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Food safety assessment of an antifungal protein from *Moringa oleifera* seeds in an agricultural biotechnology perspective

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ABSTRACT

Mo-CBP$_3$ is an antifungal protein produced by *Moringa oleifera* which has been investigated as potential candidate for developing transgenic crops. Before the use of novel proteins, food safety tests must be conducted. This work represents an early food safety assessment of *Mo*-CBP$_3$, using the two-tiered approach proposed by ILSI. The history of safe use, mode of action and results for amino acid sequence homology using the full-length and short contiguous amino acids sequences indicate low risk associated to this protein. *Mo*-CBP$_3$ isoforms presented a reasonable number of alignments (>35% identity) with allergens in a window of 80 amino acids. This protein was resistant to pepsin degradation up to 2 h, but it was susceptible to digestion using pancreatin. Many positive attributes were presented for *Mo*-CBP$_3$. However, this protein showed high sequence homology with allergens and resistance to pepsin digestion that indicates that further hypothesis-based testing on its potential allergenicity must be done. Additionally, animal toxicity evaluations (e.g. acute and repeated dose oral exposure assays) must be performed to meet the mandatory requirements of several regulatory agencies. Finally, the approach adopted here exemplified the importance of performing an early risk assessment of candidate proteins for use in plant transformation programs.

1. Introduction

Fungi are responsible for several diseases that attack plants causing damages in various important crops, decreasing their productivity (Mandal et al., 2013; Labandeira and Prevec, 2014). Furthermore, the chemical control used as strategy in combating fungal diseases is associated with toxic and hazardous effects on the environment and nontarget organisms (Meng et al., 2010; Marei et al., 2012; Yadav et al., 2013). Modern strategies to confront this problem include conventional plant breeding, which is conceptually supported by crossing of plants with desired characteristics, and genetic engineering, which aims to obtain transgenic plants expressing defined characteristics (Qaim, 2010; Zhang et al., 2010; Wang et al., 2014).

Significant efforts have been directed towards the identification of antifungal proteins that can be used for producing crops resistant to pathogens. Despite the importance of this issue, no commercial transgenic plant is available with genes that encode proteins to confer resistance against phytopathogenic fungi. On the other hand, several studies have already described the efficacy of antifungal proteins when processed on different host plants, either in laboratory or greenhouse conditions (Lacerda et al., 2014).

Moringa oleifera Lamarck is a native plant from northwest India, with distribution in various parts of the world, mainly in the tropics. *M. oleifera* is known as a multipurpose tree since it has been widely used as food and feed, as well as in the traditional medicine and industry (Ramachandran et al., 1980; Anwar et al., 2007; Ben Salem and Makkar, 2009; Kumar et al., 2010). From the seeds of this plant species, a chitin-binding protein, *Mo*-CBP$_3$ (*M. oleifera* – Chitin-Binding Protein), has been purified. *Mo*-CBP$_3$ possesses a broad spectrum of activity against important phytopathogenic fungi, such as *Fusarium oxysporum*, *Fusarium solani*,

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Rhizoctonia solani, *Colletotrichum gloesporioides* and *Colletotrichum musae* (Gifoni et al., 2012). As to its structural attributes, *Mo*-CBP$_3$ is a glycoprotein with apparent molecular mass of 18.0 kDa, presenting multiple heterodimeric isoforms composed of two different polypeptide chains linked by disulfide bonds. It is a highly stable protein, maintaining its secondary structure and antifungal activity at extremes of temperature and pH (Batista et al., 2014). In this context, *Mo*-CBP$_3$ protein may represent a promising tool for use in the development of transgenic crops resistant to phytopathogenic fungi. Indeed, the Embrapa (Brazilian Agricultural Research Corporation) group has considered the inclusion of this protein in its plant transformation programs. However, this company in a partnership with our team has agreed to perform early food safety assessments of candidate proteins before their introduction into crops. This could avoid the use of inappropriate protein for costly activities involved in the production of transgenic plants as well as may help to guide modifications in the protein structure in order to free it of its potential risks. Current strategies for food safety assessment of candidate proteins are essentially based on the guidelines of FAO and WHO compiled in the second edition of the Codex Alimentarius document (Codex Alimentarius, 2009).

The International Life Sciences Institute (ILSI) proposed a well-accepted alternative guideline (Delaney et al., 2008). It is a two-tiered evaluation, based on weight of evidence, to assess the safety of novel proteins. The steps consist of potential hazard identification of the protein (Tier I) and hazard characterization (Tier II), performed when the results of the first stage are not sufficient to define safety. Tier I includes an assessment of the biological function or mode of action and intended application of the protein, history of safe use, comparison of the amino acid sequence of the protein to other proteins, biochemical and physicochemical properties, as well as the expression level and dietary intake of the recombinant proteins. Tier II comprises acute and repeated dose toxicological studies and/or hypothesis-based testing. In fact, the ILSI guideline is a more flexible approach because it takes into account all data obtained in a holistic way. The predictive value of each piece of evidence should be well understood in order to give some data more 'weight' than others during the assessment, adding greater confidence in overall evaluation (Delaney et al., 2008).

Although the two-tiered approach, based on weight of evidence, proposed by ILSI has been initially planned for proteins in a stage of the development of the transgenic crop where the protein expression levels can be estimated, we also believe on their suitability for the safety assessment of new proteins whose use in plant transformation is still being analyzed. Thus, the present study aimed to perform an early food safety assessment of the antifungal protein *Mo*-CBP$_3$ in an agricultural biotechnology perspective, following this approach. This study allowed us to gather information about the *Mo*-CBP$_3$ risks that were crucial to formulate decisions on the immediate use of this protein for plant transformation.

2. Material and methods

2.1. M. oleifera seeds

To obtain the protein of interest, *Mo*-CBP$_3$, *M. oleifera* pods were collected from trees in the Pici Campus at the Federal University of Ceará (Fortaleza, Brazil). A voucher specimen was deposited in the Prisco Bezerra Herbarium (Fortaleza, Brazil), at this same Institution, under number EAC34591. The seeds were then separated from the pods and kept in plastic containers at room temperature until the moment of analysis.

