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Genotypic variation in the response to embryogenic callus induction and regeneration in *Saccharum spontaneum*

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Abstract

Embryogenic callus induction and regeneration are useful in many aspects of plant biotechnology, especially in the functional characterization of economically important genes. However, in sugarcane, callus induction and regeneration vary across genotypes. *Saccharum spontaneum* is an important wild germplasm that confers disease resistance and stress tolerance to modern sugarcane cultivars, and its genome has been completely sequenced. The aim of this study was to investigate the effect of genetic variations on embryogenic callus induction and regeneration in *S. spontaneum* and to screen genotypes having high tissue culture susceptibility. The study was performed using nine genotypes of *S. spontaneum* and the following five parameters were assessed to determine the response of genotypes to embryogenic callus induction and regeneration: callus induction, embryogenic callus ratio, embryogenic callus induction, embryonic callus regeneration and regeneration capacity. All the genotypes varied significantly ($P < 0.01$) in all the parameters, except for embryonic callus regeneration, which was high (>80%) for all the genotypes. High broad-sense heritability (86.1–96.8%) indicated that genetic differences are the major source of genotypic variations. Callus induction was found to be strongly positively correlated with embryogenic callus induction ($r = 0.890$, $P < 0.01$) and regeneration capacity ($r = 0.881$, $P < 0.01$). Among the nine tested genotypes, VN2 was found to be the most responsive to tissue culture and could therefore be used to characterize functional genes in *S. spontaneum*. We also suggested an approach with potential applications in facilitating the rapid identification of sugarcane genotypes susceptible to tissue culture.

Keywords: crop wild relative, embryogenic callus, genotypic variation, *Saccharum spontaneum*, sugarcane, tissue culture

Introduction

Sugarcane (*Saccharum* spp.) is an important cash crop that accounts for more than 70% of sugar production and 40% of bio-ethanol production globally (Mustafa *et al.*, 2018). In 2019, more than 1.9 billion tons and 28.2 million ha of sugarcane were harvested in 110 tropical and subtropical countries (FAOSTAT, 2020). Modern cultivated sugarcane is derived from complex interspecific hybrids mainly between *Saccharum spontaneum* and *Saccharum officinarum* (Roach, 1972; Yang *et al.*, 2019). *Saccharum spontaneum* is a wild relative with considerably higher hardiness but lower biomass and sugar content than *S. officinarum* (Roach, 1989; Zhang *et al.*, 2018). As a primary supplier of important agronomic traits such as biotic and abiotic stress tolerance, *S. spontaneum* has been widely used as a parent in conventional hybridization-based sugarcane breeding programmes (Roach, 1978; Moore *et al.*, 2014).

In vitro culture techniques, especially embryogenic callus induction and regeneration, play key roles in plant biotechnology for germplasm conservation, propagation and genetic improvement. Pathogen-free preservation of genetic lines of sugarcane by embryogenic calli requires less

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space, time and other resources (Glaszmann *et al.*, 1996). Embryogenic calli of elite cultivars are produced and proliferated for rapid micropropagation of sugarcane (Mordocco *et al.*, 2009). Artificial sugarcane seeds are also produced by encapsulating somatic embryos within alginate (Martinez-Montero *et al.*, 2008); embryogenic calli are used for mutation breeding, and mutants with desirable traits are selected (Mahlanza *et al.*, 2013).

