

# 1 Selectable and Screenable Markers for Rice Transformation

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## 1.1 Introduction

Rice transformation is a major goal in cereal biotechnology, not only because rice is the world's most important food crop but also because this species has now been recognized as the model for cereal genomics. A number of alternative transformation strategies are available, the most widely used of which are particle bombardment (Christou et al. 1991) and *Agrobacterium*-mediated transformation (Hiei et al. 1994). Regardless of the chosen strategy, transformation is a low-efficiency process. Since most foreign genes introduced into plants do not confer a phenotype that can be used conveniently for the identification or selective propagation of transformed cells, marker genes must therefore be introduced along with the transgene of interest to provide such a phenotype.

A selectable marker gene encodes a product that allows the transformed cell to survive and grow under conditions that kill or restrict the growth of nontransformed cells. Most such genes used in rice are dominant selectable markers that confer resistance to antibiotics or herbicides (Table 1.1). In recent years, public concern over the safety of such markers has been aroused in some parts of Europe. It is perceived that antibiotic-resistance markers could pose a threat to health if they "escaped" into nature (especially those conferring resistance to antibiotics used in clinical medicine), while herbicide-resistance markers could damage the ecosystem if they spread from crops to related weed species. Although research suggests that the likelihood of such horizontal gene transfer is minimal, some laboratories have focused on the development of a new breed of selectable markers whose biological activities pose no potential harmful effects. Strategies have also been developed to eliminate markers from transgenic plants. These include attempts to introduce the transgene of interest and the marker gene at separate loci, so they can be separated by sexual crossing (Komari et al. 1996), and the use of site-specific recombination systems such as *Cre-loxP* (Dale and Ow 1991; Zubko et al. 2000) or transpositional mechanisms involving *Ac-Ds* elements (Goldsbrough et al. 1993) to eliminate the markers once the transgenic line is stable.

A screenable marker gene, also called a visual marker, scorable marker or reporter gene, generates a product that can be detected using a simple and often quantitative assay (Table 1.2). Such markers are used for a variety of purposes, e.g. confirming transformation, determining transformation efficiency

**Table 1.1.** Selectable markers and selective agents that have been used or evaluated in rice

Selectable marker	Source	Principle of selection	Comments and principle references for use in rice
<i>ble</i> (glycopeptide-binding protein)	<i>Streptalloteichus hindustantus</i>	Confers resistance to the glycopeptide antibiotics bleomycin and pheomycin (and the derivative Zeocin)	Bleomycin evaluated for selection against wild-type cells. <i>ble</i> gene not yet introduced as marker; Dekeyser et al. (1989)
<i>dhfr</i> (dihydrofolate reductase)	Mouse	Confers resistance to methotrexate	Tested in cell suspension cultures; Meijer et al. (1991)
<i>hpt</i> (hygromycin phosphotransferase)	<i>Klebsiella</i> spp.	Confers resistance to hygromycin B	Christou et al. (1991); Hiei et al. (1994)
<i>nptII</i> (neomycin phosphotransferase)	<i>Escherichia coli</i>	Confers resistance to the aminoglycoside antibiotics neomycin, kanamycin and G148 (geneticin)	Toriyama et al. (1988); Raineri et al. (1990); Chan et al. (1993)
<i>bar</i> and <i>pat</i> (phosphinothricin acetyltransferase)	<i>Streptomyces hygroscopicus</i>	Confers resistance to phosphinothricin (glufosinate) and the herbicides bialaphos and Basta	Christou et al. (1991); Cao et al. (1992)
<i>csr1-1</i> (acetolactate synthase)	<i>Arabidopsis thaliana</i>	Confers resistance to chlorsulphuron	Li et al. (1992)
<i>tms2</i> (indoleacetic acid hydrolase)	<i>Agrobacterium tumefaciens</i>	Confers sensitivity to naphthaleneacetamide (NAM)	Uphadyaya et al. (2000)

