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CHARACTERISTICS OF CRY1AB PROTEIN FROM BIOINSECTICIDES AND INSECT RESISTANT GM CROPS

ABSTRACT

Biological insecticides are an effective method used in plant protection. One of the most widely used active substances in biological insecticides is Cry1Ab protein, which is toxic for lepidopteran insects. This protein is produced during bacterial sporulation by *Bacillus thuringiensis.* Other sources of Cry1Ab protein are genetically modified plants (GM) with expression of *cry1Ab* gene. Cry1Ab protein in both bioinsecticides and GM plants is present in the form of protoxin, which requires activation by enzymatic treatment in the gut of susceptible insects. So far, Cry1Ab mode of action is not fully understood, but there are 3 main concepts describing it. Two of them assume that a toxic protein after binding to receptors in the insect gut penetrates into the cells, causing pore formation in the gut, which leads to the death of the sensitive insect. In the third model Cry1Ab toxic action is a result of toxin-induced chemical processes initiating a cell death pathway. This work describes the structure and mode of action of Cry1Ab protein, present in biological insecticides and genetically modified plants.

Key words: biological insecticide; Cry1Ab protein; Cry protein mode of action; GM plant

INTRODUCTION

Biological plant protection products are a unique type of pesticides, which are derived from natural materials, such as animals, plants, bacteria or minerals. By April 2016, 299 active ingredients of biological plant protection products were registered and used in 1401 registered pesticides in the United States of

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America (USA) (US EPA, 2016). Substances containing *Bacillus thuringiensis* Berliner crystal proteins are the most commonly used biological plant protection products. Insecticidal properties of Cry1Ab protein produced by *B. thuringiensis* are known since the beginning of the 20th century. Nowadays its insecticidal activity on lepidopteran insects is used to reduce losses caused by pests in vegetable crops, orchards and forests. Although biopesticides are not currently used in the European Union (EU) for maize protection their potential in reduction of chemical insecticide usage against maize pests is large (Meissle *et al.*, 2010). The application of bacterial endospores can also help to reduce the accumulation of *Fusarium verticillioides* mycotoxins in this crop. Insecticides based on *B. thuringiensis* endospores are used for control of the European corn borer (ECB) (*Ostrinia nubilalis* Hbn., Crambidae) in other countries, e.g. the USA. Along with the increase in maize acreage and observed climate changes, the distribution area of this insect has moved to the northern regions of Europe. In Europe, the ECB larvae damage can be found in 20% (Hungary) or even up to 60% (Spain) of maize acreage, with estimated yield losses range from 5 to 30%. Economic yield losses are often recorded in Hungary, Italy, Spain, France, Germany and Poland (Meissle *et al.*, 2010). In southern Poland, where maize is intensively grown *O. nubilalis* caterpillars damage 50-80%, and locally even up to 100% of plants, causing up to 20-30% and sometimes up to 40% of direct loss in grain yield (Lisowicz and Tekiela, 2004). Yield losses are not only directly connected to insects foraging but also indirectly by increased susceptibility of damaged plants to pathogen infections, especially *Fusarium* fungi, which produce dangerous mycotoxins (Saladini *et al.*, 2008). The ECB is relatively difficult to control due to the larvae foraging inside the plant. Protection treatment is cumbersome, expensive and inefficient, and thus systemic insecticides are commonly recommended (Mrówczyński *et al.* 2005).

Cry1Ab protein has been used to protect genetically modified (GM) maize varieties, which in many countries have become an alternative to the use of chemical insecticides. The use of genetic modification allows to limit the damage caused by the ECB by up to 97 –100% (Bereś and Gabarkiewicz, 2008). The cultivation of GM crops on a large scale began in 1996 when the global planting area was equal to 1.7 million hectares. In 20 years period the area of GM crops cultivation increased more than 100-fold and reached 179.7 million hectares. In 2015, GM maize was grown on 56.6 million hectares, representing 29% of the world's total GM crops (James, 2016). In Europe, the only GM maize which has been authorized for cultivation is MON810 line with *cry1Ab* gene expression. Two additional GM events of maize (DAS 1507 and Bt11) are in the process of authorization.