These biological phenomena implicate the significance of induction and regeneration of embryogenic calli in sugarcane. Successful embryonic callus induction and plant regeneration require appropriate media containing different hormonal combinations and suitable genotypes amenable to *in vitro* culture. Although several universal tissue culture systems have been established and optimized (Barba *et al.*, 1977; Basso *et al.*, 2017), the effects of genotypic variation are difficult to overcome in sugarcane given the presence of considerable genotypic variations in response of sugarcane tissue culture to these systems, as reported in various studies (Fitch and Moore, 1990; Tolera *et al.*, 2014; Mahlanza *et al.*, 2019). Although the genetic diversity of *S. spontaneum* has been illustrated by phenotypic traits (Govindaraj *et al.*, 2014), cytological features (Panje and Babu, 1960; Zhang *et al.*, 2012) and molecular markers (Chang *et al.*, 2012; Liu *et al.*, 2016), genotypic variations in *in vitro* cultures have rarely been reported. In a recent study, the reference genome of *S. spontaneum* was fine-sequenced, and more than 35,000 genes were annotated using bioinformatics tools (Zhang *et al.*, 2018). This breakthrough provided a suitable platform for the isolation, identification and characterization of endogenous genes that account for various economically important traits in *S. spontaneum* by using different cutting-edge technologies and powerful molecular tools such as CRISPR/Cas9 system and RNA interference. All these technologies and tools are based on the requirement of screening and identification of suitable *S. spontaneum* genotypes having excellent callus induction and regeneration capacity.

In the present study, we determined the effect of genetic variations on embryogenic callus induction and regeneration in *S. spontaneum*. We aimed to investigate the genetic effect and to screen genotypes with high tissue culture susceptibility, which will be helpful in *in situ* gene function identification in *S. spontaneum*. A simplified methodology for the screening of large-scale sugarcane genotypes in *in vitro* cultures has also been suggested.

Materials and methods

Experimental design

We used a set of *S. spontaneum* germplasm conserved in the National Germplasm Repository of Sugarcane in Kaiyuan (NGRS-KY, Yunnan Province, China). Six genotypes, namely GD41, GX87-20, HNLS5, SC79-1-4, YN2011-44 and YN75-1-2, were collected from South China, whereas three genotypes, namely LAO2, S.SP2003-2 and VN2, were obtained through germplasm exchange.

A completely randomized experimental design with three replications was used. Non-contaminated plate cultures from the same batch of explants were included in a replication unit; each genotype was represented by more than 300 explants in each of the three replications.

Embryonic callus induction and regeneration

After 6–8 months of growth in the field, healthy apical portions of the shoots were sampled. The young leaf rolls were obtained by removing older leaves and through surface sterilization with 70% ethanol. We took 1 cm-long segments of leaf rolls, approximately 2 cm above the apical meristem. The young leaf rolls were excised into square slices having a width of approximately 2 mm and a length of approximately 5 mm. These slices were used as explants and abaxially cultured in induction medium containing 4.75 g/l of Murashige & Skoog (MS) salts, 0.5 g/l of casein acid hydrolysate, 0.15 g/l of citric acid, 0.1 g/l of L-cysteine, 2.0 mg/l of 2,4-dichlorophenoxyacetic acid, 30 g/l of sucrose and 5.0 g/l of agar. The cultures were incubated in the dark at 28 ± 2°C for 3 weeks. Browned and necrotic parts were removed from the calli before subculturing. After 3 weeks of subculturing, calli were classified into different types based on their morphological characteristics described by Taylor *et al.* (1992). All the calli of different types were counted, and the data were recorded.

The embryonic calli were collected and placed in differentiation medium containing 4.75 g/l of MS salts, 0.15 g/l of citric acid, 0.1 g/l of L-cysteine, 0.5 mg/l of N-(phenylmethyl)-9H-purin-6-amine, 20 g/l of sucrose and 5.0 g/l of agar. An MGC-450BP light incubator (Bluepard Instruments Co., Ltd., Shanghai, China) was used to provide the environmental conditions, with a temperature of 28 ± 2°C, a light intensity of 200 μmol/m^2/s and a photoperiod of 16/8 h (light/dark). After every 3 weeks, the embryonic calli were transferred to a fresh medium. After two rounds of cultivation, regeneration was checked. Embryogenic calli with at least one green spot or small shoots were considered as regenerated and were transferred to rooting medium containing MS salts without any hormones and incubated under the aforementioned environmental conditions.

The pH of the media was adjusted to 5.9 with NaOH prior to autoclaving. After autoclaving, when the medium temperature reached 50°C, filter-sterilized L-cysteine was added and the media were poured into sterile Petri dishes (NEST Biotechnology Co., Ltd., Wuxi, China). All the

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medium components were purchased from Coolaber Technology Co., Ltd, Beijing, China.