2.2. Mo-CBP$_3$ preparation

Mo-CBP$_3$ was purified from crude extract of *M. oleifera* mature seeds as well described previously by Gifoni et al. (2012). Briefly, the crude extract was extensively dialyzed against Milli-Q grade water and $(NH_4)_2SO_4$ added to the soluble material, denoted as the albumins, to yield 90% saturation. Albumin fraction was dissolved in and dialyzed against the extracting buffer and applied to a chitin column that had been equilibrated with the same buffer. After elution with the starting buffer of the unbound proteins from the column, the chitin-bound proteins, named P_{NAG} and P_{AC}, were eluted with 0.1 M *N*-acetyl-D-glucosamine (NAG) that was prepared in the extracting buffer and with 0.05 M acetic acid (pH 5.0), respectively. The P_{NAG} sample was dialyzed against 0.1 M acetic acid and distilled water, lyophilized and applied to a cation-exchange matrix (Resource S™) that had been previously equilibrated with 0.05 M sodium acetate buffer, pH 5.2. Four major adsorbed protein peaks (*Mo*-CBP$_2$, *Mo*-CBP$_3$, *Mo*-CBP$_4$, and *Mo*-CBP$_5$) were recovered after being selectively desorbed by stepwise elution with 0.4, 0.5, 0.6, and 0.7 M NaCl, respectively, included in the equilibrium buffer.

The production of *Mo*-CBP$_3$ was monitored through quantification of soluble proteins by Bradford method (Bradford, 1976), using a curve constructed with bovine serum albumin as standard. The purification of the protein was monitored by 17.5% SDS-PAGE (Laemmli, 1970). Briefly, *Mo*-CBP$_3$ (1 mg/mL) was mixed to 2× sample loading buffer [62.5 mM Tris–HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue] (1:1, v/v) and incubated at 100 °C for 5 min. Then, 2.5 μg of protein/well were run at 20 mA/gel for 1 h. Protein bands were visualized by coomassie brilliant blue R-250 staining.

From the SDS-PAGE profile of *Mo*-CBP$_3$, the percentage of relative purity of the protein was calculated using the Image Master 2D platinum (v.7.0, GE Healthcare) software. This methodology has been adopted by our team, and it is also widely used by chemical companies to show the purity of commercialized proteins.

Immediately after the production of the *Mo*-CBP$_3$ and up to 72 h before its use in the *in vitro* and *in vivo* tests, batches of the protein were checked to verify the maintenance of their bioactivity against fungi according to the methodology described by Gifoni et al. (2012).

2.3. History of safe use of M. oleifera species

A literature review on the history of safe use (HOSU) of *M. oleifera* species, source of the *Mo*-CBP$_3$ protein, was performed according to the principles described by Constable et al. (2007). For that search, the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) was accessed, using the following combination of keywords: (a) "*Moringa oleifera*" and "food safety"; (b) "*Moringa oleifera*" and "risk assessment"; (c) "*Moringa oleifera*" and "toxicity"; (d) "*Moringa oleifera*" and "nutrition"; (e) "*Moringa oleifera*" and "review"; and (f) "*Moringa oleifera*".

2.4. Amino acid sequence similarity

From cDNA sequences (GenBank accession numbers KF616830 until KF616833) coding for *Mo*-CBP$_3$ were deduced the amino acid sequences of four isoforms of this protein referred to as *Mo*-CBP$_3$-1, *Mo*-CBP$_3$-2, *Mo*-CBP$_3$-3, and *Mo*-CBP$_3$-4 (Freire et al., 2015). These sequences were used in a FASTA format without the signal peptide and compared with all protein sequences deposited in seven large reference public databases: NR, Refseq_Protein, SwissProt, PDB, Env_nr (http://www.ncbi.nlm.nih.gov/), UniProt SwissProt (http://www.uniprot.org/) and Uniprot-trEMBL (http://www.expasy.org/). The algorithm used was BLASTP 2.2.29+ and the scoring matrix was BLOSUM62. No keyword was used to limit the search. Specific

details of the alignment was carefully checked (e.g. *E*-value < 0.01, shared identity >50%, the size of the alignment and frequency of gaps ≤6%) to determine the significance of any similarity found, since there is no established criterion for search of toxic and/or antinutritional proteins (Hérouet-Guicheney et al., 2009). All comparisons were run in September 15–29, 2014.

An *in silico* search was performed to assess sequence similarities of *Mo*-CBP$_3$ isoforms with any known allergenic proteins using criteria established by FAO/WHO (2001). The four sequences were compared to those of allergens deposited in the following databases: (1) Structural Database of Allergenic Proteins (SDAP) (http://fermi.utmb.edu/SDAP/), updated in February 2013; (2) Allergen Database for Food Safety (ADFS) (http://allergen.nihs.go.jsp), updated in February 2014; (3) AllergenOnline version 14 database (http://www.allergenonline.com/), updated in January 2014; (4) Allermatch™ (http://allermatch.org/), updated in August 2012. The *Mo*-CBP$_3$ sequences were subjected to FASTA comparisons using as filter *E*-value cutoff of 0.01 for detection of identity >50% for the full-length sequence similarity, > 35% in a window of 80 amino acids, and short sequences of eight, seven or six contiguous amino acids identical to those of known allergenic proteins. To assess the relevance of immune peptides (8, 7 or 6 amino acids) of *Mo*-CBP$_3$, a further search in the epitope (immunogenic determinants) database of the ADFS was conducted. All comparisons were run in September 15–29, 2014.

2.5. Search of N-glycosylation sites

The full-length acid sequences of *Mo*-CBP$_3$ isoforms were analyzed for the presence of N-glycosylation sites by NetNGlyc 1.0 Server program (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.6. Mode of action and specificity

A literature review was conducted on the mode of action and specificity of *Mo*-CBP$_3$ following the recommendations of Delaney et al. (2008). The PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) was accessed, using the following combination of keywords: (1) "mode of action" and "*Mo*-CBP$_3$"; (2) "mode of action" and "*Mo*-CBP$_3$" and "specificity".

