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Identification and freedom to operate analysis of potential genes for drought tolerance in maize

Identificación y analisis de libertad de operación en genes potenciales de maíz para la tolerancia a sequía

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ABSTRACT

Drought tolerance is an important character for agricultural crops, particularly corn. Genes confering this feature can be patented, thus hindering their use. From a thorough analysis, three genes (DREB, ZAT10 and CspB) were identified and their sequences were captured in the NCBI database. From these sequences and using free software tools, expression cassettes -including regulatory regions (promoters E35S + Pleader, Ubi-1, rab17; terminators Trub, Tnos)- were designed. Patent searches were conducted in international databases (The Lens and PATENTSCOPE). Four patents and an application were found. In the Colombian national database of the Superintendence of Industry and Commerce (SIC), only the application made through PCT was identified. The claims and nucleotide sequences contained in the application were analyzed and it was found that they do not affect the expression cassettes designed. There is freedom to operate for these constructs and it is possible to continue developing drought-tolerant GM maize lines for the domestic market.

Key words: intellectual property, GM crops, gene design.

RESUMEN

La tolerancia a sequía es un carácter importante para los cultivos agrícolas, en particular para el maíz. Los genes que confieren esta característica pueden estar patentados, dificultando así su uso. A partir de un análisis exhaustivo se identificaron tres de tales genes (DREB, ZAT10 y CspB) y se capturaron las secuencias en las bases de datos del NCBI. A partir de estas secuencias y mediante herramientas de software libre se diseñaron casetes de expresión que incluyeron regiones regulatorias (promotores E35S+pLeader, Ubi-1, rab17; terminadores Trub, Tnos). A continuación se realizaron búsquedas de patentes en bases de datos internacionales (The Lens, y PATENSCOPE). Se encontraron cuatro patentes y una solicitud. En la búsqueda en la base de datos nacional de la Superintendencia de Industria y Comercio, se identificó solo la solicitud realizada por PTC. Analizadas las reivindicaciones y secuencias nucleotídicas contenidas en la solicitud, se encontró que no afectan los casetes de expresión diseñados. Se comprueba la libertad de operación para estos constructos, y la posibilidad de desarrollar líneas. GM tolerantes a sequía para el mercado nacional.

Palabras clave: propiedad intelectual, cultivos GM, diseño de genes.

Introduction

Maize (*Zea mays*) is a fundamental crop as it is a staple food for both humans and animals due to its high nutritional content and its relatively low cost when compared with other agricultural products. It is also used globally for the production of ethanol, an alternative use that may reduce its availability as a food source (Viveros, 2007). Maize is susceptible to stress due to water deficit (Boyer and Westgate, 2004), particularly during pollination, flowering and embryo development stages (Grant *et al.*, 1989; Bolaños and Edmeades, 1996). In Colombia, almost 72% of internal demand for this cereal is supplied by imports (FENALCE, 2014). The National Federation of Cereal and Legume Growers (FENALCE) has research programs for the development of tropical maize hybrids in order to increase the competitiveness of yellow maize in four regions across the country, as well as projects that promote the use of maize in combination with other crops and technical support for areas with unfavorable climatic conditions. There are, however, no known initiatives to obtain drought tolerant varieties in Colombia. In other countries, both public and private sector programs have advanced in conferring abiotic stress resistance to maize using conventional breeding, molecular markers, phenotypical characterization and transgenesis. Public sector efforts using genetic engineering are scarce and have reached only an exploratory stage so far. Most resources for research in this area come from the private sector (Edmeades, 2013). Maize event MON

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87460, Droughtgard™, from Monsanto, was the first genetically modified (GM) maize to be commercially planted in the United States with an initial planted area of 50 000 Ha in 2013. In 2014, this area increased to 275 000 Ha. In cooperation with BASF and Evogene (an Israeli company specialized in computational genomics), Monsanto continues searching for and validating genes that confer drought tolerance (Edmeades, 2013). The event MON 87460 was donated to the "Water Efficient Maize for Africa" (WEMA) project, a public-private association. The WEMA project aims to provide maize hybrids with insect resistance and drought tolerance to South Africa, Kenya, Uganda, Mozambique and Tanzania. Drought tolerance in general can reduce water requirements in irrigation systems and has the potential to increase crop yields in dry areas, as well as in dryland farming systems by reducing the negative effects derived from sporadic drought (Edgerton, 2009); therefore, it is expected to have great impact on sustainable crop systems, in particular in developing countries where drought will probably be more severe and prevalent than in industrialized countries (James, 2014).

