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Molecular Breeding of Grasses by Transgenic Approaches for Biofuel Production

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1. Introduction

Since the Industrial Revolution, fossil fuels have been consumed in massive amounts. However, little fossil fuels are estimated to remain, and the number of new oil fields in the world continues to decline. Thus, the future supply of fossil fuels will be tight and will not continue to support our levels of consumption for long. In addition, rapid consumption of fossil fuels for energy production continues to elevate the atmospheric carbon dioxide concentration, which was 280 ppm before the Industrial Revolution and is 380 ppm today. The emissions, including carbon dioxide, derived from the combustion of fossil fuels act as greenhouse gases and cause the serious problem of global warming.

In this context, society must develop beyond the consumption of our limited fossil fuels. Plant biomass, which is organic matter derived from the photosynthetic fixation of atmospheric carbon dioxide, holds much promise as a future energy source. The use of plant biomass for energy is called carbon neutral, because the combustion of plant biomass only releases carbon dioxide that was originally fixed from the atmosphere by plants. Because a sustainable society based on carbon-neutral energy must be constructed to preserve the global environment, we must develop related practical technology as soon as possible.

However, recent hikes in the global price of grain, caused by various political and economic events, has made us consider shifting toward using non-grain plant biomass to improve both energy and food-supply security. Thus, research into the production of biofuel from lignocellulose, which constitutes the bodies of woody and grass plant species, is a major field of study in modern plant science.

Needless to say, this research includes biotechnological approaches. In particular, genetic engineering is expected to be a key technology in herbaceous plant breeding, because most of the bioenergy crop species are not yet domesticated well. This chapter focuses on recent progress in the molecular breeding of herbaceous plants.

2. Herbaceous perennials for dedicated lignocellulosic biofuel crops

High biomass yields are indispensable to successful cultivate energy crops that can compete with and replace fossil fuels (Karpenstein-Machan, 2001; Lewandowski et al., 2003). Considering food-supply security, net energy yield, and environmental protection,

large and fast-growing non-food grass species are among the most promising candidates for lignocellulose production. Perennial species are more attractive than annuals because they have minimal input requirements, commonly yield more aboveground biomass in the form of stems and leaves, and their well-developed root systems can serve as carbon sinks.

Name (Scientific name)[a]	Photosystem	Yield (t DM ha^{-1} yr^{-1})[b]	Location	Citation
Switchgrass (*Panicum virgatum* L.)	C4	15.9‡	U.S.A. (Oklahoma)	Aravindhakshan et al. (2010)
Sugarcane (*Saccharum* spp. hybrids)	C4	41.3[c]	Japan (South western islands)	Terajima et al. (2010)
Miscanthus species (*Miscanthus* × *giganteus*)	C4	28.7†	Central Italy (Pisa)	Angelini et al. (2009)
		12.4‡	U.S.A. (Oklahoma)	Aravindhakshan et al. (2010)
Erianthus species (*Erianthus arundinaceus* Retz.)	C4	58.4	Japan (Northern Kanto region)	Ando et al. (2011)
Giant reed (*Arundo donax* L.)	C3	37.7†	Central Italy (Pisa)	Angelini et al. (2009)

[a] Actual scientific names of experimental cultivars or lines used in the study cited are given for *Erianthus* species, *Miscanthus* species, and sugarcane.
[b] Values followed by the same obelisk were obtained by the same study.
[c] An average value calculated with the data from three different locations.

Table 1. Summary information for perennial grasses described in this review (modified and updated from Lewandowski et al., 2003)

C4 perennial grasses show promise as lignocellulosic biofuel crops because their highly efficient C4 photosynthesis often yields more biomass than C3 grasses (Jakob et al., 2009). There are many available candidate perennial grasses, which differ in their potential productivity, chemical and physical biomass properties, environmental demands and crop management requirements (Lewandowski et al., 2003). This review will focus on noteworthy C4 perennial grasses as dedicated lignocellulosic biofuel crops, including switchgrass (*Panicum virgatum* L.), *Miscanthus* species, *Erianthus* species, and sugarcane (*Saccharum* species). In addition, the high biomass C3 grass species giant reed (*Arundo donax* L.), which has a photosynthetic capacity comparable to or higher than that of C4 grasses (Angelini et al., 2009; Rossa et al., 1998), will be discussed as well. These perennial grasses are summarized in Table 1 and will be briefly described in sections 2.1 through 2.3.

2.1 Switchgrass

Switchgrass (*Panicum virgatum* L.) is an outcrossing perennial warm-season forage grass originally from the prairies of North America. In the 1990's, the United States Department of Energy sponsored a 10-year research project to evaluate and develop this grass as a dedicated herbaceous lignocellulosic energy crop because of its potential for high fuel

yields, environmental enhancement, and ability to grow well on marginal cropland without heavy fertilizing or intensive management (Bouton, 2008; McLaughlin & Kszos, 2005; Wright & Turhollow, 2010).

Switchgrasses are divided into two ecotypes: lowland and upland (Bouton, 2008; Lewandowski et al., 2003). The lowland ecotypes are tall and robust plants adapted to wetter sites; they mature later and have longer growth periods and higher biomass yields than upland ecotypes. Although somatic chromosome numbers vary in switchgrass (2n from 2x = 18 to 12x = 108) (Hopkins et al., 1996), the lowland ecotypes are predominantly tetraploid (2n = 4x = 36) (Bouton, 2008; Lewandowski et al., 2003). The upland ecotypes have shorter and fine-stemmed morphology, and are adapted to drier habitats. They are commonly octoploid (2n = 8x = 72), or occasionally tetraploid (2n = 4x = 36) (Bouton, 2008) or hexaploid (2n = 6x = 54) (Lewandowski et al., 2003). Irrespective of ecotype, switchgrasses with the same ploidy level can be intercrossed (Lewandowski et al., 2003).

2.2 *Saccharum* complex

The term "Saccharum complex" was first coined by Mukherjee (1957) and originally encompassed four closely-related interfertile genera, *Saccharum*, *Erianthus*, *Sclerostachya*, and *Narenga*. Based on species richness and the geographic distributions of endemic species, India was considered the center of maximum variation of the *Saccharum* complex (Mukherjee, 1957). Eventually, the genus *Miscanthus* was added to the *Saccharum* complex, because it was thought to be involved in the origin of *Saccharum* (Daniels et al., 1975, as cited in Alwala et al., 2006; and Amalraj & Balasundaram, 2006).

Modern sugarcane varieties are mostly derived from interspecific hybridization within the genus *Saccharum* (Amalraj & Balasundaram, 2006), and intergeneric hybridization between *Saccharum* and other genera in the *Saccharum* complex is thought to be the primary gene pool for sugarcane breeding (Cheavegatti-Gianotto et al., 2011). Thus, the *Saccharum* complex can also be considered as the primary gene pool for breeding non-domesticated *Miscanthus* and *Erianthus* as well.