CHARACTERISTICS OF CRY PROTEINS

B. thuringiensis was isolated for the first time by Ishiwatari in 1901 from death silkworm larvae (*Bombyx mori* L.). The microorganism has been recognized as its pathogen and named *Bacillus sotto*. In 1911 Berliner isolated another strain from flour moth larvae (*Anagasta kuehniella* Zeller) in Germany, hence comes the current name - *Bacillus thuringiensis* Berliner (Bt) (Milner, 1994). The bacterium *B. thuringiensis* is a gram-positive bacterium that is commonly found in the dirt and dust of grain elevators and in the soil. Strains of this bacteria are widely distributed in the soil throughout the world but with varying intensity. Martin and Travers (1989) reported that *B. thuringiensis* in the soil occurs commonly, as it was found in 785 of 1115 analyzed soil samples (70%). In contrast, DeLucca *et al.*, (1981) analyzing 46373 soil bacterial isolates from 115 fields, found only 250 samples containing *B. thuringiensis* isolates (0.5%) from which 94 was *B. thuringiensis var. kurstaki*. During sporulation of *B. thuringiensis* Insecticidal Crystal Proteins (ICP) known as Cry proteins or delta-endotoxin are produced. These proteins are toxic to various insect groups and are encoded by *cry* gene family (Whiteley and Schnepf, 1986). According to Bravo *et al.* (2011) Cry proteins belong to the class of pore-forming toxins (PFT).

Cry proteins classification is based on their primary amino acid sequence. By 2010, more than 500 different *cry* genes have been identified and classified into 67 groups (*cry1-cry67*) (Crickmore, 2010). The nomenclature of these proteins is based solely on amino acid sequence, which is not necessarily associated with similar biological activity. The full protein name contains the word Cry (crystal), followed by Arabic number (primary rank), then the uppercase (secondary rank) and lowercase character (tertiary rank). In the name of the Cry protein the Arabic number may reoccur which means that the protein sequences are identical but independently isolated (quaternary rank). Cry proteins with the same first number, uppercase and lowercase letter, for example Cry1Ab1 and Cry1Ab2 have 95% amino acid sequence similarity, while the Cry protein with the same first number and uppercase, for example Cry1Aa and Cry1Ab have at least 78% of common amino acid sequence. Cry proteins which have the same first number, for example Cry1Ab and Cry1Ba have at least 45% of the common sequences. The current list of the Cry proteins is available on the University of Sussex website (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html).

The largest group of Cry proteins are tridomain proteins (3D) which includes 53 subgroups (Crickmore *et al.*, 2010). Their structure was first described by Li *et al.* (1991). It was shown that they are globular molecules, comprising of three different domains linked by individual connectors.

CRY1AB PROTEIN STRUCTURE AND MODE OF ACTION

Cry1Ab protein has 3 domains where domain I consists of 7 α-helices, with centrally located α-helix 5 and six other amphipathic helices surrounding it. Domain II consists of 3 antiparallel β-sheets "packed" in the form of a β-barrel (Li *et al.*, 1991). Each of the first two β-sheets consists of four chains with the Greek key motif. The third β-sheet comprises of three chains arranged in a meander motif and a short α-helix domain arranged opposite to domain I with which it interacts (Pigott and Ellar, 2007). The structure of domain II is highly variable, which indicates that this domain is responsible for the Cry proteins specificity. The domain III structure is less variable than the domain II, but more variable than domain I. The domain III is a *jelly-roll beta-sandwich* consisting of antiparallel β-sheets. Each of the sheets has 5 chains (Pigott and Ellar, 2007). Two long loops extend from domain III and interact with domain I (Grochulski *et al.*, 1995).

The full length 131 kDa of bacterial protein Cry1Ab is named a protoxin and is not toxic in the synthesized form. When this form is dissolved in an alkaline environment (pH 9 - 10) of insects gastrointestinal tract, it must be activated by proteases present in the gut of susceptible insect. Enzymes hydrolyze the domain I α1 N-terminus and catalyze the cleavage of approximately half of the C-terminus sequence, resulting in 60 - 70 kDa fragments (Gill *et al.*, 1992, Bravo *et al.*, 2002) (Fig.1). Obtained proteins act as an active toxin and are resistant to proteolytic enzymes (Haider and Ellar, 1989; Douville *et al.*, 2001). According to Höfte *et al.* (1986), the minimum length of Cry1Ab fragment ensuring insecticidal activity is 564 - 578 amino acids which corresponds to a weight of about 60 kDa. The site of action of Cry protein is a midgut membrane of the target insect where the pH is alkaline, between 9 and 11 (Milne and Kaplan, 1993). Cry1A toxins are completely soluble at pH 9.5 (Bietlot *et al.*, 1989).