One major obstacle in access to drought tolerance technologies which have been proven are patents that protect the different components involved, such as elements of expression cassettes, genetic transformation protocols, and media and conditions for *in vitro* plant regeneration (Hincapié and Chaparro-Giraldo, 2014).

To overcome this issue it is necessary to identify reported genes that confer drought resistance and,among them, select those with a greater freedom to operate (FTO) through a detailed analysis of the patents and patent applications involved. This process also entails a computer-based design of expression cassettes, the identification of genetic regulatory elements required to obtain GM lines with drought resistance, and their respective FTO analysis.

The genes selected here for the design of expression cassettes and their introduction into Colombian maize genotypes have been extensively studied and therefore can be expected, on a scientific base, to confer drought resistance and to have the maximum FTO possible.

Materials and methods

Drought tolerance genes search and selection

Genes used in this analysis were selected from scientific literature based on the relevance of the studies and their results. Nucleotide and amino acid sequences were obtained from patent databases (https://www.lens.org/lens/) and from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/gene). In total, 105 papers reporting transgenesis to confer drought tolerance to model plants or cultivated species, including maize, were considered. Papers published between 1995 and 2013 were accessed through the National University of Colombia database, mainly using Academic Search Complete, Science Direct, Springer Journal, Journal Storage and Nature.com. Genes were sorted into four groups according to their biological function in relation to drought tolerance: i) osmoprotectants, molecular chaperones and metabolites; ii) stress response transcriptional regulators; iii) post-translational modifications (phosphorylation/dephosphorylation, farnesylation); and iv) detoxification systems and genetic control. A data base was constructed considering the source organism from which the drought tolerance genes were derived, the organisms transformed by genetic engineering or target organisms, gene name, codified protein, transformation vector, and promoter region. Information concerning physiological parameters for the evaluation of drought tolerance such as germinating capacity under drought conditions or their equivalent (in soil or *in vitro*), cell damage, photosynthetic activity, osmotic potential, phenology and yield was also included, as well as a brief description of the most relevant aspects of each study, its authors and year of publication.

Freedom to operate analysis

Once the components were selected, an FTO analysis was carried out for all the elements of the expression cassettes and for the pCAMBIA 1301 transformation vector in order to determine which of them can be used freely or require some sort of licensing from the technology developers or patent holders if commercial transgenic lines are set to be developed. For the patents, their respective number was recorded, as well as the date of issue and the name of the patent holder. The claims were analyzed for the object or innovation subject of protection. Patents were searched for mainly in three databases: The Lens (https://www.lens.org/lens/), which specializes in plant genetic engineering; PATENTSCOPE, from the World Intellectual Property Organization (WIPO, http://www.wipo.int/patentscope/en/), which contains applications or issued patents in several countries through the Patent Cooperation Treaty (PCT); and the database from the Colombian Superintendence of Industry and Commerce (SIC) (http://serviciospub.sic.gov.co/~oparra/serv_57/externas/datospatente.php), which contains information on applications or granted patents in the country.

Results and discussion

Selected drought tolerance genes

The overexpression of genes that code for transcription factors (TFs) has shown positive results in conferring drought tolerance in GM plants. Some TFs regulate several genes located downstream in signaling cascades involved in stress response, so their manipulation has effects over different genes at the same time. This can also cause unwanted effects due to other biological processes that may be regulated by the same TFs. The most commonly used TFs in transgenic-induced drought tolerance are DREB/CBF and zinc-finger proteins (ZFP252, WRKI, ZPT2-3, SAP, STZ, AZF2 , ZAT10 and others). Other TFs or transcription activators such as AP37 (Oh *et al*., 2009), AREB1 (Kang *et al*., 2002; Fujita *et al*., 2005), NAC (Hu *et al*., 2006), HARDY (Karaba *et al*., 2007), MYB (Ding *et al*., 2009), and NF-Y (Nelson *et al*., 2007; Li *et al*., 2008) have been used to increase drought tolerance through genetic engineering.