In sections 2.2.1 through 2.2.3, we will address individual genera within the *Saccharum* complex. However, a comprehensive molecular breeding system with intercrossing across the complex should ultimately be undertaken in the development of novel hybrid biofuel crops.

2.2.1 Sugarcane (*Saccharum* species)

Sugarcane is a tall perennial C4 grass that is cultivated in tropical and subtropical regions of the world. Notably, this grass stores high concentrations of sucrose in the stem. Approximately, 65 to 70% of global sugar production in the form of sucrose is derived from sugarcane (FAO, 2003). The potential of sugarcane as an important energy crop was argued because of the advent of large-scale sugarcane-based ethanol production in Brazil (Tew & Cobill, 2008).

Sugarcane belongs to the genus *Saccharum*. Although six polyploid species are recognized within *Saccharum* (Table 2), modern cultivars for sugar production are mostly derived from interspecific hybridization between *S. officinarum* (2n = 8x = 80) and *S. spontaneum* (2n from 5x = 40 to 16x = 128) and are thus complex polyploids with variable chromosome numbers

(2n approx. from 100 to 120) (Henry, 2010; Piperidis et al., 2010). Of the other four species, *S. robustum*, *S. barberi*, and *S. sinense* have also provided minor contributions to the breeding of some modern sugarcane cultivars (Cheavegatti-Gianotto et al., 2011).

Species	Chromosome number (2n)	Genomic contribution to modern interspecific hybrid cultivars and elite lines (%)[a]	Note
S. barberi	From 111 to 120	-	Semi-sweet Indian cane
S. edule	From 60 to 80	-	Cultivated for edible inflorescence
S. officinarum	80	From 70 to 80	Domesticated sweet cane
S. robustum	60 or 80	-	Putative ancestor of *S. officinarum*
S. sinense	From 81 to 124	-	Semi-sweet Chinese cane
S. spontaneum	From 40 to 128	From 10 to 23	Wild cane found throughout Asia

[a] Values are the proportions of total chromosome complement reported by Piperidis et al. (2010) where the proportion of chromosomes derived from interspecific exchanges between *S. officinarum* and *S. spontaneum* are shown to be 8-13%.

Table 2. Species of *Saccharum* and their characteristics (modified from Henry, 2010)

The breeding of sugarcane as a dedicated biomass crop called "energy cane" can be categorized into three strategies with different objectives: the “sugar model”, the “sugar-and-fiber model”, and the “fiber-only model”. The fiber yield of energy cane is important because of its use for electricity generation, cellulosic ethanol production, and so forth; fiber is considered a by-product in the sugar and sugar-and-fiber models or the main product in the fiber-only model (Tew & Cobill, 2008). In the sugar model, improved sugar yield and sugar content are the main foci, so traditional sugarcane cultivars can be used. In the sugar-and-fiber and fiber-only models, Type I and Type II energy canes, respectively, would be used. Tew & Cobill (2008) defined Type I and Type II energy canes as follows:

- Type I energy cane is bred and cultivated to maximize both its sugar and fiber content.
- Type II energy cane is bred and cultivated primarily or solely for its fiber content.

Recently, Japanese breeders succeeded in developing a high-quality Type I energy cane cultivar ‘KY01-2044’ with 1.5 times the total biomass yield and 1.3 times the total sugar yield than the major Japanese sugar-producing cultivar (Asia Biomass Office, 2010; Terajima et al., 2010). The new cultivar allowed the establishment of an experimental system for simultaneous production of ethanol and sucrose from total sugar with residual fiber as a heat source. In addition to ethanol production, this system is designed to produce an amount of sucrose comparable to conventional sugar production systems.

2.2.2 *Miscanthus* species

Miscanthus is a genus of C4 perennial rhizomatous grasses widely distributed in Asia and the Pacific Islands. *Miscanthus* was thought to consist of 17 species divided into four sections (Deuter, 2000). However, recent taxonomic analyses using molecular markers revealed that the genus can be reduced to approximately 11-12 species. The species *M. sinensis* ssp. *condensatus* is sometimes recognized at specific rank as *M. condensatus* (Clifton-Brown et al., 2008) (Table 3).

Species	Chromosome number and ploidy level[a]
M. floridulus (Labill.) Warb.	2n = 2x = 38
M. intermedius (Honda) Honda	2n = 6x = 114
M. longiberbis Nakai	-
M. lutarioparius	-
M. oligostachyus Stapf.	2n = 2x = 38
M. paniculatus (B. S. Sun) Renvoize & S. L. Chen	-
M. sacchariflorus (Maxim.) Hack.	2n = 2x = 38 (in China) 2n = 4x = 76 (in Japan)
M. sinensis Anderss.	2n = 2x = 38
M. sinensis ssp. condensatus (Hackel) T. Koyama	2n = 2x = 38
M. tinctorius (Steud.) Hack.	2n = 2x = 38
M. transmorrisonensis Hayata	2n = 2x = 38
M. × *giganteus* Greef & Deuter ex Hodkinson and Renvoize (Hybrid species between *M. sacchariflorus* and *M. sinensis*)	2n = 3x = 57

[a] Major cytotypes from Deuter (2000) are shown.

Table 3. Species in the genus *Miscanthus* and their chromosome numbers

The basic chromosome number of *Miscanthus* species is 19, and polyploids and aneuploids are observed (Deuter, 2000). Of the species in Table 3, *M. sacchariflorus*, *M. sinensis*, *M. sinensis* ssp. *condensatus*, *M. floridulus*, and *M.* × *giganteus* are of interest for biomass production (Deuter, 2000). In particular, the triploid hybrid species *M.* × *giganteus* shows superior characteristics, such as high biomass yield, and is thus thought to be the most practical *Miscanthus* species for bioenergy production, especially in Europe and North America (Lewandowski et al., 2000; Pyter et al., 2007). The existence of this promising hybrid will further encourage interspecific hybridization within *Miscanthus,* because the genus has a lot of genetic diversity within and between species. In addition, the frost tolerance, growth at low temperature, and robustness against pests and diseases of *Miscanthus* make it a potential gene pool for developing widely-adaptable stress-tolerant cultivars of sugarcane through intergeneric hybridization (Clifton-Brown et al., 2008).

2.2.3 *Erianthus* species

Erianthus is a tall C4 perennial rhizomatous grass. The genus *Erianthus* was erected by André Michaux in *Flora Boreali-Americana* in 1803 for the New World species *E. saccharoides* Michaux (Tagane et al., 2011). Old World species have distinct morphology from New World species (Grassl, 1972, as cited in Tagane et al., 2011) and are characterized by the

presence of a distinctive luteolin, di-C-glycoside, that is not present in the New World species (Williams et al., 1974). The genus is widely distributed in the Americas (New World species) and in the Mediterranean, India, China, South East Asia, and New Guinea (Old World species) (Amalraj & Balasundaram, 2006).