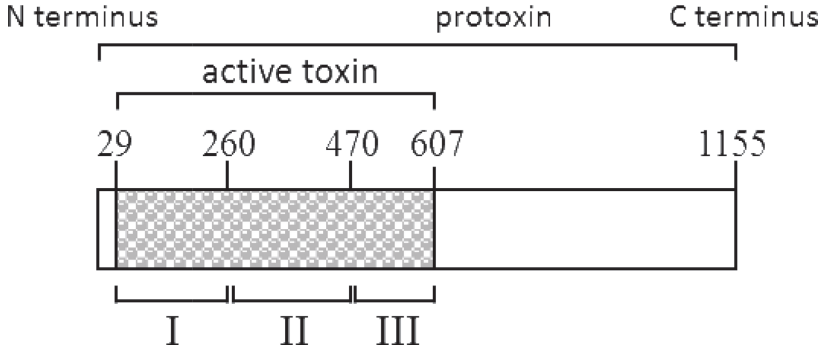

Fig. 1. Graphic representation of bacterial Cry1Ab protein amino acid sequence. Protoxin 1-1155 aa, active toxin 29-607 aa and three protein domains (I, II, III) (according to Martens *et al.*, 1995)

The domains of the Cry protein are involved in its specific insecticidal activity. Domain II plays a decisive role in the protein action specificity. Domain I is considered to have a main role in oligomerization and pore formation in the cell

membrane (Li *et al.*, 1991; Grochulski *et al.*, 1995). Hypothesis concerning the role of domain I in pore formation seems to confirm the fact that the helices from α3 to α7 are relatively large (more than 30 Å) and therefore long enough to "cover" the hydrophobic cell membrane (Pigott and Ellar, 2007). Both the domain II and III determine the protein action specificity to certain insects through the mediation of specific interaction with various proteins in insect gastrointestinal tract (most of the cell membrane receptors binding sites are located in domains II and III). These domains are responsible for binding to the target insect cell membrane, transferring domain I close to the membrane, allowing the formation of pores (Bravo and Soberón, 2007).

Several models of Cry protein action have been described in the literature. In the classical model Cry protein binds to a membrane receptor, then is taken into cells of insect gut to form pores, and thereby destabilizes the function of the cell membrane. By forming of non-selective permeable channels for cations, anions and neutral soluble substances and by water flow cells swell and consequently die. When the process includes many cells, the death of susceptible insect occurs as a result of starvation or infection, only a few days after ingestion of a toxic protein (Knowles, 1994). Besides the classical model, there are other models of Cry protein action: the so called sequential binding model which also implies that the protein toxic action is based on pore formation, and a signal path model, whereby pore formation is insignificant. In 2006 Jurat- Fuentes reported that the toxicity of Cry protein is the result of both osmotic lysis and disturbances in signaling pathway. The common element of all models is solubilisation and activation of the Cry protein in the insect gut in the form of the protoxin by proteases, and specific binding to the gut cell membrane. Such binding of proteins may lead to changes in its conformation, thereby activating its toxicity.

In the model of sequential binding, enzymatically activated monomeric Cry protein form binds to intestinal epithelial cells, resulting in protein conformation changes, necessary for oligomerization and penetration into the membrane to form pores (Soberón *et al.*, 2010). In this model, the first step is the low affinity binding of activated toxin to aminopeptidase N (APN) or alkaline phosphatase (ALP) receptors which are numerously present on the cell membrane surface. By binding Cry protein domain III with ALP and APN, the activated toxin is located within the membrane microvilli, close to cadhedrin receptor (CAD), or cadhedrin-like molecule to which it can bind. The role of ALP and APN after binding is the induction of Cry protein binding to the membrane, leading to pore formation and cell lysis. After binding of CAD with the toxin, α-helix removal is induced, resulting in Cry protein oligomers formation. Oligomerization is quicker in alkaline environment, similar to pH of the lepidopteran midgut (Russel *et al.*, 2004). Binding to CAD is a multiplex reaction when the proteolytic cleavage of the N-terminus containing α-helix I of domain I is present, resulting in the exposition of domain I hydrophobic regions. The subsequent

protein Cry1Ab oligomer binding to ALP/APN receptors allows pre-pores occurrence that penetrate the membrane and form stable pores. These pores have a high probability of opening, unlike those formed from the action of the protein monomeric form, wherein there is minimal interaction with liposomes, inducing partially closed pores. The binding of Cry oligomers to ALP or APN is reversible, these receptors are not very specific, which may lead to an accumulation of Cry oligomers near the ATP-binding cassette transporters (ABC transporter) - a membrane system allowing transfer of small particles through the membrane. Binding with the transport system facilitates oligomers introduction to the inside of the membrane.