2.7. In vitro digestion

Susceptibility to *in vitro* digestion of *Mo*-CBP$_3$ was assessed by incubating this protein in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) (Sigma, USA) at a concentration of 0.5 mg/mL. The SGF is a mixture of 0.034 M NaCl, 0.7% HCl, pH 1.0–2.0, and 3.2 mg/mL of pepsin (Sigma P7012), while SIF is composed of 0.05 M potassium phosphate, pH 7.5 and 10 mg/mL of pancreatin (Sigma P7545), both prepared according to the recommendations of Roesler and Rao (2001). The mixture of *Mo*-CBP$_3$ in SGF or SIF was incubated at 37 °C, under constant agitation, and aliquots of 200 μL removed after 0, 15, 30, 60 s, 2, 5, 10, 20, 30 min, 1 and 2 h. The tests were run in triplicate in the different fluids. Bovine serum albumin (BSA) was used as a digestible protein control. The digestibility of the proteins in SGF and SIF was monitored by 17.5% SDS-PAGE and Western blot analyses using anti-*Mo*-CBP$_3$ rabbit polyclonal antibodies (1:10,000).

3. Results

3.1. Mo-CBP3 preparation

The protein yield of *Mo*-CBP$_3$ preparations used in the present study was 10.89 ± 1.65 mg protein/g defatted seed flour. The batches were homogeneous and free of contaminants as shown by SDS-PAGE. A single protein band with an apparent molecular mass of 18.0 kDa, under non-reducing condition, was observed. This protein when treated with 2-mercaptoethanol migrated as two protein bands with apparent molecular masses of 4.1 and 8.1 kDa. To be sure that the protein remained active after all purification steps performed, *Mo*-CBP$_3$ protein batches were assessed for their bioactivity against fungi. All protein samples presented activity against *F. solani* spores in the expected magnitude. The SDS-PAGE protein profile and antifungal activity data confirmed the findings previously reported by Gifoni et al. (2012). Additionally, the purity of the produced protein was calculated as >99.9%.

3.2. History of safe use of M. oleifera species

The scientific literature search using a series of keyword combinations on the HOSU of *M. oleifera* species revealed a large number of reports for all parts (leaves, seeds, bark, roots, sap, and flowers) of this widely used medicinal plant and source of food products. As recently reviewed by Stohs and Hartman (2015), a considerable number of human and animal as well as *in vitro* studies indicate that various preparations of *M. oleifera* leaves and other plant parts have demonstrated a very high degree of safety. On the other hand, some reports on adverse effects of *M. oleifera* isolated compounds or preparations have been described in animal or *in vitro* studies, such as effects in female reproductive system (Sethi et al., 1988; Shukla et al., 1988; Prakash et al., 1987), geno- and cytotoxic activities (Villasenor et al., 1989; Rolim et al., 2011; Asare et al., 2012; Araújo et al., 2013), hypotensive effects (Faizi et al., 1994, 1998), source of antinutritional factors (Igwilo et al., 2013, 2014), hepatic and renal damage (Oyagbemi et al., 2013), and acidosis (Omabe et al., 2014). In parallel to the scientific data, for hundreds of years several parts of *M. oleifera* have been consumed by humans and animals in many tropical and subtropical countries with no reports of toxic effects (Fahey, 2005; Thurber and Fahey, 2009).

3.3. In silico analysis

In silico analysis of the amino acid sequences of *Mo*-CBP$_3$ isoforms revealed no significant similarity with sequences of any known toxic, antinutritional and/or allergenic protein deposited in seven general protein databases. Likewise, the comparison of the full-length sequences of all isoforms of *Mo*-CBP$_3$ with protein sequences deposited in the ADFS, Allermatch™, AllergenOnline and SDAP allergen databases showed no identity >50% (Table 1). On the other hand, the *Mo*-CBP$_3$ isoforms showed a reasonable number of significant alignments (>35% identity) when a window of 80 amino acids was applied as search parameter in the same allergen databases (Table 1). Table 2 shows minutely all identified hits for each *Mo*-CBP$_3$ isoform. Many well-known allergens were identified such as Sin a 1 from *Sinapis alba*, Ber e 1 from *Bertholletia excelsa*, Ric c 1/3 from *Ricinus communis* and Cor a 14 from *Corylus avellana*, and among the different isoforms the identities detected throughout the databases accessed varied from 35 until 46.25%. The search for identity with eight contiguous amino acids revealed no identity of *Mo*-CBP$_3$ with any known allergen (Table 1). However, identity of seven contiguous amino acids with known allergens was found for two isoforms of *Mo*-CBP$_3$ (*Mo*-CBP$_3$-2 and *Mo*-CBP$_3$-3) in ADFS, Allermatch™ and SDAP databases. Evidences of identity with short sequences of six contiguous amino acids were found for the four isoforms of this protein in ADFS and Allermatch™ database. In the SDAP database, identity with six contiguous amino acids was found only for *Mo*-CBP$_3$-2 and *Mo*-CBP$_3$-3 isoforms (Table 1). In fact, the various peptides of six contiguous amino acids account only for six

Table 1
Search of similarity between the full-length amino acid sequence of the four isoforms of *Mo*-CBP$_3$ and known allergenic proteins in specific databases.[a].