The seven Old World species comprise the section *Ripidium* (Table 4). The basic chromosome number of these *Erianthus* species is x = 10, the same as *Saccharum officinarum*. Polyploids are also observed, as in other genera in the *Saccharum* complex.

Species	Chromosome number[a]
E. arundinaceus (Retz.) Jesw.	2n = 30, 40, 60
E. bengalense (Retz.)	2n = 20, 30, 40, 60
E. elephantinus Hook. f.	2n = 20
E. hostii Griseb.	2n = 20
E. kanashiroi Ohwi	2n = 60
E. procerus (Roxb.) Raizada	2n = 40
E. ravennae (L.)	2n = 20

[a] Chromosome numbers shown by Amalraj & Balasundaram (2006) are shown.

Table 4. Species in the genus *Erianthus* sect. *Ripidium* and their chromosome numbers

The Old World species are increasingly used in sugarcane breeding because of their high biomass yields, drought tolerance, and resistance to pests and diseases. Two species in particular, *E. arundinaceus* and *E. procerus,* are considered most useful due to their disease resistance, ratooning ability, vigor, and environmental stress tolerance (Tagane et al., 2011). Recently, Ando et al. (2011) reported the wintering ability and high biomass yields of the *E. arundinaceus* clone JW630 grown experimentally in Nasushiobara, Japan, where the monthly mean minimum air temperatures from December to March are below freezing, indicating its potential as breeding stock for cold-tolerant sugarcane and as a dedicated lignocellulosic biofuel crop (Table 1).

2.3 Giant reed

Giant reed (*Arundo donax* L.) is a rhizomatous C3 perennial grass that evolved in Asia but is also considered a native to the Mediterranean region (Lewandowski et al., 2003). The grass is used to make reeds for woodwind musical instruments, in construction, and as a source of cellulose for rayon manufacture and paper pulp production (Perdue, 1958).

Recently, giant reed has gained attention as a source of lignocellulose for bioenergy production because of its high yield and strong pest resistance (Lewandowski et al., 2003). Studies of the giant reed in Central Italy demonstrated biomass production equivalent to or higher than *Miscanthus* × *giganteus* (Angelini et al., 2009) (Table 1). Because giant reed exhibits heavy metal tolerance, it could be used simultaneously for both phytoremediation and bioenergy production (Papazoglou, 2007; Papazoglou et al., 2005).

Giant reed is highly polyploid, with a possible base chromosome number x = 12 [(2n = 10x = 120 - 8 or 2n = 9x = 108 + 4) (Gorenflot et al., 1972) or (2n = 110) (Lewandowski et al., 2003)]. Although isozyme and DNA analyses have revealed genetic diversity in giant reed (Khudamrongsawat et al., 2004; Lewandowski et al., 2003), the plant is thought to produce

no viable seed, owing to aberrant division of the megaspore mother cell (Bhanwra et al., 1982). This behavior suggests that conventional breeding through sexual hybridization cannot be performed.

3. Plant regeneration systems

In most case, effective plant regeneration systems with *in vitro* cell cultures are required to establish reliable transformation systems in plants. In this section, we summarize the regeneration systems of switchgrass, sugarcane, *Miscanthus*, *Erianthus*, and giant reed.

3.1 Switchgrass regeneration systems

Biotechnology research on the molecular breeding of switchgrass began in 1992 at the University of Tennessee with the support of Oak Ridge National Laboratory (Vogel & Jung, 2001). This work yielded the first reported plant regeneration system through embryogenesis and organogenesis (Denchev & Conger, 1994). In the experiments, mature caryopses and young leaf segments were used as explants for callus induction. Then, the influences of the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (picloram) in combination with 6-benzyladenine (BA) were examined on the plant regeneration system. The combination of 2,4-D and BA led to best results in experiments with mature caryopses (Denchev & Conger, 1995). Alexandrova et al. (1996a) increased the efficiency of switchgrass regeneration by using immature inflorescences obtained from node cultures in aseptic conditions; one immature inflorescence can produce hundreds of spikelets of the same genotype in a single Petri dish that can be easily used as explants for callus induction. Most recently, a new medium, LP9, for the production, maintenance, and regeneration of switchgrass callus was reported (Burris et al., 2009). Callus produced on LP9 can be easily propagated, maintains its regenerability for at least six months, and is adaptable to *Agrobacterium*-mediated transformation (Burris et al., 2009).

Embryogenic suspension cultures initiated from embryogenic callus were also established in switchgrass (Gupta & Conger, 1999), and osmotic pre-treatment with 0.3 M each of sorbitol and mannitol was effective for plant regeneration from suspension cultures (Odjakova & Conger, 1999). Another type of culture system that is highly attractive for gene transfer experiments was also established: multiple shoot clumps were induced from intact seedlings with various combinations of 2,4-D and 1-phenyl-3-(1,2,3-thidiazol-5-yl) urea (TDZ) (Gupta & Conger, 1998).

Most of the research mentioned above used the lowland cultivar 'Alamo'. However, because switchgrass is outcrossing and self-infertile, genetic variation exists not only among cultivars but also within a cultivar, indicating that the establishment of a totipotent tissue culture is not highly dependent on the cultivar itself but rather on screening highly regenerable genotypes as in other outcrossing plants, like ryegrasses (Takahashi et al., 2004). Therefore, Odjakova & Conger (1999) and Burris et al. (2009) used specific single genotypes Alamo 2702 and Alamo 2, respectively, in their experiments, and Alexandrova et al. (1996b) developed a micropropagation procedure with node culture for the multiplication of such selected genotypes. Interestingly, the selected line HR8, having high somatic embryogenic capacity, was recently bred using recurrent tissue culture selection to allow easy induction of embryogenic callus from mature seeds (Xu et al., 2011).

3.2 Sugarcane regeneration systems

The first sugarcane regeneration system via callus culture was reported in 1964 (Nickell, 1964, as cited in Lakshmanan, 2006) and was followed by numerous reports resulting from a great deal of research activity. These early reports have been well-reviewed elsewhere (Lakshmanan, 2006; Suprasanna et al., 2008a). Although many papers on plant regeneration from sugarcane calli are still being published, they generally vary only in reporting specific culture conditions, which must be optimized for each cultivar and genotype because of the outcrossing nature of sugarcane. Most callus cultures are supplemented with the auxin 2,4-D, but sometimes other synthetic auxins, such as picloram and 3,6-dichloro-2-methoxybenzoic acid (dicamba) are used, with or without the cytokinins BA, TDZ, or kinetin. Research on sugarcane regeneration systems may be stalled. To spark innovation, here we update the previous reviews (Lakshmanan, 2006; Suprasanna et al., 2008a) by describing innovative reports published since 2007.

In addition to plant hormones, exogenous amino acids may positively or negatively influence somatic embryogenesis in plants. In sugarcane tissue culture, the addition of arginine to culture media was found to significantly induce somatic embryogenesis and to promote plant regeneration (Nieves et al., 2008). Similarly, glycine, arginine, and cysteine positively affected somatic embryogenesis and subsequent plant regeneration; 0.75 mM glycine was the most effective treatment studied (Asad et al., 2009).