Oligomers forming pre-pores can be formed from monomers of the same protein type (homo-oligomers) or from a combination of different types of monomers (hetero-oligomers). One explanation for the synergistic effect of Cry3D protein is that if Cry proteins linked in the oligomer are toxic, the toxicity induced by hetero-oligomer may be greater than the toxicity of each of the homo-oligomers respectively (Soberón et al, 2000). But there are also studies indicating that the lack of Cry protein mutants toxicity may be a dominant negative (DN) feature. Assuming that DN-mutant forms hetero-oligomers with wild type, they are non-toxic.

An extremely important step in the study of Cry protein action mechanism is the way of penetration to the target insect gut membrane and pore formation. There are two proposed mechanisms of pore formation – umbrella model (Li *et al.*, 1991) and penknife model (Hodgmann and Ellar, 1990). Most accepted model of Cry protein binding to the membrane is the model assuming that the hydrophobic structure described as a hairpin consisting of a centrally located α-helix 5 and α-helix 4 penetrates the membrane phospholipid bilayer, while the rest of the amphipathic domain I helices are distributed on the gut membrane surface in umbrella-like conformation. Formed pores have a size from 1 to 2.6 nm in diameter and, depending on the pH, may be closed or open. In the penknife model the domain I with central, highly hydrophobic helix α5 and α6 linked by loop "opens" the membrane in a manner similar to a penknife and penetrates inside. The remaining part of Cry protein molecule binds receptors on the membrane surface (Hodgman and Ellar, 1990). In both models, the central position of the domain I (α-helix 5) is responsible for the pores formation. In contrast to proposed models some authors claim that the whole protein molecule penetrates the insect gut membrane (Nair and Dean, 2008).

Hypothesis about protein toxicity which would result from leaks caused by the pores in insects gut membrane was questioned by Zhang *et al.* (2006). In signaling pathway model described by these authors, the protein molecules do not form pores in the gut cell membrane but bind to the membrane receptor, initiating chemical processes leading to cell death. By attaching and integration of Cry protein with cadhedrin, the intracellular signaling pathway is initiated that leads to G protein activation (signal dependent on the presence of Mg^{2+}).

Activated G protein stimulates a membrane-bound adenylate cyclase (AC) which catalyse cAMP production and activates the kinase A which causes protrutiions in the gut cells, cell swelling, lysis and subsequent death. According to this model, the oligomers are not formed from protein monomeric forms and no pores are formed.

The model described by Jurat-Fuentes (2006) assumes that the cytotoxicity is due to the combined influence of cell osmotic lysis and signaling pathway - elements of both described above models. Firstly activated Cry protein monomer binds to the cadhedrin-like receptor, then activates the signaling pathway regulated by phosphatase. Signaling is directly dependent on Cry proteins interaction with actin (cytoskeletal protein). Upon binding to the cadhedrin-like receptor, Cry monomers are oligomerized and bind to APN receptor. As a result of pore formation, osmotic shock and signal pathway activation occurs, leading to cell death.

Recent studies show that Cry proteins, considered to be specific to only one insects order, may also have activity against other insects groups (Frankenhuyzen, 2013). Author demonstrated the existence of cross-reaction for 27 Cry proteins which are active against more than one insects order. However, the toxic effect is much less pronounced for those insects than for insects with a basic range of specificity for a particular protein. For example Cry1Ba protein manifests main activity against Lepidoptera order but also exhibits toxic effects against beetles (Coleoptera) and flies (Diptera). It was indicated that for Cry protein its specific activity is highly influenced by the pretreatment upon ingestion by an insect, whereby it is dissolved and treated with digestive enzymes (Frankenhuyzen, 2013).

BIOINSECTICIDES WITH *B. THURINGENSIS* ENDOSPORES

The first formulation with natural isolate of *B. thuringiensis* was Sporeine-insecticide registered in France in 1938. In 1961, the bacterial extract of *B. thuringiensis var. kurstaki* (with Cry proteins, including Cry1Ab) was registered by the Federal Environmental Protection Agency of the United States (US EPA) (Kumar *et al.*, 1996; US EPA, 2016). Products based on *B. thuringiensis* are approved for use in agriculture in many countries of the world (APVMA 2010; UE DG SANCO 2010), mostly they are a mixture of bacterial endospores, including Cry proteins, thus the range of activity against insects is large.

Formulations based on *Bacillus thuringiensis* are the most successful commercial biopesticides in the biological control market accounting for 90% of all biopesticides sold all over the world (Glare and Callaghan, 2000).