Database	*Mo*-CBP$_3$ isoforms											
	Mo-CBP$_3$-1			*Mo*-CBP$_3$-2			*Mo*-CBP$_3$-3			*Mo*-CBP$_3$-4		
	CS[b]	80 aa[c]	6/7/8 aa[d]	CS	80 aa	6/7/8 aa	CS	80 aa	6/7/8 aa	CS	80 aa	6/7/8 aa
AllergenOnline	0	15	[e]/0	0	19	[e]/0	0	24	[e]/0	0	16	[e]/0
ADFS	0	6	4/0/0	0	4	8/4/0	0	7	8/4/0	0	5	4/0/0
Allermatch™	0	7	3/0/0	0	11	2/1/0	0	9	2/1/0	0	8	3/0/0
SDAP	0	1	0/0/0	0	5	2/1/0	0	2	1/1/0	0	2	0/0/0

[a] The results are represented as number of similar sequences found in accordance with the specifications for each parameter analyzed.
[b] The search was done considering the complete sequence (CS) of each protein isoform. To be relevant the identity should be greater than 50% (Aalberse, 2000).
[c] To be considered relevant the identity must be greater than 35%, on a window of 80 amino acids (Codex Alimentarius, 2009).
[d] To be considered relevant identity must be 100%, on sequences of 6, 7 or 8 contiguous amino acids.
[e] In this database it is not possible to do a search for 6 or 7 contiguous amino acid sequences.

distinct peptides ("QQQQGQ", "DEVDEI", "RCCQQL", "CCQQLR", "CQQLRN" and "QQCRQQ"). Likewise, only two different peptides were found in the search with seven contiguous amino acids ("RCCQQLR" and "CCQQLRN"). In addition, the peptides found were not present in epitopes of any known allergenic protein after analysis in the ADFS.

The search for N-glycosylation sites indicated the absence of these consensus sequences in *Mo*-CBP$_3$ isoforms.

3.4. Mode of action and specificity

Studies on the mode of action of *Mo*-CBP$_3$ protein is still little explored since *Mo*-CBP$_3$ has just been discovered by our research team. The protein was shown to be able to cross and damage the plasma membrane and to inhibit the acidification of the medium induced by glucose in *F. solani* spores. This suggests its influence on proton pump (H^+-ATPase) present in cell membrane of the fungus responsible for maintaining intracellular pH and electrochemical gradient necessary for food absorption (Gifoni et al., 2012).

Regarding protein specificity, Gifoni et al. (2012) have also shown that the protein was inactive against the oomycete *Pythium oligandrum*, which has greater amount of cellulose in the cell wall rather than chitin, demonstrating so the specificity of this protein to chitin.

3.5. In vitro digestibility

Mo-CBP$_3$ protein showed high resistance to degradation by SGF, even after 2 h of incubation (Fig. 1A). However, when in contact with SIF the protein proved to be more susceptible to degradation, being totally hydrolyzed after 2 h of incubation (Fig. 2A). The protein was detectable by anti-*Mo*-CBP$_3$ antibody after SGF treatment (Fig. 1B), whereas it could not be detected after 1 h in SIF (Fig. 2B). The digestible protein control, BSA, was completely degraded in the first minutes of incubation in both fluids (data not shown).

4. Discussion

Genetic engineering techniques together with conventional plant breeding have become the main approach for improving important agronomic crops. The introduction of exogenous genes into plant genome through genetic engineering adds specific characteristics to these crops (Parrott et al., 2010; Gong; Wang, 2013; Hammond et al., 2013). In order to prevent any adverse effects on human health and other non-target animals, novel proteins should be rigorously tested for their food and feed safety. Ideally, the risk assessment should be performed at an early stage in the timeline of the development of genetically modified plant (Rice et al., 2007).

Several guidelines to investigate the safety of candidate proteins for the development of transgenic crops have been developed by international organizations. In this context, the antifungal protein *Mo*-CBP$_3$ was analyzed for food safety using a two-tiered approach, based on weight of evidence, proposed by ILSI (Delaney et al., 2008). This is a more flexible approach which contemplates different analysis and the results are interpreted holistically. Moreover, this evaluation is consistent with the recommendations provided by authorities and international organizations such as FAO/WHO and OECD (OECD, 1996; FAO/WHO, 2001; Codex Alimentarius, 2009).

The first step in the safety assessment includes a search on the history of safe use of the candidate protein. According to Constable et al. (2007), the history of safe use of traditional foods constitutes the reference point for the comparative safety assessment of novel foods and foods derived from GMO. In the case of proteins derived from plants, the latter becomes the source of comparison. Important factors in establishing a history of safe use include the exposure time of humans or animals to the food, the way the food was processed and its expected levels of ingestion, potential hazard associated with consumption and reports of animal and human exposure and its consequences. Regarding *M. oleifera* species, its leaves, pods, seeds and roots have historically been used as food by humans and animals in many parts of the world with no reports of adverse effects (Fahey, 2005; Thurber and Fahey, 2009). Another factor that reduces the risk associated with the ingestion of this species is its millennial use in traditional medicine and its incorporation in herbal formulations (Mehta et al., 2003; Goyal et al., 2007; Mbikay, 2012). Allied to that, its seeds (the source of *Mo*-CBP$_3$ protein) have been successfully used in developing countries as a natural coagulant in water treatment for human consumption (Katayon et al., 2006; Vieira et al., 2010; Mangale et al., 2012). Likewise, the scientific literature has accumulated a reasonable certainty about the safe use of *M. oleifera* preparations based on the results of human, animal and *in vitro* studies (Stohs and Hartman, 2015). As similarly described for other beneficial medicinal/food plant species, purified substances and extracts of different parts of *M. oleifera* were capable to cause some adverse effects detected by animal and *in vitro* tests (previously cited in the Results section). In general, the popular and scientific knowledge have pointed out for no or low toxic effects related to the ingestion of *M. oleifera* parts. However, further toxicological studies would be required to better clarify the synergistic effect of different constituents and their mode of action.

Another important aspect in the food safety assessment of a novel protein consists in comparing its primary amino acid sequence with allergenic, toxic and/or antinutritional proteins. As there is no recognized definition of a toxic and/or anti-nutritional protein based on its primary sequence, the comparison should be

Table 2
Identified hits (>35% identity) for *Mo*-CBP$_3$ isoforms in four allergen databases using as search parameter a window of 80 amino acids.