The moisture status of *in vitro* cultures influences plant regeneration in sugarcane. Partial desiccation has been reported to enhance plant regeneration from calli (Garcia et al., 2007; Kaur & Gosal, 2009). A similar phenomenon was observed even in irradiated embryogenic cultures (Suprasanna et al., 2008b).

A fundamental but important aspect of sugarcane tissue culture is the selection of the initial explants. In most previous reports, explants were obtained from field-grown plants. However, Garcia et al. (2007) recommended using sugarcane plants grown *in vitro* because they provided a year-round source of physiologically uniform explants and could be prevented from releasing large amounts of phenolic compounds, which can hamper tissue culture (Garcia et al., 2007). Basnayake et al. (2011) recommended using explants obtained from donor plants with good water supplies, especially when working with genotypes that are recalcitrant to tissue culture due to phenolic compounds.

3.3 *Miscanthus* regeneration systems

To the best of our knowledge, the first preliminary regeneration experiments in *Miscanthus* were conducted with immature inflorescences, mature leaves, immature leaves, nodal segments, internodal segments, meristematic regions, and ovules as explants, which were cultured on media containing a wide range of auxin types (Gawel et al., 1987, as cited in Gawel et al., 1990). In these experiments, immature inflorescences were the only explants that produced calli, and only media containing 2,4-D or picloram with no cytokinin produced a regeneration response (Gawel et al., 1987, as cited in Gawel et al., 1990). The research group thus focused on immature inflorescences as explants and published that 9.0 μM of 2,4-D was effective for callus induction and subsequent plant regeneration in *M. sinensis* cultivars 'Gracillimus', 'Variegatus', and 'Zebrinus' (Gawel et al., 1990). In these experiments, callus induction and plant regeneration were not distinguished from one

another; shoot formation occurred during successive culture on the same culture medium as callus induction (Gawel et al., 1990).

Many researchers have focused on the high-biomass species *M.* × *giganteus*. The auxins 2,4-D or 2,4,5-trichlorophenoxyacetic acid in combination with the cytokinin 6-furfurylaminopurine were best for inducing regenerable embryogenic callus from immature inflorescences (Lewandowski & Kahnt, 1992). Although immature inflorescences emit fewer browning substances than shoot tips or leaf primordia (Lewandowski & Kahnt, 1993), browning substances from the explants were detrimental to tissue culture (Lewandowski & Kahnt, 1992). Ascorbic acid, cysteine, and watering the explants were not effective treatments, but liquid culture gave better results because the browning substances did not accumulate around the explants or calli (Lewandowski & Kahnt, 1992).

The effects of different explants, such as shoot apices, leaves and root sections of *in vitro*-propagated plants and leaf and immature inflorescence sections from greenhouse-grown plants, were examined in *M.* × *giganteus*. Shoot apices had the highest percentage of embryogenic callus formation, while immature inflorescence-derived callus had the highest regenerability (Holme & Petersen, 1996). The growth conditions of donor plants influence tissue culture (Creemers-Molenaar et al., 1988), as has been observed in *M.* × *giganteus* (Holme & Petersen, 1996). Leaf explants from *in vitro*-propagated shoots and from greenhouse-grown plants showed differences in embryogenic callus formation; the best results were obtained from leaves of *in vitro*-propagated shoots and older leaves of greenhouse-grown plants (Holme & Petersen, 1996). However, supplying BA to the callus induction medium led to different results: a higher percentage of regenerable shoot-forming callus was formed on shoot apices compared with leaf sections of *in vitro*-grown shoots when 0.4 μM BA was supplied (Petersen, 1997). Also, small immature inflorescences of *M.* × *giganteus*, between 2.5 and 8 mm, were more suitable for embryogenic callus formation than explants from shorter or longer inflorescences, shoot apices or leaf explants, indicating that the size and type of explant influence culture responses (Petersen et al., 1999). Furthermore, different carbon sources, and their sterilization methods, influenced *M.* × *giganteus* tissue culture. Significant differences were reported for carbon sources and their sterilization methods in tissue cultures of various explant-derived calli. Leaf explants were more affected by the carbon sources than were shoot apices or immature inflorescences, and both callus proliferation and plant regeneration were generally improved by the use of filter-sterilized carbon sources (Petersen et al., 1999).

As mentioned above, severe browning is a major problem in *Miscanthus* tissue culture, but supplying proline to callus induction and suspension cultures effectively prevented browning in *M.* × *giganteus* (Holme et al., 1997). Proline is thought to inhibit polyphenol oxidase, which causes enzymatic browning of cultured tissues (Öztürk & Demir, 2002). The addition of proline to callus induction media increased embryogenic callus formation on shoot apices and leaf explants. However, results varied with proline concentration and the basal salts in the medium. Specifically, 12.5 to 50 mM proline in callus induction media with Murashige & Skoog (MS) salts (Murashige & Skoog, 1962) increased embryogenic callus formation more than media with N6 salts (Chu et al., 1975). The inhibitory effects of proline on tissue browning in *Miscanthus* were confirmed by another group (Głowacka et al., 2010). Conversely, adding honey instead of sucrose to callus induction media inhibited browning of cultured immature inflorescences, and a combination of the honey and banana pulp was best for inducing regenerable callus in *M.* × *giganteus* (Płażek & Dubert, 2009, 2010).

Media component (mg L^{-1} final conc.)	Media		
	HB[a]	MS	N6
Macroelements			
KNO_3	2000	1900	2830
NH_4NO_3	1500	1650	
$(NH_4)_2SO_4$			463
$CaCl_2 \cdot 2H_2O$	200	440	166
$MgSO_4 \cdot 7H_2O$	300	370	185
KH_2PO_4	300	170	400
Microelements			
Na_2-EDTA	37.25	37.3	37.25
$FeSO_4 \cdot 7H_2O$	27.85	27.8	27.85
$MnSO_4 \cdot 4H_2O$	11.25	22.3	4.4
$ZnSO_4 \cdot 7H_2O$	4.3	8.6	1.5
$CuSO_4 \cdot 5H_2O$	0.0125	0.025	
$CoCl_2 \cdot 6H_2O$	0.0125	0.025	
H_3BO_3	3.1	6.2	1.6
KI	0.415	0.83	0.8
Vitamins			
Inositol	100	100	
Nicotinic acid	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5
Thiamine HCl	0.4	0.1	1
Another organic			
Glycine	2	2	2

[a] Composition presented in Sun et al. (1999).

