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GENETICS AND PLANT BREEDING

Increased transient genetic transformation in immature embryos of Brazilian BR 451 maize co-cultivated with *Agrobacterium tumefaciens*

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ABSTRACT. Genetic engineering has amplified the possibilities of crop breeding and supported sustainability ideals. *Agrobacterium tumefaciens* is the preferred transformation system, since it produces transgenic plants with more stable transgene expression and inheritance. The agrobacteria and plant tissue must be co-cultivated in conditions that allow gene transfer. This study aimed to evaluate how co-cultivation time and temperature affect the transformation of immature maize embryos of the Hi-II hybrid (model genotype) and the Brazilian BR 451 variety with *A. tumefaciens* EHA101:pTF102. The pTF102 plasmid carries a *uidA* reporter gene that enables transient transformation to be quickly verified by GUS histochemical assays. Increasing the co-cultivation period from three to five days at 20°C resulted in a higher number of GUS positive embryos and blue spots per embryo in the BR 451 Brazilian variety, indicating better bacterial T-DNA transfer into the target explant cells. This condition raised the BR 451 response level to match the response level of the Hi-II control genotype, indicating that this Brazilian variety is suitable for genetic transformation.

Keywords: agrobacteria; GUS histochemical assay; genetic engineering; *Zea mays*.

Aumento da transformação genética transiente em embriões imaturos do genótipo brasileiro de milho BR 451 co-cultivados com *Agrobacterium tumefaciens*

RESUMO. A engenharia genética ampliou as possibilidades do melhoramento genético vegetal, apoiando ideais de sustentabilidade. A *Agrobacterium tumefaciens* é o sistema de transformação preferido, uma vez que permite a produção de plantas transgênicas com maior estabilidade na expressão gênica e na herdabilidade. A Agrobactéria e o tecido vegetal devem ser co-cultivados em condições que permitam a transferência de genes. Este estudo teve como objetivo avaliar como o tempo e a temperatura durante o co-cultivo afetam a transformação de embriões imaturos de milho do híbrido Hi-II (genótipo modelo) e da variedade brasileira BR 451 com *A. tumefaciens* EHA101:pTF102. O plasmídeo pTF102 carrega o gene repórter *uidA* permitindo que a transformação transiente seja prontamente verificada pelo ensaio histoquímico de GUS. O aumento do período de co-cultivo de três para cinco dias a 20°C resultou num maior número de embriões GUS positivos e maior número de pontos azuis por embrião na variedade brasileira BR 451, indicando uma melhor transferência do T-DNA da bactéria para as células do explante alvo. Esta condição elevou o nível de resposta da BR 451 chegando a coincidir com o nível de resposta do controle Hi-II, indicando que esta variedade brasileira é adequada para transformação genética.

Palavras-chave: agrobactéria; ensaio histoquímico de GUS; engenharia genética; *Zea mays*.

Introduction

Maize (*Zea mays* L.) is a highly important crop for food production, occupying 13% of the agricultural area in the world (USDA, 2014) and being extensively used for human and animal nutrition. Maize has been a target species for biotechnological innovation, being the first genetically modified cereal released in the world market. In Brazil, 88.4% of maize is genetically modified (ISAAA, 2016), but there is no Brazilian transgenic maize commercially released in the current market.

Despite the unmatched commercial success of maize transformation and significant progress made in the molecular techniques for this species, the effective rate of genetic transformation of maize is still insufficient (Yadava et al., 2016). The inefficiency of genetic transformation is attributable to various limitations of the available maize tissue culture and transformation protocols.

Genetic transformation by *A. tumefaciens* allows large DNA segments to be transferred and inserted into the plant genome with a low gene copy number (Zhao et al., 2001). The first transgenic maize plants obtained by agrobacteria were reported by Ishida et al. (1996) followed by other groups (Zhao et al., 2001; Frame et al., 2002; Ombori, Muoma, & Machuka, 2014; Lee & Zhang, 2016; Souza et al., 2017). To succeed in maize transformation, it is necessary to use target tissues with high rates of cell division, such as immature zygotic embryos. Maize transformation also requires use of highly *in vitro* responsive maize genotypes and specific *A. tumefaciens* strains, since this bacterium does not naturally infect monocotyledonous plants.