Mo-CBP$_3$ isoforms	Identified hit				
	Accession number	Description/Species	Identity (%)	*E*-value	Database
Mo-CBP$_3$-1	P80208	Napin 3/*Brassica napus*	45.03	4.4e-005	AllergenOnline
			35.20	9.2e-5	ADFS
			46.25	3.4e-07	Allermatch™
	P15322	Allergen Sin a 1/*Sinapis alba*	43.40	6.1e-008	AllergenOnline
			36.10	1.1e-7	ADFS
			40.24	1.4e-05	Allermatch™
			35.19	-[a]	SDAP
	AAK15088	Albumin 2S/*Sesamum indicum*	42.50	1.5e-008	AllergenOnline
	CAA62911.1	Allergen Sin a 1.0106/*Sinapis alba*	41.90	2.6e-007	AllergenOnline
	CAA62910.1	Allergen Sin a 1.0105/*Sinapis alba*	41.20	3.4e-007	AllergenOnline
	ACI41244.1	Albumin 2S/*Sesamum indicum*	41.20	3.4e-005	AllergenOnline
	CAA62912.1	Allergen Sin a 1.0107/*Sinapis alba*	41.20	2.2e-007	AllergenOnline
	CAA62909.1	Allergen Sin a 1.0104/*Sinapis alba*	41.20	9.3e-008	AllergenOnline
	CAA62908.1	Allergen Sin a 1.0108/*Sinapis alba*	40.70	3.7e-007	AllergenOnline
	P80207	Allergen Bra j 1-E/*Brassica juncea*	39.00	0.0058	AllergenOnline
			39.02	0.00014	Allermatch™
	P04403	2S Albumin Ber e 1/*Bertholletia excelsa*	37.54	0.0004	AllergenOnline
			41.30	1.7e-4	ADFS
	2LVF	2S Albumin Ber e 1 - Chain A/*Bertholletia excelsa*	37.54	0.00028	AllergenOnline
	P01089	2S albumin - Allergen Ric c 1/3/*Ricinus communis*	36.20	1.3	AllergenOnline
	Q7Y1C2	2S albumin seed storage protein/*Juglans nigra*	36.20	0.007	AllergenOnline
			35.00	0.00027	Allermatch™
	Q9AUD1	2S Albumin - Allergen Ses i 1/*Sesamum indicum*	41.00	2.4e-5	ADFS
			42.50	1e-07	Allermatch™
	B6EBI1	2S Albumin - Allergen Ses i ?/*Sesamum indicum*	40.20	5.9e-5	ADFS
	Q8GT96	Recombinant Ib pronapin/*Brassica napus*	36.10	–	AllergenOnline
			36.90	6.7e-4	ADFS
	Q42473	2S storage protein - Allergen Bra r 1/*Brassica rapa*	38.75	2.5e-10	Allermatch™
	D0PWG2	2S albumin - Allergen Cor a 14/*Corylus avellana*	35.00	0.00011	Allermatch™
Mo-CBP$_3$-2	AAK15088	Albumin 2S/*Sesamum indicum*	42.50	1.6e-005	AllergenOnline
			40.31	–	SDAP
	CAA62911.1	Allergen Sin a 1.0106/*Sinapis alba*	41.40	1.9e-005	AllergenOnline
			41.44	–	SDAP
	P15322	Allergen Sin a 1/*Sinapis alba*	43.40	1.2e-005	AllergenOnline
			36.40	3.1e-5	ADFS
			39.02	0.0016	Allermatch™
	CAA62910.1	Allergen Sin a 1.0105/*Sinapis alba*	41.28	5.8e-005	AllergenOnline
	CAA62912.1	Allergen Sin a 1.0107/*Sinapis alba*	41.28	4e-005	AllergenOnline
	CAA62909.1	Allergen Sin a 1.0104/*Sinapis alba*	41.28	1.9e-005	AllergenOnline
			40.96	–	SDAP
	ACI41244.1	Albumin 2S/*Sesamum indicum*	41.20	3.5e-005	AllergenOnline
	P80208	Napin 3/*Brassica napus*	41.20	0.0037	AllergenOnline
			42.50	3.9e-05	Allermatch™
	CAA62908.1	Allergen Sin a 1.0108/*Sinapis alba*	40.20	2.2e-005	AllergenOnline
	P01089	2S albumin - Allergen Ric c 1/3/*Ricinus communis*	38.76	2.4	AllergenOnline
	Q8H2B8	2S Albumin - Allergen Ana o 3/*Anacardium occidentale*	37.50	0.43	AllergenOnline
			37.50	0.022	Allermatch™
	P80207	Allergen Bra j 1-E/*Brassica juncea*	37.50	0.078	AllergenOnline
			36.59	0.0021	Allermatch™
	Q7Y1C2	2S albumin seed storage protein/*Juglans nigra*	36.20	0.0053	AllergenOnline
			37.50	6.4e-05	Allermatch™
	D0PWG2	2S albumin - Allergen Cor a 14/*Corylus avellana*	36.20	0.0019	AllergenOnline
			36.25	1.7e-05	Allermatch™
	Q8GT96	Recombinant Ib pronapin/*Brassica napus*	36.20	0.0041	AllergenOnline
			35.80	0.0038	ADFS
	2LVF	2S Albumin Ber e 1 - Chain A/*Bertholletia excelsa*	35.02	0.00043	AllergenOnline
	P04403	2S Albumin Ber e 1/*Bertholletia excelsa*	35.02	0.00082	AllergenOnline
			35.00	4.5e-06	Allermatch™
			36.25	–	SDAP
	ABG73108.1	2S albumin - Pis v 1 allergen/*Pistacia vera*	35.01	0.00077	AllergenOnline
	Q84XA9	Putative allergen I1/*Carya illinoinensis*	35.00	0.0046	AllergenOnline
			35.00	5.6e-05	Allermatch™
	Q9AUD1	2S Albumin - Allergen Ses i 1/*Sesamum indicum*	40.80	2.9e-5	ADFS
			42.50	3.3e-08	Allermatch™
	B6EBI1	2S Albumin - Allergen Ses i ?/*Sesamum indicum*	40.00	2.4e-4	ADFS
	Q42473	2S storage protein - Allergen Bra r 1/*Brassica rapa*	41.25	3.5e-09	Allermatch™
			40.83	–	SDAP
	P93198	Albumin seed storage protein - Jug r 1/*Juglans regia*	35.00	0.00015	Allermatch™
Mo-CBP$_3$-3	AAK15088	Albumin 2S/*Sesamum indicum*	41.20	1.6e-007	AllergenOnline
			38.00	–	SDAP
	2LVF	2S Albumin Ber e 1 - Chain A/*Bertholletia excelsa*	40.20	2e-005	AllergenOnline
	P80208	Napin 3/*Brassica napus*	40.04	0.0011	AllergenOnline