Table 5. Composition of HB, MS, and N6 basal media

Recently, studies involving tissue cultures of *M. sinensis* have been reported. Głowacka & Jeżowski (2009a, 2009b) reported culturing anthers of *M. sinensis* and subsequently demonstrated the effects of inflorescence developmental stage on callus induction and plant regeneration (Głowacka et al., 2010). Mature seeds can also serve as explants for tissue culture in *M. sinensis*. Mature seeds of 18 accessions from various sites in Japan were subjected to callus induction, and a combination of a relatively high 2,4-D concentration (5 mg L^{-1}) and a relatively low BA concentration (0.01 mg L^{-1}) efficiently induced regenerable calli that could be used to produce transgenic *Miscanthus* plants (Wang et al., 2011). Interestingly, there was a correlation between the average annual air temperature at accession collecting sites and the frequency of embryogenic callus induction; seeds collected from warmer regions formed a higher percentage of embryogenic calli (Wang et al., 2011). Most recently, Zhang et al. (2011) germinated seeds collected in China and compared explants from the epicotyls, young leaves, and radicles. The epicotyl was best for embryogenic callus formation and plant regeneration from the callus. In the experiments, Holley & Baker (HB) medium (Holley & Baker, 1963, as cited in Zhang et al., 2011) was better for callus induction than other common basal media, such as MS medium, N6 medium (Chu et al., 1975), or half strength of the MS medium. Zhang et al. surmised that HB medium would work well for callus induction in other grass species, because the medium had been effective for callus culture of wheat (Sun et al., 1999). The

components of HB medium will be of interest to many researchers, so we compare HB, MS, and N6 media in Table 5. We were unable to obtain the original publication (Holley & Baker, 1963, as reported in Zhang et al., 2011), so we give the composition of HB basal medium presented in Sun et al. (1999).

3.4 *Erianthus* regeneration systems

To date, only one published report concerns the plant regeneration system of *Erianthus* species. Callus induction and plant regeneration from calli was achieved in *E. elephantinus* (Jalaja & Sreenivasan, 1999). Calli were induced from expanding leaves, leaf sheaths, and immature inflorescences as explants on MS basal medium containing 2 mg L^{-1} 2,4-D. As in *Miscanthus* species, tissue browning occurred, especially in explants from leaf sheaths (Jalaja & Sreenivasan, 1999). Unfortunately, detailed data on the frequencies of callus induction and plan regeneration from the callus were not reported because the study focused mainly on somaclonal variation, such as morphology, pollen fertility, and chromosome aberrations, of the regenerants (Jalaja & Sreenivasan, 1999).

3.4.1 Protocol for plant regeneration system of *Erianthus arundinaceus*

We have recently succeeded in establishing a plant regeneration system in *E. arundinaceus*. Here, we present the simplified protocol. All culture media used were based on MS medium containing 3% (w v^{-1}) sucrose, adjusted to pH 5.8, and solidified with 0.25% (w v^{-1}) Gelrite (Wako, Osaka, Japan). Components of the culture media are shown in Table 6.

Media	Components[a]
Plant maintenance medium	0.3% (w v^{-1}) activated charcoal 3%(w v^{-1}) sucrose
Callus induction medium	5 mg L^{-1} 2,4-D 25 mM L-proline 750 mg^{-1} $MgCl_2 \cdot 6H_2O$ 3%(w v^{-1}) sucrose
Multiple shoot formation medium	1 mg L^{-1} BA 3 mg L^{-1} NAA 3%(w v^{-1}) sucrose
Shoot elongation medium	0.3 mg L^{-1} GA 3%(w v^{-1}) sucrose

[a] Additives added to MS basal medium are shown for each medium.

Table 6. Culture media used in our plant regeneration system for *Erianthus arundinaceus*

- Establishment of *in vitro*-grown plants

We obtained *in vitro*-grown plants of the *E. arundinaceus* clone JW630 (Ando et al., 2011). Shoot tips, each containing an apical meristem, of greenhouse-grown plants (approx. 3 cm long) were used as explant sources. After removing the mature outer leaves, the shoot tips were submerged in 70% ethanol for 1 min, surface-sterilized in 10% (v v^{-1}) sodium hypochlorite solution (1% available chlorine) for 20 min, rinsed twice in sterile distilled

water under aseptic conditions, aseptically stripped of more outer leaves, and cultured on plant maintenance medium under long-day conditions [16 h light (80 μmol m^{-2} s^{-1})/8 h dark; 28°C]. The explants were subcultured every two weeks until rooting.

- Callus induction from axillary buds of *in vitro*-grown plants

Axillary buds at the proximal end of each leaf were isolated from *in vitro*-grown plants under a stereomicroscope. The isolated buds were placed on callus induction medium, and cultured under continuous fluorescent light (40 μmol m^{-2} s^{-1}) at 25°C. They were subcultured every two weeks.

- Plant regeneration from calli

For plant regeneration, calli were transferred to the plant maintenance medium and cultured under long-day conditions [16 h light (80 μmol m^{-2} s^{-1})/8 h dark; 28°C].

- Results and tips

As with other C4 plants, such as switchgrass, sugarcane, and *Miscanthus*, explant browning was severe at first. Rooting of the explants took about two months, but once rooted, the *in vitro* plants grew vigorously on the plant maintenance medium (Figure 1A). Calli were easily induced from axillary buds of *in vitro*-grown plants (Figure 1B), and plant regeneration was observed from the calli (Figure 1C). However, calli induced from explants from field-grown plants were rare due to severe bacterial contamination and tissue browning, even when we used apical meristems, immature inflorescences, and young leaf rolls (data not shown). The *in vitro*-grown plants produced multiple shoot clumps when cultured on multiple shoot formation medium (Figure 2A), and the newly formed short shoots maintained their morphological states and could elongate to normal morphology after being transferred to shoot elongation medium containing gibberellic acid (GA) (Figure 2B).

Fig. 1. Plant regeneration system for *Erianthus arundinaceus*. An *in vitro*-grown plant cultured for three months (A), a callus induced from an axially bud of the *in vitro*-grown plants (B), and plant regeneration from calli (C).

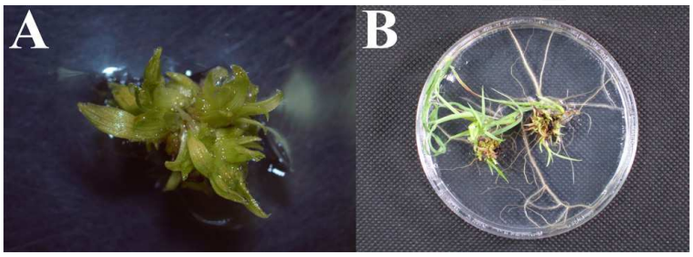

Fig. 2. Multiple shoot formation in *Erianthus arundinaceus*. A multiple shoot clump derived from an *in vitro*-grown plant (A), and elongated shoots from the multiple shoot clumps (B).

3.5 Giant reed regeneration system

To the best of our knowledge, only one scientific article on the regeneration of giant reed has been published so far (Takahashi et al., 2010). In the system, *in vitro* propagation was first optimized for the year-round production of explants (Figure 3).