While several maize genotypes have been effectively transformed (Ji, Xu, & Wang, 2013, Ombori et al., 2014; Souza et al., 2017), Hi-II hybrid maize has been widely used for genetic transformation (Zhao et al., 2001; Frame et al., 2002; Frame, Main, Schick, & Wang, 2011; Lee & Zhang, 2016; Nahampun, Lopez-Arredondo, Xu, Herrera-Estrella, & Wang, 2016) and is considered the model for maize plant transformation. However, the poor agronomic traits of the Hi-II hybrid (Ishida, Hiei, & Komari, 2007; Que et al., 2014) motivate the search for other maize genotypes that respond well to transformation. Since the Brazilian BR 451 maize variety presents high protein quality, good agronomical performance and adaptation to different regions of Brazil (Guimarães, Parentoni, & Pacheco, 2004), as well as a suitable *in vitro* response (Carvalho, Bohorova, & Bordallo, 1997; Petrillo et al., 2008), it offers a Brazilian alternative to the American Hi-II hybrid.

Co-cultivation is a critical step in the maize genetic transformation protocol (Ishida et al., 2007), and a three-day co-cultivation period is most often used (Frame et al., 2002; 2011; Ishida et al., 2007; Nahampun et al., 2016). In addition, the co-cultivation temperature also influences transformation efficiency (Huang & Wey, 2005).

This study aimed to test whether modifying co-cultivation period and temperature can improve *Agrobacterium*-mediated maize genetic transformation of Hi-II hybrid and BR 451 maize and verify if the Brazilian BR 451 variety is suitable for genetic transformation.

Material and method

Experiments were conducted in the Plant Biotechnology Laboratory at the University of Passo Fundo (CQB 272/08). The BR 451 variety (Guimarães et al., 2004), courtesy of Embrapa Maize and Sorghum, and the Hi-II American hybrid (Al88 x B73) were established at a greenhouse for zygotic embryo production.

The *A. tumefaciens* disarmed strain EHA 101 containing the pTF102 binary plasmid was used. This plasmid harbors the *uidA* reporter gene under control of CaMV35S constitutive promoter and contains an intron in the coding region (Paz et al., 2004). Bacteria from a glycerol stock were grown in YEP solid medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 5 g L^{-1} NaCl, and 15 g L^{-1} Bacto-agar, pH 6.8) supplemented with 100 mg L^{-1} spectinomycin and 50 mg L^{-1} kanamycin for two days at 28°C. Isolated colonies from this main board were cultured in the same medium supplemented with 100 μM acetosyringone for three days at 19°C. Two loops of this culture were suspended in 5 mL infection medium (pH 5.2) (Frame et al., 2006) containing N6 salts (Chu et al., 1975), vitamins (0.5 mg L^{-1} thiamine HCl, 2.0 g L^{-1} glycine, 0.5 mg L^{-1} pyridoxine HCl, 0.5 mg L^{-1} nicotinic acid), 100 mg L^{-1} hydrolyzed casein, 2.88 g L^{-1} L-proline, 2.0 mg L^{-1} 2,4-D, 68.5 g L^{-1} sucrose, 36 g L^{-1} glucose, supplemented with 100 μM acetosyringone. Cell density was adjusted to OD_{550nm} = 0.3 - 0.4, and bacteria were incubated for two hours at room temperature in a horizontal shaker at 100 rpm.

Approximately 11 days after pollination, 1.2 to 1.8 mm immature zygotic embryos of Hi-II and BR 451 were collected and immersed in 1 mL infection medium containing *A. tumefaciens* for five min in the dark. After infection, explants were transferred to solid co-cultivation medium containing the same infection medium formulation with the following modifications: without glucose; sucrose reduced to 30 g L^{-1}, with 0.5 g L^{-1} MES; 10 μM silver nitrate; 8.0 g L^{-1} agar; 5.8 pH (Frame et al., 2006). Embryos were placed in Petri dishes with scutella in contact with the culture medium. Petri dishes were sealed with porous tape and kept in the dark. Four treatments were conducted, with co-cultivation time and temperature variations, as follows: (T1) three days at 20°C, (T2) three days at 28°C, (T3) five days at 20°C, and (T4) five days at 28°C. After the co-cultivation, all the agrobacteria co-cultured explants were transferred to the resting medium, containing the same co-cultivation medium formulation, with the following modifications: without acetosyringone, with 100 mg L^{-1} cefotaxime and with 100 mg L^{-1} vancomycin. Petri dishes were sealed with porous tape and kept in the dark at 28 °C for seven days. This step aimed to eliminate the bacteria and allow embryogenic callus development.