**Table 1.2.** Screenable markers used in rice

Screenable marker	Source	Principle of assay	Comments and principle references for use in rice
<i>gusA</i> ( $\beta$ -glucuronidase, GUS)	<i>Escherichia coli</i>	Catalyses hydrolysis of $\beta$ -glucuronides; variety of colorimetric, fluorometric and chemiluminescent assay formats; can be used for in vitro and in vivo nondestructive assays	Sensitive and versatile. Stable protein; Christou et al. (1991)
<i>luc</i> (firefly luciferase)	The firefly <i>Photinus pyralis</i>	Light produced in the presence of luciferase, its substrate luciferin, $Mg^{2+}$ , oxygen and ATP	Sensitive; unstable protein useful for inducible/repressible systems; Baruah-Wolff et al. (1999)
<i>gfp</i> (green fluorescent protein)	The jellyfish <i>Aequorea victoria</i>	Spontaneous fluorescence under UV or blue light.	Sensitive, substrate independence means it can be used in living plant cells for real-time imaging; Vain et al. (1998)

and monitoring gene or protein activity. The ability of screenable markers to form fusion genes at the transcriptional level can be used to assay the activity of regulatory elements. This property can be exploited, in the form of gene-trap vectors, to isolate and characterize new genes as part of a functional genomics strategy. Screenable markers can also form translational fusion products, which allows them to be used to monitor protein localization in the cell or at a whole-plant level.

We begin this chapter by considering the benefits and potential disadvantages of individual marker genes used in transgenic rice. It is desirable to have available a range of different markers so that techniques such as multi-step transformation (with different selectable markers), or the simultaneous monitoring of the activities of several genes (with different visual markers) are possible. We conclude with a brief discussion of strategies for marker-gene delivery and expression.

## 1.2 Dominant Selectable Markers for Rice

### 1.2.1 Aminoglycoside 3'-Phosphotransferase (Neomycin Phosphotransferase)

The first reports of gene transfer to rice involved the transformation of protoplasts, either by electroporation or mediated by polyethylene glycol (Zhang et al. 1988; Zhang and Wu 1988; Datta et al. 1990). In these studies, the selectable marker gene *nptII* (also known as *aph(3')II*, *kan* and *neo*) was used, conferring resistance to aminoglycoside antibiotics such as neomycin, kanamycin and geneticin (G418).

Kanamycin is an effective selective agent for transformed rice protoplasts, but rice callus shows natural resistance to this antibiotic and survives on medium containing levels of kanamycin up to ten times higher than that sufficient to kill many other species (Caplan et al. 1992). It has also been found that protoplast-derived callus selected on kanamycin is very inefficient in terms of regeneration, and that a large number of albino plants arise from such experiments (Toriyama et al. 1988). The earliest reports of rice transformation using *Agrobacterium* also involved kanamycin selection. Raineri et al. (1990) used 200 mg l<sup>-1</sup> kanamycin to select rice callus derived from mature embryos, but no transgenic plants were recovered.

Selection on G418 is more effective than kanamycin, perhaps because G418 is more toxic than kanamycin to rice cells. Furthermore, G418 selection also results in the recovery of a higher proportion of fertile, green, transgenic plants (Ayes and Park 1994). Even so, the overall efficiency of the procedure is low. Chan et al. (1992, 1993) used this antibiotic in combination with the *nptII* gene following *Agrobacterium*-mediated transformation of rice roots and immature embryos. In both cases, it was possible to generate G418-resistant callus, but

transgenic plants could be recovered only when using embryos as the target tissue. However, a total of just four transgenic plants was produced following selection on 40 mg G418 l<sup>-1</sup>.