TFs from the DREB/CBF family modulate a group of genes that define the main metabolic pathways related to stress tolerance (Long and Ort, 2010). Different types of DREB have been described, and their role in stress response seems to be different too. Expression of DREB1 is strongly regulated by low temperature, while that of DREB2 responds more specifically to drought, salt (Liu *et al*., 1998) and heat (Sakuma *et al*., 2006b). Even though DREB2 has been described to play an important role in drought-related gene expression, DREB2 from *Arabidopsis*, which is a member of group IV from the DREB/CBF subfamily, is only a weak inductor of downstream genes when overexpressed and causes a minimum increase in drought tolerance, apparently due to post-translational modifications or its rapid degradation by proteasome 26S (Qin *et al*., 2008). Nonetheless, the DREB2 protein can be made constitutively active through the deletion of specific residues, in which case the overexpression of DREB2ACA significantly increases drought tolerance in *Arabidopsis*, despite a delay in growth, an effect that has also been found by the constitutive expression of other DREBs and its association to delayed growth (Sakuma *et al*., 2006a). Overexpression of DREB1A improves cold- and dehydration-stress tolerance, while DREB2ACA improves dehydration tolerance and only slightly cold tolerance in transgenic plants (Nakashima *et al*., 2014). In contrast with these results, constitutive ectopic expression, or stress-induced expression of maize ZmDREB2A (Qin *et al*., 2008) and soybean GmDREB2 (Chen *et al*., 2008) in A*rabidopsis* results in better drought, heat and salt stress tolerance in transgenic plants, without growth defects. This suggests that ZmDREB2A and GmDREB2 do not require modification (Yang *et al*., 2010). Barley plants transformed independently with TaDREB3 and TaDREB2 and evaluated for drought and frost tolerance showed similar results (Morran *et al*., 2011). A transgenic population constitutively overexpressing these factors showed slow growth, a delay in flowering and less grain yield than their untransformed counterparts. However, plants transformed with either of these two genes showed better survival than control plants under severe drought. This study also evaluated the expression of TaDREB2 and TaDREB3 in well-hydrated plants and under different types of stress. Under no biotic stress, their expression was low, while they were highly induced by drought, especially TaDREB2; cold conditions only activated them weakly; TaDREB2 was strongly activated in seeds by mechanical damage; no activation was detected by saline stress; and TaDREB3 was weakly induced in leaves by ABA (Morran *et al*., 2011). Taking all these results into account, TaDREB2 was chosen for the design of an expression cassette using the drought-inducible promoter rab17 (Al-Abed *et al*., 2007) to avoid the deleterious effects described by Wang *et al.* (2003).

A second selected gene is ZAT10, which acts as a transcriptional regulator and seems not to cause any negative effects in transformed plants that express it constitutively. ZAT10 is a member of a group of transcriptional suppressors from the zinc finger gene family C2H2, which have a characteristic ERF-associated amphiphilic repression (EAR) motif and appear to have a key role in modulating plant response to abiotic stress (Meissner and Michael, 1997; Englbrecht *et al*., 2004; Ciftci-Yilmaz and Mittler, 2008). Zinc finger proteins play a critical role in many cellular functions, including transcriptional regulation, RNA binding, apoptosis regulation and protein-protein interactions. A study by Xiao *et al*. (2009) thoroughly evaluates drought resistance in transgenic rice (var. Zhonghua 11) under field conditions using plants independently transformed with seven genes that had been previously documented for their relation to drought tolerance (CBF3, SOS2, NCED2, NPK1, LOS5, ZAT10 and NHX1), under the control of the Actin 1 constitutive promoter and the stress-inducible promoter of a rice HVA22 homolog. The main criteria to evaluate the performance of plants and their tolerance to drought were their relative yield and the relative fertility of the panicle. In general, plants from transgenic families with the constructs HVA22P:CBF3, HVA22P:NPK1, Actin1:LOS5, HVA22P:LOS5, Actin1:ZAT10, and HVA22P:ZAT10 showed significantly higher yield and fertility than untransformed plants, while transgenic families with the remaining four constructs (Actin1:SOS2, Actin1:NCED2,