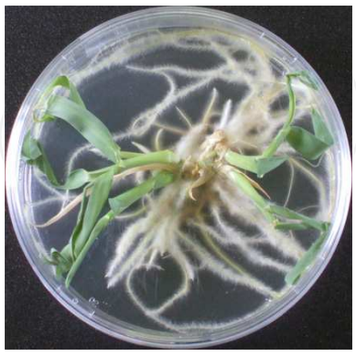

Fig. 3. An *in vitro*-grown giant reed plant. The photo was taken two months after subculture (adapted from Takahashi et al., 2010).

Calli were induced from axillary buds isolated from the *in vitro*-grown plants. Several combinations of 2,4-D and BA were used for callus induction. They influenced both callus induction frequency and later plant regeneration on hormone-free media because of possible carryover effects of plant hormones in the original callus induction media. Media supplemented with 3 mg L^{-1} 2,4-D appeared to stabilize callus formation and subsequent shoot formation on hormone-free medium (Figure 4).

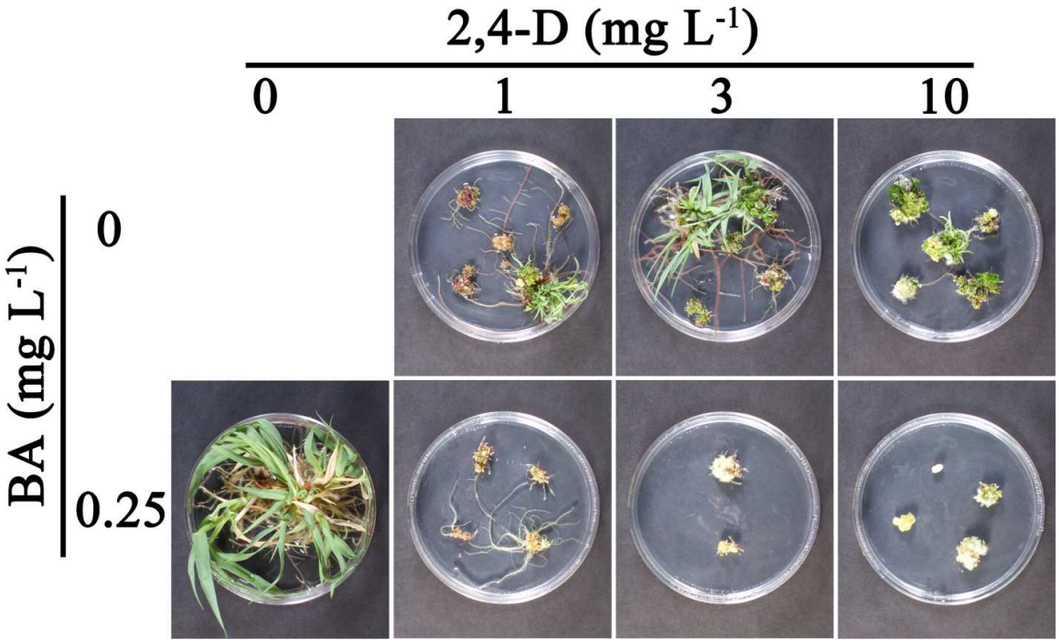

Fig. 4. Effect of original callus induction media on subsequent shoot formation on hormone-free media in giant reed. The concentrations of 2,4-D and BA are those in original callus induction media (modified and adapted from Takahashi et al., 2010).

In total, 11 genotypes were treated under optimized conditions, and data suggest genotypic effects in tissue culture response, although the giant reed is thought to propagate asexually because its seeds are non-viable. Ex-callus induction frequencies and shoot formation frequencies ranged from 82.9 to 100% and from 0 to 37.5%, respectively.

4. Transformation systems

We summarize here several earlier reports on transformation systems in switchgrass, sugarcane, *Miscanthus*, and giant reed. We know of no such reports for *Erianthus*.

4.1 Switchgrass transformation systems

There are some reports on the establishment of transformation systems in switchgrass. Gene transfer methods and names of transgenes used so far are shown in Table 7.

Transgenes[a]	Gene transfer methods	Reference
sgfp, [*bar*]	Gene gun	Richards et al. (2001)
uidA, [*bar*]	*Agrobacterium*	Somleva et al. (2002)
pporRFP, [*hph*]	*Agrobacterium*	Burris et al. (2009)
uidA, *GUSPlus*, [*hph*]	*Agrobacterium*	Xi et al. (2009)
sgfp, [*hph*]	*Agrobacterium*	Li & Qu (2011)
sgfp, *GUSPlus*, [*bar*][b], [*hph*]	*Agrobacterium*	Xu et al. (2011)
uidA, [*bar*], [*hph*], [*nph*]	*Agrobacterium*	Song et al. (2011)

[a] Selectable marker genes in brackets.
[b] Not used as a selectable marker.

Table 7. A list of transgenes and gene transfer methods in used in reported switchgrass transformation systems

The first transgenic switchgrass plants were produced by the gene gun method (Richards et al., 2001). Tungsten particles coated with a plasmid harboring the *sgfp* and *bar* genes were introduced into immature inflorescence-derived embryogenic calli. In total, 2,430 calli were subjected to the gene transfer, and 97 plants eventually showed tolerance, conferred by the *bar* gene, to 0.1% Basta herbicide (Richards et al., 2001). Basta tolerance was also observed in T_1 transgenic progeny resulting from crosses between transgenic and nontransgenic control plants, indicating inheritance of the *bar* gene (Richards et al., 2001).

All subsequent reports on switchgrass transformation have used *Agrobacterium*-mediated transformation (Table 7). The first such report involved sophisticated experiments that examined the effects of various explants of different genotypes for callus induction and of various concentrations of acetosyringone during inoculation and cocultivation on gene transfer efficiency (Somleva et al., 2002). The gene transformation efficiencies ranged from 0 to 97.3%, but the efficiencies were clearly depended on genotype and kind of explant. Improvements in basal media should lead to efficient transformation in switchgrass. Recently, switchgrass Type II calli were induced from newly-developed LP9 medium (see section 3.1) and were subjected to the *Agrobacterium*-mediated transformation with a final transformation efficiency of 4.4% (Burris et al., 2009).

Because *Agrobacterium* is the causal agent of crown gall, interactions between *Agrobacterium* and plant cells during gene transfer often trigger an undesired plant defense mechanism, called hypersensitive cell death, that leads to lower gene transfer efficiency. Thus, highly compatible *Agrobacterium* strains must be selected for plant transformation. Indeed, different strains of *Agrobacterium*-strain varied in their transient transgene expression efficiencies (Chen et al., 2010) and in stable transformation rates in switchgrass (Song et al., 2011; Xi et al., 2009). For stable transformation of switchgrass, the most noteworthy *Agrobacterium* strain so far has been EHA105; two different research groups found it to be more effective than other strains, such as AGL1, GV3101, and LBA4404 (Song et al., 2011; Xi et al., 2009).