Transient transformation was verified by *uidA* reporter gene expression in immature embryos by

the β-glucuronidase (GUS) histochemical assay, performed in accordance to Jefferson, Kavanagh, and Bevan (1987) five days after infection in all treatments. Embryos were incubated in a solution containing X-glucuronidase substrate for 16 hours at 37°C. After incubation, the reaction was removed and embryos were stored in 70% ethanol until analysis time. The *uidA* gene encodes the GUS enzyme that produces an indigo blue color dimer in the presence of the substrate, enabling transformation events to be visualized and counted.

The frequency of embryos with blue spots (GUS positive embryos), and the number of blue spots per embryo were accessed. Embryos not subjected to infection were co-cultivated in each treatment and used as control.

The genotypes (Hi-II and BR 451) were evaluated in independent experiments carried out in the same manner, as follows. The experimental design was completely randomized, with five replicates per treatment (co-cultivation time and temperature). The experimental unit was a Petri dish containing 30 explants. For transient transformation analysis, a random sample of five explants per repetition was taken, totaling 25 embryos per treatment evaluated by GUS histochemical assay. Means ± 1 standard deviation was used for treatment comparison. The experiment was repeated three times.

Result and discussion

Differences were not observed in the number of GUS positive embryos or number of blue sports per embryo in Hi-II maize when the co-cultivation was performed in different conditions. On average, 72% of embryos were transformed, and 14.6 blue spots per embryo were observed (Figure 1A and B). The blue spots represent the location where the reporter gene was inserted in the embryo and the number of blue spots per embryo represents the intensity of the transient genetic transformation. The most commonly used co-culture conditions for Hi-II maize genetic transformation protocols are three-day co-cultivations at 20°C (T1) (Frame et al., 2002; 2006; 2011; Omer, Matheka, Ali, & Machuka, 2013; Ombori et al., 2014; Nahampun et al., 2016). In our analysis, a longer period of co-cultivation (five days) or higher temperature did not increase the T-DNA transfer from bacteria to Hi-II embryos.

The overall transformation frequency observed in the Brazilian BR 451 variety was lower (43%) compared to Hi-II (72%). Likewise, the number of transformation sites (blue spots per embryo) was also lower for the BR 451 (11.7) compared to Hi-II (14.6) (Figure 1). Differences among maize genotypes in the capacity to be infected have been widely reported in the literature (Huang & Wei, 2005; Frame et al., 2006; Omer et al., 2013; Ombori et al., 2014; Souza et al., 2017), with genotype dependence being the main limitation in maize genetic transformation by this method (Hiei, Ishida, & Komari, 2014). Genotypic differences can be explained by compatibility variation between maize genotype and the bacterial strain employed in the transformation or the T-DNA transference machinery encoded by the bacteria and plant cell genomes (Ombori et al., 2014; Lowe et al., 2016). The molecular and chemical signaling that allow agrobacteria to connect and insert T-DNA into the host plant cell is regulated by turning genes on and off, which is strongly influenced by the plant genotype (Tzfira, Li, Lacroix, & Citovsky, 2004).

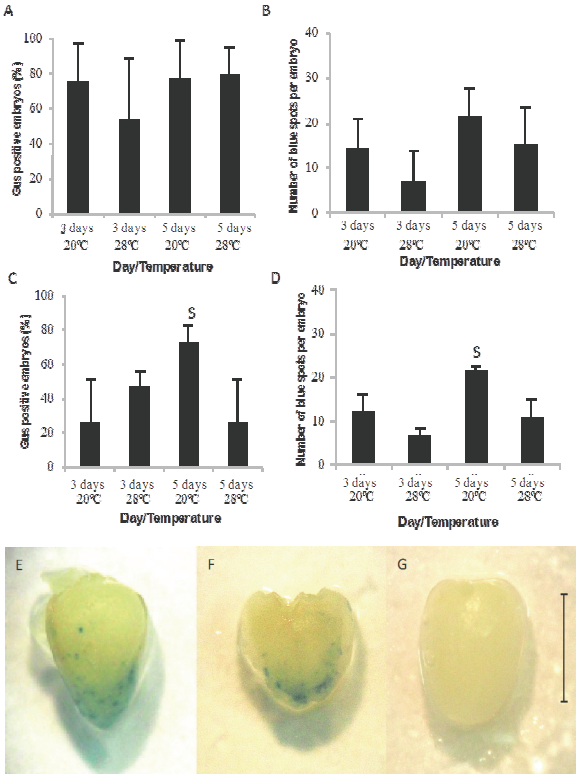

Figure 1. Transient expression of GUS observed in immature zygotic maize embryos co-cultivated with *A. tumefaciens* at different conditions: (A) Frequency of GUS positive Hi-II embryos, (B) mean number of blue spots per Hi-II embryo, (C) Frequency of GUS positive BR 451 embryos, (D) mean number of blue spots per BR 451 embryo, (E) Gene *uidA* expression in Hi-II immature embryos co-cultivated for five days at 20°C, (F) Gene *uidA* expression in BR 451 embryos co-cultivated for five days at 20°C, and (G) negative control not infected with agrobacteria. Bar = 1 mm, S = (superior) Means +1 SD.