HVA22P:NCED2, and HVA22P:NHX1) showed no drought tolerance according to the criteria used. Genes LOS5 and ZAT10 showed better effects than the other five, and no negative phenotypic effects were reported when ZAT10 was constitutively expressed. The product of this gene acts in the stress signaling cascades, downstream from DREB (Yang *et al*., 2010), apparently regulating the expression of genes involved in the ROS defense system, mainly ascorbate peroxidase 2 (APX2) and ferro-superoxide dismutase (FSD1) (Mittler *et al*., 2006).

The third selected gene is CspB, which codes for a cold-shock induced protein involved in cellular adaptation to low temperature in *Escherichia coli* and *Saccharomyces cerevisiae* and has also been identified in *Bacillus subtilis* (Willimsky *et al*., 1992). Cold-shock proteins are nucleic acid-binding chaperones that are induced to stabilize mRNA after a significant drop in temperature (Mazzon *et al*., 2012). The transcriptome from *B. subtilis* was analyzed by Kaan *et al.* (2002) using DNA macrochips at different times after a temperature drop from 37 to 18°C, and they found a cold-shock induced increase in the mRNA levels of genes CspB, CspC and CspD. The gene CspB was used in the first drought tolerant GM crop that was commercially released in the United States in 2013 by Monsanto, the event MON 87460 or Droughtgard™ maize, which provides strong empirical evidence of its effectiveness.

The three genes selected for the FTO analysis, CspB, DREB and ZAT10, come from B. subtilis, wheat (*Triticum aestivum*) and *Arabidopsis thaliana*, respectively. The criteria used to select them were: i) strong empirical evidence of their role in drought tolerance according to physiological parameters evaluated in vitro, in greenhouse or in the field; ii) yield values measured in field studies for crops; iii) gene products that o not act in early stages of stress signaling cascades and absence of undesired pleiotropic effects; and iv) availability of recent studies.

Expression cassettes were designed *in silico*. In general terms, they were composed of a promoter sequence, an open reading frame (ORF) and a 3' untranslated terminator region, which, in eukaryotes, contains a polyadenylation site (Cai *et al*., 2008). The following constructs with the genes CspB, ZAT10 and DREB were designed (Tab. 1): i) CspB with an enhanced 35S Cauliflower Mosaic Virus (CaMV E35S) promoter and a petunia leader sequence (Pleader), ii) ZAT10 with the Zea mays ubiquitin 1 gene promoter region (Ubi-1), and iii) DREB with the drought-inducible promoter rab17 followed by a Guanine to adjust the reading frame. For CspB and DREB the RuBisCo terminator sequence (Trub) was used and for ZAT10 the nopaline synthase terminator sequence (Tnos). Design of the expression cassettes was done using Gene Designer 2.0 (https://www.dna20.com/resources/genedesigner) (Villalobos *et al*., 2006). As a transformation vector, the plasmid pCAMBIA 1301 (http://www.snapgene.com/resources/plasmid_files/plant_vectors/pCAMBIA1301/) was selected.

Freedom to operate analysis

A proper FTO analysis reduces the risk of infringing third-party rights and possible future lawsuits, which can

TABLE 1. Elements of the constructs designed to confer drought resistance to maize. # ID, corresponds to the GenBank accessions for the sequences.