(*continued on next page*)

Table 2 (*continued*)

Mo-CBP$_3$ isoforms	Identified hit				
	Accession number	Description/Species	Identity (%)	*E*-value	Database
			40.00	8.1e-06	Allermatch™
	ACI41244.1	Albumin 2S/*Sesamum indicum*	40.00	3.6e-007	AllergenOnline
	P04403	2S Albumin Ber e 1/*Bertholletia excelsa*	40.00	4e-005	AllergenOnline
			35.00	6.4e-6	ADFS
			37.50	3.7e-07	Allermatch™
	CAA62911.1	Allergen Sin a 1.0106/*Sinapis alba*	39.10	0.00011	AllergenOnline
			42.50	–	SDAP
	P15322	Allergen Sin a 1/*Sinapis alba*	39.10	0.00017	AllergenOnline
			35.70	1.9e-4	ADFS
			40.00	0.0041	Allermatch™
	D0PWG2	2S albumin - Allergen Cor a 14/*Corylus avellana*	38.80	0.00088	AllergenOnline
			35.40	3.4e-4	ADFS
			38.75	8.1e-06	Allermatch™
	CAA62910.1	Allergen Sin a 1.0105/*Sinapis alba*	38.60	0.00029	AllergenOnline
	CAA62912.1	Allergen Sin a 1.0107/*Sinapis alba*	38.40	0.00025	AllergenOnline
	CAA62909.1	Allergen Sin a 1.0104/*Sinapis alba*	38.40	0.00027	AllergenOnline
	CAA62908.1	Allergen Sin a 1.0108/*Sinapis alba*	37.90	0.00015	AllergenOnline
	Q7Y1C2	2S albumin seed storage protein/*Juglans nigra*	36.24	0.0078	AllergenOnline
			36.25	0.00014	Allermatch™
	P80207	Allergen Bra j 1-E/*Brassica juncea*	36.24	0.069	AllergenOnline
			36.25	0.0016	Allermatch™
	Q8H2B8	2S Albumin - Allergen Ana o 3/*Anacardium occidentale*	36.21	0.17	AllergenOnline
			36.25	0.0069	Allermatch™
	AAA34275.1	alpha-type gliadin precursor protein/*Triticum aestivum*	35.40	3.6	AllergenOnline
	P01089	2S albumin - Allergen Ric c 1/3/*Ricinus communis*	35.30	–	AllergenOnline
			35.30	1.4e-4	ADFS
	Q8GT96	Recombinant Ib pronapin/*Brassica napus*	35.30	–	AllergenOnline
			35.30	0.0014	ADFS
	CAR82265.1	D-type LMW glutenin subunit, partial/*Triticum aestivum*	35.04	88	AllergenOnline
	CAA25593	unnamed protein product/*Triticum aestivum*	35.03	5.5	AllergenOnline
	AAA17741.1	alpha-gliadin/*Triticum aestivum*	35.03	4.7	AllergenOnline
	BAA12318.1	alpha-gliadin, partial/*Triticum aestivum*	35.03	1.9e+002	AllergenOnline
	AAA34280.1	alpha/beta-gliadin precursor/*Triticum aestivum*	35.03	5	AllergenOnline
	CAA26384.1	unnamed protein product/*Triticum aestivum*	35.03	5	AllergenOnline
	Q9AUD1	2S Albumin - Allergen Ses i 1/*Sesamum indicum*	40.20	1.2e-7	ADFS
			41.25	1.3e-10	Allermatch™
	B6EBI1	2S Albumin - Allergen Ses i ?/*Sesamum indicum*	39.03	2.7e-7	ADFS
	Q42473	2S storage protein - Allergen Bra r 1/*Brassica rapa*	41.25	3.5e-09	Allermatch™
Mo-CBP$_3$-4	P15322	Allergen Sin a 1/*Sinapis alba*	44.60	9.7e-008	AllergenOnline
			36.80	7.3e-8	ADFS
			40.24	6.6e-05	Allermatch™
			37.15	–	SDAP
	P80208	Napin 3/*Brassica napus*	43.75	0.00018	AllergenOnline
			45.00	4.4e-06	Allermatch™
	CAA62911.1	Allergen Sin a 1.0106/*Sinapis alba*	43.00	3.9e-007	AllergenOnline
	CAA62910.1	Allergen Sin a 1.0105/*Sinapis alba*	42.50	5e-007	AllergenOnline
	CAA62912.1	Allergen Sin a 1.0107/*Sinapis alba*	42.50	3.3e-007	AllergenOnline
	CAA62909.1	Allergen Sin a 1.0104/*Sinapis alba*	42.50	1.5e-007	AllergenOnline
	CAA62908.1	Allergen Sin a 1.0108/*Sinapis alba*	41.90	5.4e-007	AllergenOnline
	AAK15088	Albumin 2S/*Sesamum indicum*	41.20	4.1e-005	AllergenOnline
			40.00	–	SDAP
	ACI41244.1	Albumin 2S/*Sesamum indicum*	40.00	9.4e-005	AllergenOnline
	2LVF	2S Albumin Ber e 1 - Chain A/*Bertholletia excelsa*	38,71	0.00026	AllergenOnline
	P04403	2S Albumin Ber e 1/*Bertholletia excelsa*	38.71	0.00037	AllergenOnline
			42.90	8.6e-5	ADFS
	P80207	Allergen Bra j 1-E/*Brassica juncea*	37.80	0.011	AllergenOnline
			37.80	0.00055	Allermatch™
	D0PWG2	2S albumin - Allergen Cor a 14/*Corylus avellana*	36.24	0.0033	AllergenOnline
			36.25	0.00017	Allermatch™
	P01089	2S albumin - Allergen Ric c 1/3/*Ricinus communis*	36.20	1.7	AllergenOnline
	Q7Y1C2	2S albumin seed storage protein/*Juglans nigra*	36.20	0.0078	AllergenOnline
			36.25	0.00048	Allermatch™
	Q8GT96	Recombinant Ib pronapin/*Brassica napus*	35.20	–	AllergenOnline
			35.20	5.3e-4	ADFS
	Q9AUD1	2S Albumin - Allergen Ses i 1/*Sesamum indicum*	40.20	3.3e-5	ADFS
			41.25	9.8e-07	Allermatch™
	B6EBI1	2S Albumin - Allergen Ses i ?/*Sesamum indicum*	39.30	8.1e-5	ADFS
	Q42473	2S storage protein - Allergen Bra r 1/*Brassica rapa*	38.75	8.7e-10	Allermatch™
	P93198	Albumin seed storage protein - Jug r 1/*Juglans regia*	35.00	0.00086	Allermatch™