Li & Qu (2011) recently developed the most high-through-put system to date, with a stable transformation efficiency of over 90%. Although most previous transformation systems employed the cultivar 'Alamo', the authors a found higher transformation efficiency with the cultivar 'Performer'. Their modifications included infection under vacuum, co-cultivation in desiccation conditions, resting between co-cultivation and selection, and L-proline supplementation in the callus culture and selection media (Li & Qu, 2011). Interestingly, this system also employed *Agrobacterium* strain EHA105, suggesting that it may be highly compatible with 'Performer'. Switchgrass transformation using a EHA105 with the switchgrass selected line HR8, which has high somatic embryogenic capacity (Xu et al., 2011) (see section 3.1), may be a promising avenue for future research.

Transgene inheritance and phenotypic expression in progeny derived from *Agrobacterium*-mediated transformation were observed in some of these reports (Li & Qu, 2011; Somleva et al., 2002; Xi et al., 2009).

4.2 Sugarcane transformation systems

Numerous reports on transformation systems in sugarcane have been previously reviewed (Hotta et al., 2010; Lakshmanan et al., 2005; Suprasanna et al., 2008a). Here, we summarize two key studies published in 2011.

Sophisticated adjustment of culture conditions for several cultivars have resulted in transformation systems widely-adaptable to different genotypes. Basnayake et al. (2011) investigated the effects of 2,4-D levels during callus proliferation, geneticin concentrations during selection, and/or light intensity during regeneration in 16 Australian sugarcane cultivars destined for microprojectile-mediated transformation. This study will be a useful guide for the rapid optimization of key tissue culture variables for efficient genetic transformation of other sugarcane cultivars.

Microprojectile-mediated gene transfer is the most common gene transfer method in sugarcane (Hotta et al., 2010), but the method often causes complex transgene integration that may result in gene silencing. However, minimal expression cassettes lacking vector backbone sequences have been reported to support simple transgene integration in plants. Most recently, a linear minimal expression cassette for the *npt* gene was introduced into embryogenic callus by microprojectile-mediated gene transfer of different amounts of the expression cassette DNA (Kim et al., 2011). Genomic DNA from transgenic sugarcane plants displayed two to 13 *npt* hybridization signals on Southern blots, and the authors observed a trend toward reduced transgene integration complexity and reduced transgene expression levels when lower amounts of the minimal expression cassette were used per shot. This

suggests that backbone free minimal expression cassettes might be efficiently integrated and expressed in sugarcane and other plant species.

4.3 *Miscanthus* transformation systems

The first transgenic *Miscanthus* plants produced via particle bombardment were reported in *Miscanthus sacchariflorus* (Zili et al., 2004, as cited in Jakob et al., 2009), although we were unable to obtain the original reference and cannot report the details. Later, transgenic *M. sinensis* plants were produced by particle bombardment methods (Wang et al., 2011); four transgenic plants containing a foreign *hph* gene with or without the *gfp* were recovered from 120 bombarded calli, and foreign gene expression was confirmed by reverse transcription-PCR analyses. So far, there are no other reports on *Miscanthus* transformation.

4.4 Giant reed transformation systems

An optimized particle bombardment protocol for gene transfer with embryogenic calli was recently reported in giant reed (Dhir et al., 2010). In the study, embryogenic calli were induced from inflorescence segments collected from field-grown mature plants, and several physical parameters, such as helium pressure, bombardment distance to target tissue, and vacuum pressure, together with other factors such as gold microparticle size, DNA concentration, and the number of bombardments, were examined with transient expression of beta-glucuronidase (GUS) and green fluorescent protein (GFP) genes (Dhir et al., 2010). Unfortunately, however, no transgenic plants were obtained due to the lack of regeneration potential of the tissue culture system (Dhir et al., 2010).

We also tried to produce transgenic giant reed plants by using particle bombardment-mediated gene transfer in combination with the plant regeneration system described above (see section 3.5). We obtained more than 100 hygromycin-resistant calli from ca. 5,000 bombarded calli (Figure 5A). Transformation of these calli with foreign *hph* was confirmed by PCR analyses (data not shown). However, we could not obtain transgenic plants from the resistant calli, because the calli had lost their shoot formation potential and produced only adventitious roots on regeneration medium (Figure 5B). Thus, improvements in these tissue culture systems are needed to produce transgenic giant reed plants.

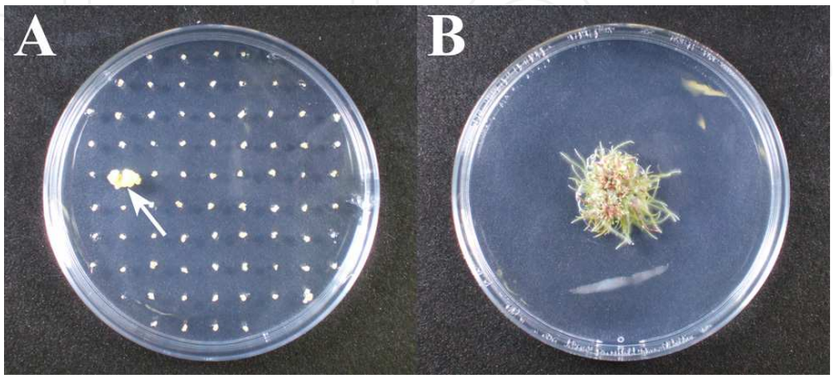

Fig. 5. Transgenic calli of giant reed. A hygromycin-resistant callus propagated during selective culture with the antibiotic hygromycin (arrow) (A), and adventitious root formation from the calli on plant regeneration medium (B).

5. Transgenic plants with improved traits

Here, we summarize reports of transgenic switchgrass and sugarcane plants having distinctive improved traits that will be of significance for biofuel production. In switchgrass, we know of only four such reports, as shown in Table 8. However, in sugarcane, several reports exist on the metabolic engineering of value-added sugarcane via carbohydrate biosynthesis and increased sucrose accumulation; the target genes and the resulting transgenic plants have been discussed in previous reviews (Hotta et al., 2010; Lakshmanan et al., 2005; Suprasanna et al., 2008a; Waclawovsky et al., 2010). Thus, for transgenic sugarcane, we focus here on reports published from 2007 to 2011 (Table 8). No studies have yet been reported for improved traits in *Miscanthus*, *Erianthus*, or giant reed.