Construct		Promoter	Drought tolerance gene		Terminator	
CSPB		E35S+Pleader	*CspB*			Trub
	# ID	HC051893.1, sequence 28 from patent WO2009126896	**Gene # ID and size**	X59715.1 (660bp)		
			ORF size	204 bp	**# ID and size**	HC510453 (643bp) sequence 43 from patent WO2010036946
	Size	1156bp	**Protein # ID and size**	CAA42235.1 (67 A.A)		
DREB		rab17+PLeader	*TaDREB*			Trub
	# ID	JA780212.1 sequence 1 from patent EP2421979 or from patent US20120102592	**Gene # ID and size**	DQ35385.(1004bp)		
			ORF size	675bp		
	Size	628bp	**Protein # ID and size**	ABC86564.1 (224 A.A).	**# ID and size**	HC510453 (643bp) sequence 43 from patent WO2010036946
ZAT10		Ubi1	AtZaT10			Tnos
	# ID	JX947345.1	**Gene # ID and size**	X98671.1 (1231 bp)		
			ORF size	684 bp		
	Size	2015bp	**Protein # ID and size**	CAA67229.1 (227A.A)	**# ID and size**	HC510454.1 (253bp) sequence 44 from patent WO2010036946

be expensive and may even impede the commercialization of an innovation (Kowalski, 2002; Wolff, 2008). Some authors argue that doing an FTO analysis from the onset of a research project allows developers to plan intellectual property (IP) rights management strategies, which can lead to the redesign of expression cassettes, negotiate royalty-free licenses for humanitarian purposes, negotiate individual licenses or the formation of consortia (Kryder *et al*., 2000; Chi-Ham *et al*., 2010). Several aspects need to be taken into account for an FTO analysis: patents are issued for specific jurisdictions, there are no international patents; regulation on what can and cannot be patented may vary from one country to another; patents expire after a certain time or if a maintenance fee is not payed; there are exceptions and limitations for certain uses of patents; and patents from different countries may have a varying range of claims (Hincapié and Chaparro-Giraldo, 2014). An FTO analysis must be periodically updated as at a given time there may be applications under study that are not yet published. In analyzing a patent, special attention must be given to the claims, as they define the scope of the protection and the rights of the patent holder (Hincapié and Chaparro-Giraldo, 2014).

Four granted patents and one PCT application for the elements of the expression cassettes designed for drought tolerance were found in the databases. One of them (US5510474A) protects the Ubi1 promoter and expires in 2016, as 20 years have passed since its initial application. The remaining three (US7,786,353B2; US20120102592 and US7663025 B2) are more recent and should expire in 2030.

Only one patent application through PCT was found (PCT/US2013/036011 WO2013158442 A1), which was also submitted in Colombia on November 12th 2014 (SIC file 14-250283) and refers to plant regulatory elements and uses thereof. This invention "provides DNA molecules and constructs, and their nucleotide sequences, useful for modulating gene expression in plants". It also claims the "transgenic plants, plant cells, plant parts, and seeds comprising the DNA molecules operably linked to heterologous transcribable polynucleotides", and methods of their use (SIC file 14-250283). The PCT patent application (PCT/US2013/036011) presents a "transgene selection cassette used for selection of transformed plant cells that confers resistance to the herbicide glyphosate driven by the CaMV 35S promoter, EXP- CaMV.35S: 1 : 1 (SEQ ID NO: 15), and a left border region from *A. tumefaciens*. The resulting plasmid was used to transform corn plants." The claims protect any transformed plant (monocotyledonous or dicotyledonous plant cells): "comprising a heterologous DNA molecule while comprising a sequence selected from the group consisting of: (a) a sequence with at least about 85 percent sequence identity to any of SEQ ID NOs: 1, 2, 3, 4, 6, or 8; (b) a sequence comprising any of SEQ ID NOs: 1, 2, 3, 4, 6, or 8; and (c) a fragment of any of SEQ ID NOs: 1, 2, 3, 4, 6, or 8, wherein the fragment has gene- regulatory activity, wherein said sequence is operably linked to a heterologous transcribable polynucleotide molecule." (PCT/US2013/036011)

The patent application in Colombia (SIC file 14-250283) apparently does not include the EXP CaMV35S or the E35S+Pleader sequences used in the design of the CspB expression cassette, because, even though they are mentioned in the field of the invention as SEQ ID NO:15, they are not included in the claims. The claims do include a sequence referred to as 3' UTR region T-AGRtu.nos-l : l : 13 (SEQ ID NO: 10) which could affect the ZAT10 expression cassette. However, when the sequence used in the design of the expression cassette is compared to the sequence in the patent application by a Blastn (Zhang *et al*., 2000), no significant identity is found. This is also the case when the sequence E35S+Pleader is compared to SEQ ID NO:15.