[a] Not provided.

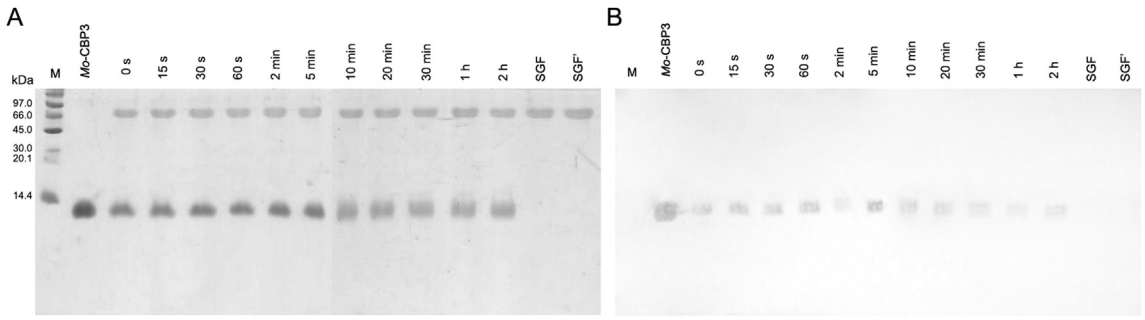

Fig. 1. SDS-PAGE (17.5%) (A) and Western Blot (B) profiles of *Mo*-CBP$_3$ (<14.4 kDa) at a concentration of 0.5 mg/mL in SGF digestion. M: molecular mass markers (phosphorylase B−97 kDa; bovine serum albumin−66 kDa; ovalbumin−45 kDa; carbonic anhydrase−29 kDa; soybean trypsin inhibitor−20.1 kDa and lactalbumin −14.4 kDa); SGF: SGF without incubation; SGF′: SGF with incubation for 2 h.

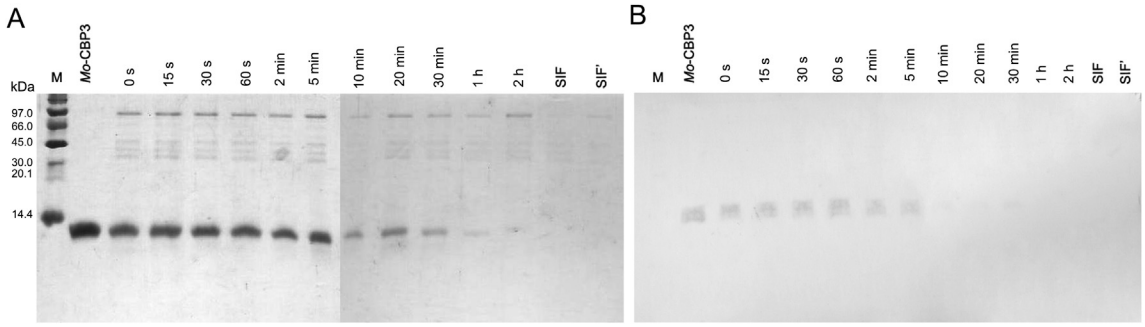

Fig. 2. SDS-PAGE (17.5%) (A) and Western Blot (B) profiles of *Mo*-CBP$_3$ (<14.4 kDa) at a concentration of 0.5 mg/mL in SIF digestion. M: molecular mass markers (phosphorylase B−97 kDa; bovine serum albumin−66 kDa; ovalbumin−45 kDa; carbonic anhydrase−29 kDa; soybean trypsin inhibitor−20.1 kDa and lactalbumin −14.4 kDa); SIF: SIF without incubation; SIF′: SIF with incubation for 2 h.

made in general databases with all protein sequences available as a conservative approach. The identified proteins with significant identity using a local alignment algorithm are then evaluated for their relevance in causing adverse effects (Codex Alimentarius, 2009; Madduri et al., 2012). *Mo*-CBP$_3$ protein showed no identity with any allergenic, toxic and/or antinutritional protein sequences deposited in seven large databases.

The allergenic potential of a protein can be assessed by comparing its primary amino acid sequence with those of known allergens (FAO/WHO, 2001; Ladics, 2008; Codex Alimentarius, 2009; Cressman and Ladics, 2009; Ladics et al., 2011). Comparison of the full-length sequences of the four *Mo*-CBP$_3$ isoforms in ADFS, Allermatch™, AllergenOnline and SDAP allergen databases showed no similarity with any known allergenic proteins. According to Aalberse (2000), alignments with high identity scores may indicate a potential for allergenic cross-reactions. Cross-reactivity is not likely for proteins with less than 50% identity over the entire protein sequence and it is fairly common above 70% identity.