Statistical analysis

We assessed the following five parameters to determine the response of different *S. spontaneum* genotypes to embryogenic callus induction and regeneration:

$$\text{Callus induction} = \frac{\text{Totalnumber of calli}}{\text{Totalnumber of explants}} \times 100$$

$$\text{Embryonic callus ratio} = \frac{\text{Number of embryonic calli}}{\text{Total number of calli}} \times 100$$

$$\text{Embryonic callus induction} = \frac{\text{Number of embryonic calli}}{\text{Total number of explants}} \times 100$$

$$\text{Embryonic callus regeneration} = \frac{\text{Number of regenerated embryonic calli}}{\text{Number of embryonic calli cultured for regeneration}} \times 100$$

$$\text{Regeneration capacity} = \frac{\text{Number of regenerated embryonic calli}}{\text{Total number of explants}} \times 100$$

One-way analysis of variance and Pearson's correlation analysis were performed using SPSS 24.0 software (IBM Corp, Armonk, NY, USA). Broad-sense heritability, a quantitative genetics parameter, defined as the percentage of genetic variance in total phenotypic variance, was determined.

Results

Calli were induced on explant surfaces and edges 3–5 days after cultivation. Three types of calli were induced with different frequencies from each genotype (Table S1). Type I calli were sticky and watery, mostly with serious browning (Fig. 1(a), (e) and (i)); Type II calli were semi-translucent and incompact with a relatively wet surface (Fig. 1(b), (f) and (j)); Type III calli were dry and compact with a nodular surface and were recognized as embryonic calli (Fig. 1(c), (g) and (k)). After the transfer of calli to regeneration medium, multiple green spots and shoots were observed on the surface of embryonic calli (Fig. 1(d), (h) and (l)). The regenerated embryonic calli developed into plantlets on blank MS medium after 6–8 weeks (data not shown).

Five parameters were assessed to investigate the *in vitro* culture response of different *S. spontaneum* genotypes. Statistical analysis revealed that responses of all the genotypes to callus induction, embryogenic callus ratio, embryogenic callus induction and regeneration capacity vary significantly (all $P < 0.01$, Table 1). The highest broad-sense heritability was noticed for callus induction (96.8%), followed by embryogenic callus induction (96.3%), embryogenic callus ratio (93.5%) and regeneration capacity (86.1%). No significant difference was observed among all the genotypes in embryogenic callus regeneration, which also displayed the lowest broad-sense heritability of 5.1% (Table 1).significant correlation was observed between embryonic callus regeneration and all the other parameters (Table 2).

The highest values of the four parameters with significant genotypic variation were observed for the VN2 genotype. Moreover, the highest regeneration capacity was observed for VN2 (51.5%), followed by that for YN2011-44 (39.0%) and HNLS5 (27.0%) (Table 1).

Discussion

The strong regeneration capacity and high transformation suitability of embryonic calli make them essential in many aspects of *S. spontaneum* research, particularly regarding the functional characterization of useful genes (Birch, 2014). In the present study, we observed significant variations in response to embryogenic callus induction and regeneration between the nine genotypes of *S. spontaneum* that can be attributed to genetic differences between these genotypes.

Other reports on sugarcane cultivars (Gandonou *et al.*, 2005; Raza *et al.*, 2010; Basnayake *et al.*, 2011; Mahlanza *et al.*, 2019) are in agreement with our findings. Moreover, similar results have been reported for other crops such as wheat progenitors (*Aegilops* sp. and *Triticum* sp., Özgen *et al.*, 2015) and rice (*Oryza sativa*, Hoque and Mansfield, 2004). Although the universality of the genetic effect on tissue culture responses has been proven by extensive research, only a few studies have quantitatively assessed this effect. In the present study, by estimating broad-sense heritability, we found that the genetic factor mainly accounts for embryogenic callus induction-related parameters (>93%) and regeneration capacity (~86%). The results provide evidence on the role of genetic factor in determining tissue culture responses of different *S. spontaneum* genotypes, highlighting the importance of genotype screening in tissue culture and in determining the model or pioneer genotype in *S. spontaneum*.