### 1.2.2 Hygromycin Phosphotransferase

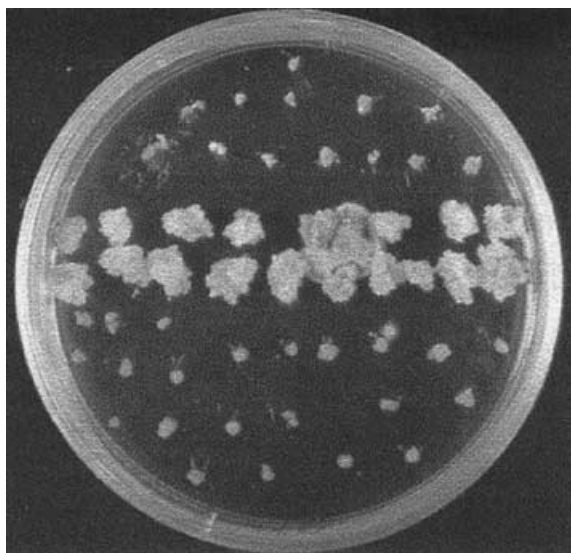
The gene for hygromycin phosphotransferase (*hpt* or *aphIV*) confers resistance to aminoglycoside antibiotics such as hygromycin B (van den Elzen et al. 1985). This antibiotic is much more effective than kanamycin and G418 for the selection of transformed rice tissues because there is no innate resistance, thus providing strong discrimination between transformed and nontransformed cells (Christou and Ford 1995). Furthermore, this antibiotic does not appear to inhibit regeneration, nor affect the fertility of transgenic plants (Aldemita and Hodges 1996).

Hygromycin in combination with the *hpt* marker gene has therefore been used as a selection system in a large number of transformation experiments, encompassing both particle bombardment and *Agrobacterium*-mediated procedures. Indeed, *hpt* was used for the first experiments involving the stable transformation of rice by particle bombardment. Immature rice embryos were bombarded and plated on regeneration medium supplemented with 50 mg hygromycin B l<sup>-1</sup> (Christou et al. 1991). Continuous selection resulted in the appearance of transformed embryogenic callus, from which transgenic plants were recovered. Nontransformed callus, and callus transformed with alternative markers did not survive when plated on hygromycin-supplemented medium (Fig. 1.1).

The first report of high-efficiency transformation of rice using *Agrobacterium* also involved hygromycin selection (Hiei et al. 1994). Callus derived from immature embryos, scutella and suspension cells of various Japonica cultivars was transformed and selected on medium containing between 50 and 100 mg hygromycin l<sup>-1</sup>, resulting in the recovery of large numbers of fertile transgenic plants. Hygromycin at 50 mg l<sup>-1</sup> was also used for the first successful *Agrobacterium*-mediated transformation of Indica and Javanica rice cultivars (Dong et al. 1996; Rashid et al. 1996). Efficient recovery of transgenic rice plants following *Agrobacterium*-mediated transformation has also been reported using 30 mg hygromycin l<sup>-1</sup> (Aldemita and Hodges 1996).

### 1.2.3 Phosphinothricin Acetyltransferase

The *bar* and *pat* genes from *Streptomyces hygroscopicus* encode phosphinothricin acetyltransferase, an enzyme that provides resistance to phosphinothricin (PPT) and derivatives such as bialaphos, which are competitive inhibitors of glutamine synthesis (de Block et al. 1987). PPT, or more correctly its ammonium salt glufosinate, is the active component of the herbicide Basta.



**Fig. 1.1.** Rice callus selected on 50 mg hygromycin ml<sup>-1</sup>. As shown, this gives strong discrimination between transformed and non-transformed tissue. (Sudhakar et al.1998)

An important advantage of this selection strategy is that rice plants can be sprayed with the selective agent in the glass-house or in the field. Rice plants transformed with this marker gene can grow when sprayed with up to 2,000 ppm of the herbicide, a concentration that is sufficient to kill nontransformed plants (Fig. 1.2).

The *bar* gene was evaluated as a selectable marker for rice in the earliest particle bombardment experiments and does not adversely affect regeneration (Christou et al. 1991). The effective concentration of PPT ranged from approximately 0.5 to 10 mg l<sup>-1</sup>. Cao et al. (1992) used *bar* and PPT for the selection of transformed callus generated by particle bombardment, and showed that PPT was at least as effective as hygromycin for the selection of transformed cells. Park et al. (1996) were the first to use *bar* for the selection of rice tissue (in this case shoot apices) transformed by *Agrobacterium*. However, transgenic plants were recovered at a low frequency and the marker was subject to transgene silencing in R2 plants. Basta has also been used in combination with *bar* for the regeneration of transgenic rice plants from transformed protoplasts (Rathore et al. 1993).

#### 1.2.4 Other Dominant Selectable Markers

A wide range of dominant selectable markers is available for plants, including genes providing resistance to other antibiotics (e.g. bleomycin, methotrexate,