As recommended by Codex Alimentarius (2009), the possibility of a cross-reaction between a novel protein and a known allergen is considered when the identity between them is >35% on a window of 80 amino acids. Proteins sharing high identity by local alignment programs, such as FASTA or BLASTP, are highly likely to cause cross-reactions (Goodman et al., 2005, 2008). Hence, they must be subjected to *in vitro* experiments with sera from atopic patients (allergic) to evaluate their potential in triggering allergic reactions (Thomas et al., 2009; Randhawa et al., 2011). According to this criterion, *Mo*-CBP$_3$ isoforms showed identity with several known allergens. Among them, stand out 2S albumins from several plant species such as *B. excelsa*, *Brassica napus*, *C. avellana, Sesamum indicum*, and *Si. alba*. Thus, this result provides sufficient evidences to indicate that *Mo*-CBP$_3$ protein must be submitted to further hypothesis-based testing on its potential allergenicity.

The allergenic potential of proteins is also evaluated through research with small segments of identical contiguous amino acids. FAO/WHO (2001) recommends searches of six to eight contiguous amino acids. The research by using six and seven contiguous amino acids revealed identity of known allergens with *Mo*-CBP$_3$ isoforms. However, with the criterion of eight contiguous amino acids no identity could be observed. The approach using six contiguous amino acids is often criticized for generating high rate of false positives (König et al., 2004). Many random results that show no risk of cross-reaction is observed when a short sequence of amino acids is used (Hileman et al., 2002). A research with eight contiguous amino acids is probably more effective in detection of immunogenic epitopes (ILSI HESI, 2001; Hileman et al., 2002). In addition, the peptides found for *Mo*-CBP$_3$ were not present in any known allergenic protein epitopes. For the allergic response to be triggered, at least two IgE-binding epitopes on the same protein are required (Huby et al., 2000; Ladics, 2008).

Many allergens are glycosylated proteins, raising the possibility that the glycosyl groups may contribute to its allergenicity (Jenkins et al., 1996). *Mo*-CBP$_3$ did not present potential *N*-glycosylation sites by *in silico* sequence analysis. On the other hand, sites of O-glycosylation in *Mo*-CBP$_3$ have been detected (Freire et al., 2015). In addition, experimental results revealed that *Mo*-CBP$_3$ is a glycoprotein with 2.5% carbohydrate in its structure (Gifoni et al., 2012). However, the current knowledge about the structures and possible epitopes of oligosaccharides linked to allergenic glycoproteins show that *N*-glycans are more often involved in the structures of IgE epitopes (Fötisch and Vieths, 2001; Altmann, 2007).

It is known that the antifungal properties of *Mo*-CBP$_3$ are triggered by alterations in the cell surface (Gifoni et al., 2012). In fact,

Mo-CBP$_3$ was able to permeabilize the plasma membrane (using propidium iodide as probe) of *F. solani* spores. Moreover, this protein appeared to interfere directly or indirectly with the plasma membrane H+-ATPase pump. Similar mode of action has also been demonstrated by other antifungal proteins like soybean toxin (SBTX) (Morais et al., 2010) and *Pv*D$_1$ of *Phaseolus vulgaris* (Mello et al., 2011). The proton pump present in the fungal membrane is responsible for maintenance of intracellular pH and electrochemical gradient which are required for absorption of food (Monk and Perlin, 1994). Disruption of plasma membrane caused by *Mo*-CBP$_3$ can affect the function of this proton-translocating ATPase enzyme and the traffic of substances across the membrane, which could lead to cell death, as suggested for other plant proteins (Ben-Josef et al., 2000; Mello et al., 2011). It is noteworthy that the presence of chitin in the fungi cell wall is crucial for *Mo*-CBP$_3$ action, which confers a margin of safety for mammals. The mechanism of action and specificity of *Mo*-CBP$_3$ is a target of intense studies by our group and soon more details about this topic can be revealed.

With few exceptions, all allergenic proteins have high stability to digestive enzymes. Thus, this stability is used as an indicator of the allergenic potential of a protein (Astwood and Fuchs, 1996). *Mo*-CBP$_3$ showed to be highly resistant to degradation by SGF, even after 2 h of incubation, but was susceptible to SIF. Therefore, *Mo*-CBP$_3$ could be a concern related to trigger an allergenic response, especially when this result is grouped with the findings of the *in silico* search on a window of 80 amino acids. In addition, it has been recently reported that *Mo*-CBP$_3$ is highly stable at temperatures (100 °C) and pH extremes for up to 1 h, maintaining its three-dimensional structure and antifungal activity almost unchanged (Batista et al., 2014). Thermal stability has been associated with the allergenic potential of many proteins. This feature comes to reinforce the concerns about the allergenic potential of *Mo*-CBP$_3$.

The set of tests that comprises the Tier I from the two-tiered approach proposed by ILSI for risk assessment of novel proteins gathered weight of evidence that *Mo*-CBP$_3$ possesses a potential to trigger allergies. Thus, a Tier II composed by a guided study with sera of allergic patients must be conducted in order to confirm this hypothesis.

5. Conclusions

The obtained results contributed to gather a relevant dataset on food safety of the *Mo*-CBP$_3$, an antifungal protein isolated from *M. oleifera* seeds. Many positive attributes were presented for this protein. However, *Mo*-CBP$_3$ showed high sequence homology with allergens that indicates that further hypothesis-based testing on its potential allergenicity must be done before its use for plant transformation. Additionally, animal toxicity evaluations (e.g. acute and repeated dose oral exposure assays) must be performed to meet the mandatory requirements of several regulatory agencies. Finally, the approach adopted here exemplified the importance of performing an early risk assessment of candidate proteins for use in plant transformation programs. This may avoid huge economic and time consuming efforts, as well as may help to guide modifications in the protein structure in order to free it of its potential risks.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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