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Intragenic modification of maize

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ABSTRACT

The discovery of plant DNA recombination techniques triggered the development of a wide range of genetically modified crops. The transgenics were the first generation of modified plants; however, these crops were quickly questioned due to the artificial combination of DNA between different species. As a result, the second generation of modified plants known as cisgenic and/or intragenic crops arose as an alternative to genetic plant engineering. Cisgenic and/or intragenic crops development establishes the combination of DNA from the plant itself or related species avoiding the introduction of foreign genetic material, such as selection markers and/or reporter genes. Nowadays it has been made successful cisgenic and/or intragenic modifications in crops such as potato and apple. The present study shows the possibility of reaching similar approach in corn plants. This research was focused on achieve intragenic overexpression of the maize Rubisco activase (Rca) protein. The results were compared with changes in the expression of the same protein, in maize plants grown after 23 cycles of conventional selection and open field planting. Experimental evidence shows that maize intragenic modification is possible for increasing specific gene expression, preserving plant genome free of foreign DNA and achieving further significant savings in time and man labor for crop improvement.

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1. Introduction

Genetic engineering induced the development of many transgenic crop plants; however, this originally promising method for crop improvement has been controversial since transgenic plants contain nucleotide sequences from species that are sexually incompatible in nature (Ryffel, 2014). The widespread application of transgenic techniques in comestible plants raised public concerns mainly about health safety (Domingo, 2016; Kamthan et al., 2016), although there is no scientific evidence that genetically modified crops harm human health (Fahlgren et al., 2016). Nevertheless, its use continues to be a topic of debate due to questions concerning intellectual property and biosafety issues involved in open field planting (Lucht, 2015; Ryffel, 2014; Yaqoob et al., 2016). To surmount these deficiencies, another generation of Genetically Modified Organisms (GMO) is being developed, known as cisgenic and/or intragenic crops. This methodology, in contrast to transgenics, only allow combinations of DNA sequences originated from the original plant and/or naturally compatible species (Ricroch and Hénard-Damave, 2015; Rommens, 2004; Schouten et al., 2006).

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The term cisgenesis initially specified the use of entire genes, i.e. regulatory and coding regions in a sense orientation. Later, the intragenesis concept was used to describe the combination of genes or intergenic fragments from the same or related plants. However, although each of these terms establishes different specifications, both plant breeding approaches share the principle of transgenes absence in the improved final product (Espinoza et al., 2013; Schouten and Jacobsen, 2008; Singh et al., 2015).

Most cisgenic and/or intragenic plants described so far have been obtained using *Agrobacterium* plasmid to transfer the desired unit of DNA cloned on the T-DNA or P-DNA region, -a variation of T-DNA made exclusively from plant DNA- (Rommens et al., 2005). However, besides the desired sequence, small DNA fragments of the plasmid backbone can be detected in some lines of the improved plants, therefore these plants are considered not-truly cisgenic (Vanblaere et al., 2014). In other cases, cisgenic and/or intragenic plant production has been achieved by bombarding two linear DNA sequences, one with the desired gene and another with a marker gene from a non-plant organism to allow the recovery of successful DNA transfer events. Later, the descendants that inherited only the desired gene are selected through guided segregation (Romano et al., 2003; Yao et al., 2006). This strategy is successful in crop plants with short propagation life cycles. In the case of maize, this method would require a longer time and depends on the use of a previously established in vitro propagation system

(Holme et al., 2013). There have been few DNA transformation studies in tropical maize lines (Assem, 2015), which means that maize experimentation is limited to a few model maize lines that are not used worldwide. However, our research group has established the proliferation and regeneration of maize embryonic calli from the Tuxpeño race, Costeño variety (Garrocho-Villegas et al., 2012). This is important because many maize varieties from the Tuxpeño race have been adapted to grow in different countries.

In this research, the intragenic model was adapted in order to modify specific protein expression in maize plants. We look forward for to the transgene absence principle, not only in the final product, but also even during the transformation process. In this study, regulatory sequences originated from the maize genome were used to achieve maize Rubisco activase (Rca) protein overexpression.

Rca is a protein expressed in the chloroplast that determines the rate of atmospheric CO_2 fixation in the plant (Hazra et al., 2015). Rca kinetics have been documented in different studies, so Rca has become the focus of researchers aiming to improve plant photosynthetic capacity to increase yield as well as plant adaptation to harsh climatic conditions (Kurek et al., 2007; Sage et al., 2008; Salvucci et al., 2006; Thieulin-Pardo et al., 2015; von Caemmerer et al., 2005; Yamori et al., 2012). Previous phenotypic and molecular analysis in a maize population monitored for 23 cycles of conventional open field seeding, showed Rca expression changes during this period. In those studies, Rca content was analyzed in the original crop Z_0 and in the cultivars obtained through agronomic selection cycles aimed to produce greater yield: Z_5, Z_{10}, Z_{15}, Z_{20} y Z_{23}. Rca exhibited four-fold higher expression in Z_{23} with respect to Z_0 (Morales et al., 1999).

