O, Koyama Y, Abe K (2004) Efficient gene disruption in the koji-mold *Aspergillus sojae* using a novel variation of the positive-negative method. Mol Genet Genomics 272:344–352

Takahashi T, Masuda T, Koyama Y (2006) Enhanced gene targeting frequency in *ku70* and *ku80* disruption mutants of *Aspergillus sojae* and *Aspergillus oryzae*. Mol Genet Genomics 275:460–470

Tilburn J, Scazzocchio C, Taylor GG, Zabicky-Zissman JH, Lockington RA, Davies RW (1983) Transformation by integration in *Aspergillus nidulans*. Gene 26: 205–221

Trogisch GD, Kocher H, Ullrich WR (1989) Effects of glufosinate on anion uptakein *Lemna gibba* G1. Z Naturforsch 44:33–38

Ullrich WR, Ullricheberius CI, Kocher H (1990) Uptake of glufosinate andconcomitant membrane-potential changes in *Lemna-Gibba* G1. Pestic Biochem Physiol 37:1–11

Varadarajalu LP, Punekar NS (2005) Cloning and use of *sC* as homologous marker for *Aspergillus niger* transformation. J Microbiol Methods 61:219–224

Weld RJ, Plummer KM, Carpenter MA, Ridgway HJ (2006) Approaches to functional genomics in filamentous fungi. Cell Res 16:31–44

Yamada O, Lee BR, Gomi K (1997) Transformation system for *Aspergillus oryzae* with double auxotrophic mutations, *niaD* and *sC*. Biosci Biotechnol Biochem 61:1367–1369

Yanai K, Horiuchi H, Takagi M, Yano K (1991) Transformation of *Rhizopus niveus* using bacterial blasticidin S resistance gene as a dominant selectable marker. Curr Genet 19:221–226