Transgenes[a]	Gene transfer methods	Reference
Switchgrass		
phaA, *phaB*, *phaC*, [*bar*]	*Agrobacterium*	Somleva et al. (2008)
PvCAD[b] [*hph*]	*Agrobacterium*	Fu et al. (2011b)
PviCAD2[b] [*hph*]	*Agrobacterium*	Saathoff et al. (2011)
COMT[b] [*hph*]	*Agrobacterium*	Fu et al. (2011a)
Sugarcane		
phaA, *phaB*, *phaC*, [*npt*]	Gene gun	Petrasovits et al. (2007)
mds6pdh, *zmglk*, [*npt*]	Gene gun	Chong et al. (2007)
*HIS*CaneCPI-1, [*npt*]	Gene gun	Ribeiro et al. (2008)
avidin[c], [*adhA*]	Gene gun	Jackson et al. (2010)
mutB, [*npt*]	Gene gun	Hamerli & Birch (2011)

[a] Selectable marker genes in brackets.
[b] Partial inverted repeat sequences for RNAi technology.
[c] Several signal peptides were fused for subcellular localization.

Table 8. A list of transgenic plants with improved traits

5.1 Transgenic switchgrass plants

A pioneering study of transgenic switchgrass involved the successful production of value-added transgenic plants that could synthesize polyhydroxybutyrate (PHB), a biodegradable alternative to standard consumer plastic (Somleva et al., 2008). Primary transgenic plants containing up to 3.72% dry weight of PHB in leaf tissues and 1.23% dry weight of PHB in whole tillers were obtained. Polymer accumulation was also analyzed in the T1 generation. This achievement resulted from the successful expression of a functional multigene pathway involving complex metabolic engineering (Somleva et al., 2008).

In the first half of 2011, three reports were published on the downregulation of lignin biosynthesis genes using RNAi technology to improve ethanol production from lignocellulosic biomass (Fu et al., 2011a; Fu et al., 2011b; Saathoff et al., 2011). The first two reports were published at almost the same time and both targeted the gene encoding cinnamyl alcohol dehydrogenase (CAD), a key enzyme for catalyzing the last step of lignin

monomer biosynthesis. The resulting transgenic plants showed significantly fewer transcripts of the target gene, reduced CAD activity, lower lignin content (Fu et al., 2011b; Saathoff et al., 2011), and altered lignin composition (Fu et al., 2011b). Furthermore, these modified lignin biosynthesizers had improved sugar release from cell walls with or without chemical pre-treatment (Fu et al., 2011b; Saathoff et al., 2011). In another study, the expression of the lignin biosynthesis-related *O*-methyltransferase gene was similarly downregulated, and the resultant transgenic plants had reduced lignin content, digestibility by cellulase, and up to 38% more efficient ethanol yield using conventional biomass fermentation processes (Fu et al., 2011a).

5.2 Transgenic sugarcane plants

PHB production is also targeted in sugarcane molecular breeding. The same gene set and the same strategy used with transgenic switchgrass (Somleva et al., 2008) were also employed for producing transgenic sugarcane (Petrasovits et al., 2007). In the study, PHB accumulated in leaves of transgenic plants to a maximum of 1.88% of dry weight without obvious deleterious effects. The PHB concentration in culm internodes of the transgenic plants was much lower (0.0033%) than in leaves (Petrasovits et al., 2007).

The same research group also examined sugar manipulation in sugarcane by engineering a new carbon sink for the six-carbon sugar alcohol sorbitol; sorbitol has intrinsic value as a non-caloric sweetener and is also used to manufacture ascorbic acid (Chong et al., 2007). Transgenic sugarcane plants expressing the *mds6pdh* gene accumulated the sorbitol. The average amounts of sorbitol detected in the most productive line were 120 mg g^{-1} dry weight (equivalent to 61% of the soluble sugars) in the leaf lamina and 10 mg g^{-1} dry weight in the stalk pith, but the accumulation caused evident necrosis in expanding leaves and reduced growth (Chong et al., 2007). More recently, another group focused on production of the sucrose isomer trehalulose (Hamerli & Birch, 2011). The transgenic sugarcane plants accumulated the trehalulose up to 600 mM in juice from mature nodes. Contrary to the case of the sorbitol-accumulating sugarcane, the trehalulose-accumulating transgenic plants were vigorous, and trehalulose production in selected lines was retained over multiple vegetative generations under glasshouse and field conditions (Hamerli & Birch, 2011).

A high value-added dedicated lignocellulosic biofuel crop could also be developed by accumulating heterologous functional proteins that could be used for therapeutic, industrial, or other purposes. This concept is adaptable to the production of cystatin, a natural inhibitor of cysteine proteinases, that can be used to protect against insect attacks (Ribeiro et al., 2008). A transformed sugarcane plant expressing high levels of the His-tagged cystatin gene $_{HIS}$*CaneCPI-1* was reported to be useful for production and purification of functional $_{HIS}$CaneCPI-1 protein (Ribeiro et al., 2008). The $_{HIS}$CaneCPI-1 protein purified through affinity chromatography in a nickel column was able to inhibit the catalytic activity of midgut cysteine proteinase purified from the sugarcane weevil *Sphenophorus levis* and human cathepsin L in nanomolar amounts, indicating that this system can be used for the production of functional recombinant proteins (Ribeiro et al., 2008). The accumulated recombinant proteins may disturb cell metabolism. If so, subcellular targeting of the recombinant proteins would be necessary to optimize protein yield and avoid detrimental effects of the accumulated protein. Subcellular targeting peptides are thus considered a useful tool for compartmentalization of recombinant proteins. To find ideal subcellular

targeting for recombinant protein accumulation in sugar cane, Jackson et al. (2010) used the glycoprotein avidin, which is potentially toxic to cells, as a test protein fused with several subcellular targeting peptides. Accumulation of avidin was directed to the apoplast, endoplasmic reticulum, and lytic and delta type vacuoles. The study identified the delta type vacuole as a promising target, but the efficiency may depend on tissue type. If the protein is resistant or can be protected by proteolytic attack, the lytic vacuole would be a preferable target (Jackson et al., 2010).

6. Conclusions

We described recent progress in the identification of candidate herbaceous perennials for dedicated lignocellulosic biofuel crops. The biotechnological approaches for molecular breeding of these plants as dedicated biofuel crops are still in immature stages. Biotic and abiotic stress tolerance and herbicide resistance are the minimum required traits. Other traits and related technologies will be important and will make large impacts in the molecular breeding of biofuel crop; these include low-fertilizer needs, sophisticated manipulation technology of secondary cell wall biosynthesis, high-efficiency photosynthesis for high productivity, promoted and synchronized flowering for hybridization, accelerated generation and, especially, organellar transformation for the effective accumulation of high-value chemicals and proteins. These traits and candidate target genes are reviewed in other works (Jakob et al., 2009; Vega-Sánchez & Ronald, 2010; Vogel & Jung, 2001).

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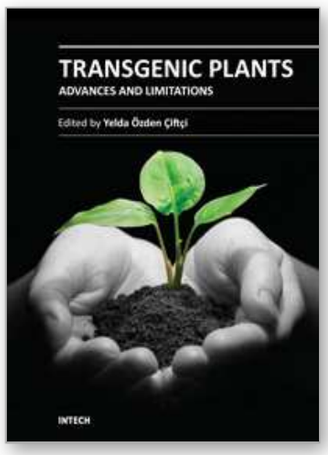

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