Although the set of clones tested in this study was only a small subsample of *S. spontaneum* collections, the role of genetic factor in tissue culture responses is evident. Three clones exhibited high regeneration capacity, and the highest value was observed for VN2. These clones are suitable for future tissue culture-related studies because of their high tissue culture susceptibility.

The present data support the assumption that embryogenic calli differentiation is less critical than calli production (Bower and Birch, 1992; Mahlanza *et al.*, 2019). High

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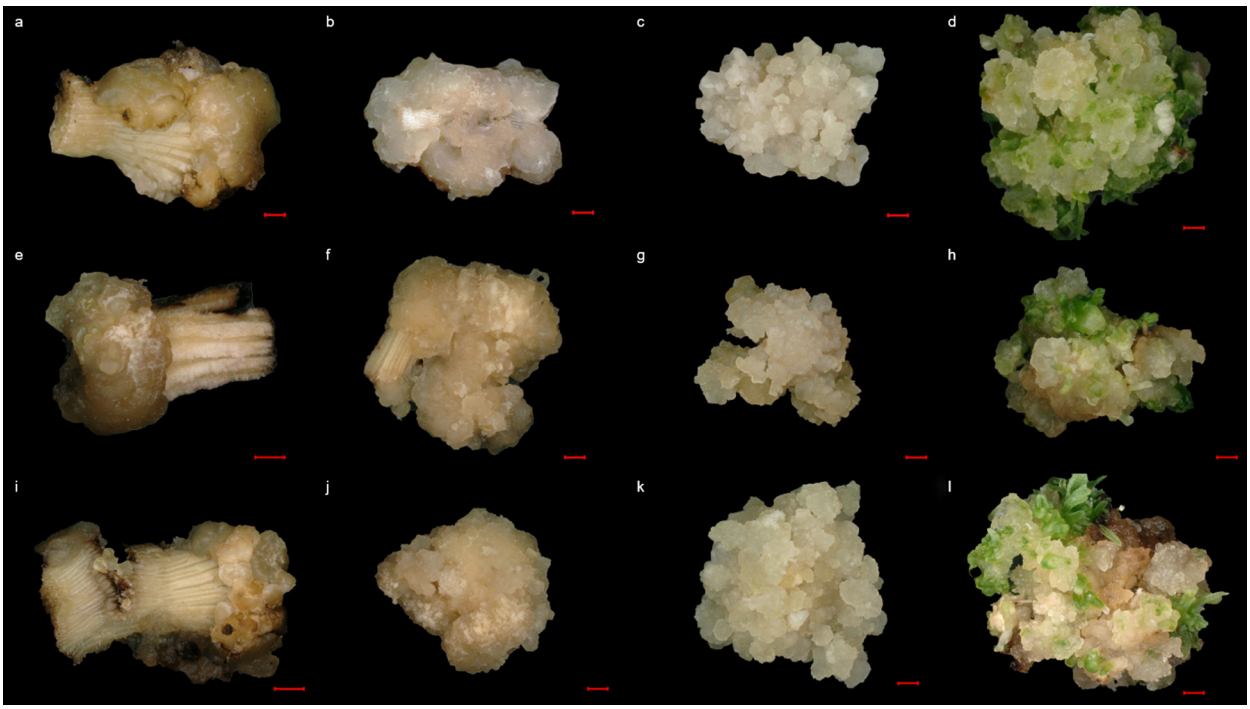

Fig. 1. Three types of calli and differentiation of embryonic calli for *Saccharum spontaneum* genotypes. VN2 (a–d), YN2011-44 (e–h) and HNLS5 (i–j) were shown. Tissue culturing for induction and regeneration of the callus is described in the Materials and methods section. Scale bar = 1 mm.