The co-cultivation conditions affected transient transformation of BR 451 embryos. The T3 treatment (five days at 20°C) promoted superior GUS positive embryos (73%) (Figure 1C), as well as the superior number of blue spots observed per embryo (23.1) compared to other treatments (Figure 1D). Compared with the standard co-cultivation conditions used for maize (three days at 20°C), two more days in co-cultivation doubled the number of embryos transformed and the number of blue spots per embryo. Increased transformation suggests that prolonged co-cultivation increases the chance of agrobacteria binding to the embryo surface, which increases the chance of T-DNA transference from agrobacteria to the plant cell nuclei. In contrast, Huang and Wey (2005) found no influence of co-cultivation period (from three to six days) on bacterial T-DNA transient transformation in immature embryos from three elite maize strains.

In accordance with Alimohammadi and Gherieh-Najjar (2009), co-cultivation temperature should be investigated, as there are different responses among monocots, genotypes, explants and agrobacteria strains. Low temperatures (19°C) favor agrobacteria T-pili formation, and higher temperatures (28°C) inhibit T-pili formation (Fullner, Lara, & Nestert, 1996). The T-pilus is an appendix or filamentous channel attached to the surface of agrobacteria, composed of Ti plasmid-encoded proteins. It is thought that this secretion system is the primary means of T-DNA transport from the bacterial cell to the plant cell (Mccullen & Binns, 2006).

According to Hiei et al. (2014), high temperature during co-cultivation may also result in agrobacteria overgrowth in the co-cultivation medium. The present study showed that high temperature (28°C) was unfavorable for transient transformation but did not promoted bacterial overgrowth even during a prolonged co-culture period. Bacterial overgrowth is undesirable because it can cause plant tissue death.

In our experiment, five day co-cultivation at 20°C increased the BR 451 response level to the level of Hi-II, since in these same conditions, the Hi-II genotype produced 71% GUS positive embryos with approximately 21 blue spots per embryo. Therefore, by modifying the BR 451 co-cultivation condition, it was possible to match the transformation performance of the Hi-II model genotype. The *uidA* gene expression in Hi-II and BR 451 immature embryos co-cultivated under these conditions is shown in Figure 1E and F.

The success of obtaining genetically transformed maize plants depends on the plant genotype, which must respond well to both *in vitro* culture and genetic transformation. The Hi-II maize hybrid and its source lines (A188 and B73) have these characteristics and have been used in transgenic plants production worldwide (Frame et al., 2002; 2011; Zhao et al., 2001). However, the Hi-II hybrid presents poor agronomic performance (Ishida et al., 2007; Que et al., 2014). The use of a genotype showing good *in vitro* cultivation response and good agronomic characteristics, such as the BR 451 (Grando et al., 2013; Petrillo et al., 2008), accelerates commercial transgenic plant production, since the gene inserted in the transgenic event can be transferred to elite lines without the transfer of additional unwanted traits. Therefore, improvement in the genetic transformation of BR 451 opens the possibility of implementing its use in genetic engineering.

Genetic engineering is an invaluable tool that enables the introduction of new traits, endogenous gene overexpression and silencing of undesirable features in important crop plants. Hence, the "New Breeding Techniques" era, including the newer genome editing technologies, such as the CRISPR/Cas9 system, is opening up a new dimension in transformation protocols and workflow (Yadava et al., 2016). The utilization of these newly arising molecular techniques to explore and modify the genome will require optimization of DNA delivery, plant *in vitro* regeneration and a more efficient transformation platform (Char et al., 2017).

This paper reports an increase in agrobacteria gene transfer for the BR 451 Brazilian genotype, enabling its use for genetically modified maize production. This aspect is particularly important for Brazil, where there are few research groups applying this technology for maize.

Conclusion

By prolonging the period of co-cultivation of immature embryos with agrobacteria, it is possible to increase the transient genetic transformation of the Brazilian genotype BR 451, enabling its use in genetic transformation studies.

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