There were no other patent applications and no issued patents in Colombia (SIC) that relate to the elements of the expression cassettes that may confer drought resistance (Tab. 2).

Conclusions

Carrying out a FTO analysis is a key part of research and design of a technological innovation with the possibility of reaching a commercial stage. Here, we show the design of expression cassettes with genes that have the potential to confer drought tolerance for maize in Colombia. Most of the elements of the expression cassettes that were designed are protected by patents, but not in Colombia. Only one PCT patent application was found (PCT/US2013/036011 WO2013158442 A1), which is under study in the country (SIC file 14-250283) and corresponds to regulatory elements that may cover the promoter of the CspB and the T-NOS terminator region of the ZAT10 expression cassettes. An analysis of the claims in this application and a comparison of the nucleotide sequences through a Blastn reveal that they do not cover the sequences used in the design of the expression cassettes.

A preliminary FTO analysis of the elements of the designed expression cassettes shows that, although most are protected in other jurisdictions, in Colombia there are no

TABLE 2. Summary of the FTO analysis of the elements of the expression cassettes that may confer drought resistance.

Element	Patent / application number	Application date	Issue date	Assignee	PCT applications	Status in Colombia
Ubi1 promoter	Patent US5510474A	25/08/1994	23/04/1996	Mycogen Plant Science, Inc., San Diego, Calif.	WO1999043838 ó PCT/ US1999/003863 NOT IN COLOMBIA	NO patent NO application
CspB y E35S+Pleader	Patent US7786353B2	29/10/2004	31/09/2010	Monsanto Technology LLC, St. Louis, MO (US)	EP1668141 ó WO/2005/033318 or PCT/US2004/031856 COLOMBIA	NO patent NO application
EXP CaMV35S and leader	US20130283478 application	24/10/2013		Monsanto Technology LLC, St.	PCT/US2013/036011 WO2013158442 A1 COLOMBIA	Application under study, file 14-250283
TaDREB and RAB17 promoter	Patent US20120102592	23/04/2010	26/04/2012	Australian Centre For Plant Functional Genomics Pty Ltd	WO2010/121316 or PCT/ AU2010/000460 COLOMBIA	NO patent NO application
ZAT10 (G545)	Patent US7663025B2	15/05/2006	16/02/2010	Mendel Biotechnology, Inc., Hayward CA (US)	WO2005047516 or PCT/ US2004/037584 COLOMBIA	NO patent NO application

granted patents or pending requests that may restrict their use. As patent rights are strictly territorial, this analysis suggests that it is therefore feasible to continue with the research process in order to obtain commercial transgenic maize lines with drought tolerance for the country without infringing third-party rights.

This FTO analysis reduces the risk of infringing an existing patent during research activities and the eventual commercialization of drought-tolerant maize, an important step in this type of development (Hincapié and Chaparro-Giraldo, 2014). It is also important to consider the legal and IP landscape as dynamic and evolving, so FTO analyses must be continuous in time and their results should be validated and updated periodically. Change can come from expired, published, invalidated or reassigned patents; licenses can be issued or terminated (Kowalski, 2002).

This preliminary analysis of the IP status of the selected genes and all related elements in the designed expression cassettes suggests that they can be used in Colombia. Commercial development of GM crops from domestic varieties by public-sector institutions or universities is enabled and could be successful considering that tested inventions are used and adapted to specific needs. These inventions may be protected, but have no current valid patents in Colombia or have soon-to-expire patents. If this were not the case, however, patents can be licensed.

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