Table 1. Response to embryogenic callus induction and regeneration of *Saccharum spontaneum* genotypes

Genotype	Callus induction (%)	Embryogenic callus ratio (%)	Embryogenic callus induction (%)	Embryogenic callus regeneration (%)	Regeneration capacity (%)
GD41[a]	46.5	43.8	20.3	82.1	16.7
GX87-20[a]	41.8	42.5	17.7	81.1	14.4
HNLS5[a]	61.9	51.9	32.1	84.0	27.0
LAO2[b]	33.1	45.7	15.1	83.0	12.5
S.SP2003-2[b]	52.1	43.1	22.4	80.6	18.1
SC79-1-4[a]	58.6	45.8	26.8	81.3	21.8
VN2[b]	74.3	83.8	62.3	82.7	51.5
YN2011-44[a]	66.6	66.2	44.3	87.8	39.0
YN75-1-2[a]	20.7	45.1	9.6	81.8	7.9
Mean	50.6	52.0	27.9	82.7	23.0
Fold change	3.59	1.97	6.47	1.09	5.38
F-genotype	93.655**	24.415**	69.828**	0.854ns	47.677**
Hb (%)	96.864	93.457	96.308	5.117	86.129

[a,b]Originated from China (a) and other countries (b).
**Statistically significant at the level of 0.01; ns, not significant.

regeneration frequency is a distinguishing feature of embryonic calli (Barba *et al.*, 1977; Taylor *et al.*, 1992; Gandonou *et al.*, 2005; Birch, 2014; Basso *et al.*, 2017), and in this study, the embryonic callus regeneration frequency was found to be high for all the clones (>80%, Table 1). Regeneration frequency of embryonic callus is not affected by genetic variation as evident by statistical analysis and low broad-sense heritability value (Table 1). These results are in agreement with those of previous studies on sugarcane cultivars (Gandonou *et al.*, 2005) and

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Table 2. Correlations among tissue culture susceptibility parameters

	Callus induction	Embryogenic callus ratio	Embryogenic callus induction	Embryogenic callus regeneration	Regeneration capacity
Callus induction	–				
Embryogenic callus ratio	0.690**	–			
Embryogenic callus induction	0.890**	0.937**	–		
Embryogenic callus regeneration	0.202ns	0.354ns	0.281ns	–	
Regeneration capacity	0.881**	0.939**	0.996**	0.360ns	–

Pearson' s correlation coefficients for each measurement are shown.
Statistical significance of the correlations was two-tail tested. **Statistically significant at the level of 0.01; ns, not significant.

wheat progenitors (Özgen *et al*., 2015) and suggest a possibility that regenerated plantlets can be obtained when embryogenic calli are well inducted. The strong intrinsic differentiation ability of the embryonic callus, which is independent of genotypic variation, could account for some aspects of high embryonic callus regeneration frequency observed in all the genotypes.

Our results facilitate the optimization of tissue culture susceptibility screening experiments. Callus induction was closely correlated with embryonic callus induction ($r = 0.890$, $P < 0.01$) and regeneration capacity ($r = 0.881$, $P < 0.01$) (Table 2), indicating that the embryonic callus induction and regeneration of *S. spontaneum* genotypes can accurately be predicted on the basis of the callus induction frequency. We therefore suggest that the simple investigation of callus induction is sufficient to represent the entire tissue culture susceptibility, including embryonic callus induction and plant regeneration. This approach represents an effective strategy for genotype screening, especially for large-scale projects.

We found a significant variation in responses of different genotypes to embryonic callus induction and regeneration in *S. spontaneum*. Several elite genotypes were identified to be of practical value in plant genomics and advanced biotechnology. As callus induction and regeneration capacity are genetically controlled phenomena, the genetic characters of the VN2 genotype can be incorporated in the modern recalcitrant cultivars of sugarcane through conventional breeding, making them amenable to tissue culture and suitable for transgenic development. High-throughput RNA sequencing of embryogenic and regenerated calli of the VN2 genotype can help explore and identify the candidate genes responsible for regeneration, which in turn will facilitate the rapid identification of modern cultivars susceptible to tissue culture by using molecular techniques.

Supplementary material
The supplementary material for this article can be found at https://doi.org/10.1017/S1479262121000198.

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Conflict of interest
None.

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