In this context, the efforts of the present research were oriented towards the molecular characterization of intragenic Rca overexpression changes in maize, in order to examine whether the increment response is comparable with the Rca expression levels reached after conventional selection and culture of maize plants.

2. Materials and methods

2.1. Plant material

To obtain intragenic Rca-overexpressing plants, immature Tuxpeño corn embryos of the Costeño variety were used to generate calli culture (Garrocho-Villegas et al., 2012). As controls, unmodified plants of the same type of corn were used. This material was provided by the Instituto Forestal de Investigaciones Agropecuarias, Zacatepec, México and in vitro cultivated in the Plant Tissue Culture Lab from Faculty of Chemistry, UNAM.

Maize plants with increased Rca expression obtained through traditional agronomic selection were obtained by planting seeds of Cónico Norteño corn, Zacatecas 58 variety, from the Z_0 and Z_{23} cultivars. The seeds were kindly provided by Dr. José Molina Galán from the Colegio de Postgraduados, Montecillo, Estado de México (Morales et al., 1999).

2.2. Intragenic construction

Intragenic construction (Fig. 1) generated a linear cassette of 2827 bp composed of the promoter sequence (969 bp) of the small subunit Rubisco (Rco) enzyme encoded by *ZmRBSC-m3* in maize, followed by the *ZmRca* gene coding sequence, with its own peptide signal for chloroplast entry (1302 bp) (access number **AAG22094.3**) (Ayala-Ochoa et al., 2004) and the terminator sequence (488 bp) from the same source as the promoter (Schäffner and Sheen, 1991; Viret et al., 1994). In addition, the intragenic cassette contained 68 bp that correspond to restriction enzyme sites to facilitate cloning and purification. Those sites were set between the extremes of the three sequences. The cassette was synthesized and subcloned using *GenScript USA* (Piscataway, NJ, USA) in the puc 57 plasmid (5530 bp, PM = 3,594,500 g/mol). This plasmid was replicated in *E. coli* cells. The intragenic cassette was isolated from the puc 57 plasmid prior to bombardment (1 μg/μl) using digestion reactions with PstI and DraI to eliminate the plasmid fractions that are not required for the plant.

2.3. Corn transformation with intragenic construct

Maize embryogenic calli were twice bombarded using particle preparation and standard biolistic process (Sanford et al., 1993) with an effective delivery of 0.8 μg of the intragenic construct precipitated on 0.5 mg tungsten particles (0.4 μm diameter) per shot. The composition of all media culture were made according to previously described by Garrocho-Villegas et al. (2012). For each assay, 0.3 g calli were placed in the center of each one of 10 Petri dishes of N_6P medium solidified with 3.5 g/L gelrite. As control, the same quantity of calli was treated with only tungsten particles. After 24 h of bombardment, the calli were transferred to N_6P medium with 2.5 g/L gelrite. They were maintained at room temperature in the dark for one week. The calli were subsequently transferred 3 times, every 15 to 20 days, to a fresh N_6P medium. Plant regeneration occurred throughout another 3 subcultures diminishing the auxin concentration in the N_6P medium (50%, 25% and 0%, respectively) without adding a selection agent. The resultant plants were maintained in MS medium enriched with 1 mg/L of indole butyric acid to aid the rooting process. At this stage, subcultures were generated every 25 to 35 days in glass bottles. Finally, when plants developed approximately 7 cm of primary root, they were planted in organic substrate (Sunshine No 3) in 1 L pots. They were covered with transparent plastic and kept in the greenhouse at 25–35 °C with 12-h light/dark photoperiods. The plastic covers were gradually perforated until they were removed completely.

2.4. Identification of intragenic corn plants

The intragenic plants were identified through end-point PCR using Taq polymerase Kapa 3G, with oligonucleotide Pairs 1 and 2 designed to detect the overexpression cassette at two different union fragments. Table 1 describes the oligonucleotides used. The results were visualized in agarose gels. The *18 S* gene was used as an internal control.

2.5. Analysis of Rca expression

During the flowering stage, 1 cm^2 samples were harvested from the leaves immediately above the ear from the identified intragenic plants and their controls, as well as from plants of the Z_0 and Z_{23} cultivars. The Rca mRNA was quantified using real-time RT-PCR with TRR oligos (Table 1). Total RNA was isolated by Quick–RNA MiniPrep (Zymmo Research) with DNase. Starting with 1 μg of RNA, cDNA was synthesized with dT oligos (Thermo Scientific Maxima H Minus First Strand), quantified with NanoDrop 2000 Thermo Scientific, and the real-time PCR reaction was performed with SYBR GreenER qPCR SuperMix Universal Life Sciences according to manufacturer instructions. The Rca mRNA expression was normalized with respect to the reference high mobility group gene (*Hmg*), access number AJ131373. The data were analyzed statistically using t Student's test for independent samples ($p = 0.05$).

The Rca protein amount was analyzed by triplicate using Western blots (Agrisera antibody, product code: AS10 700), according to Yamori and von (2011). Gels of total protein extracts were analyzed

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