The first bioinsecticide based on *B. thuringiensis ssp. thuringiensis* endospores was present in the form of powders for the suspensions preparation but their "adhesion" to the plant surface was very limited. According to Bechtel and Bulla (1976) parasporal crystals constitute from 20 to 30% of endospores dry

weight. According to Schnepf *et al.*, (1998) Cry proteins are about 20 - 30% of bacterial cells dry weight in bioinsecticides with *B. thuringiensis* endospores, of which almost 80% are Cry1A (a, b or c), and about 20% Cry2 A or B (Abbott Laboratories, 1992).

In the 70's of the last century strain HD-l was isolated having up to 200-fold higher insecticidal activity than previously used *B. thuringiensis ssp. thuringiensis* (Frankenhuyzen *et al.*, 1993). This strain was used for the Dipel insecticide production which is produced so far. Except Dipel WG insecticide (Sumitomo Chemical, Japan), Foray 76B SC (Sumitomo Chemical Agro Europe S.A.S., France) is registered in Poland. The active substance of Foray 76B SC is *B. thuringiensis var. kurstaki* endospores and is used in the forests protection against nun moth caterpillars (*Lymantria monacha* L.). Bioinsecticides with *B. thuringiensis* endospores are not currently registered for maize protection in Poland. Insecticides containing *B. thuringiensis* endospores show selective and high effectiveness against crops pests. They do not affect the non-target organisms, but their regular use in the long-term may result in development of resistance in target insects (Malinowski, 1999).

GM PLANTS WITH *CRY1AB* GENE

Bacterial endospores used in the form of insecticides are rapidly degraded by light, they are washed off by rain and do not show systemic action, therefore *cry* genes have been transferred by genetic engineering methods to a number of crop species (Smith, 2005). There are 27 maize modifications with *cry1Ab* gene registered in the world, as well as 4 cotton modifications. Among the GM maize varieties there are single modification events like MON810, Bt11, Bt176, MON802 or MON809. From those, only varieties based on MON810 event can be used in European agriculture, while MON810 and Bt11 varieties can be used as food and feed in the EU. Stacking of traits by classical breeding led to the production of varieties where *cry1Ab* gene is combined with another modification e.g. *cp4-epsps* herbicide tolerance gene, eg. MON810 x NK603 or *pat* gene, eg. T25 x MON810. GM varieties with stacked traits constantly increase in numbers. There are 10 known double modifications with *cry1Ab* gene, six lines of triple modifications, four containing four genes and one modification of five genes (until November 2016).

MON810 MAIZE

MON810 maize was first authorized GM maize in the USA (1996). In 1998, it was also authorized for cultivation and use as food and feed in the EU. In 2015, maize MON810 was cultivated on an area of about 116 867 ha in 5 EU countries: the Czech Republic (997 ha), Spain (107 749 ha), Portugal (8017 ha),

Romania (2.5 ha) and Slovakia (104 ha) (Monsanto, 2016). Genetic transformation of MON810 was performed via biolistic techniques using PV - ZMBK07 plasmid which consisted of: *cry1Ab* gene from *B. thuringiensis var. kurstaki ssp*. HD - 1 - encoding a Cry1Ab protein, 35S promoter of cauliflower mosaic virus (CaMV), the maize hsp70 intron, untranslated 3' region of nopaline synthase gene (NOS) from Ti plasmid of *Agrobacterium tumefaciens*, sequence encoding neomycin phosphotransferase (*nptII*). A construct integrated into the genome for transformation event MON810 contains a 3.6 kb version of *cry1Ab* gene. Studies of Hernandez *et al.* 2003 confirmed a shortening of the sequence between positions 2235 and 2571 and the total loss of NOS terminator sequence which were confirmed by the results of Rosati *et al.* (2008). Cry1Ab protein in transgenic plants is synthesized in a truncated form, with a weight of 92 kDa. It has no C- or N-terminal fragments present in bacterial protoxin, its insecticidal action requires enzymatic activation. *CaMV 35S* (P-35S) promoter used for the MON810 transformation is a constitutive promoter, however, there are reports showing that the P-35S is unevenly active in different cell types and at different developmental stages of the plant (Sunilkumar *et al.*, 2002).

Cry1Ab protein content may vary in different plant tissues and between MON810 varieties. The results of Cry1Ab protein content analyses in leaves of 5 maize MON810 varieties indicate different content per gram of fresh weight: Agrigold A6609Bt 0.76-2.30 μg/g; Asgrow RX799Bt 0.77-2.39 μg/g; Monsanto Novelis 0.44-11.07 μg/g and 0.32-11.07 μg/g; Pioneer P31B13Bt 0.66-2.20 μg/g; Pioneer P33V08Bt 0.66-2.17 μg/g and 0.35-0.53 μg/g (Abel and Adamczyk, 2004). It is estimated that climate and soil conditions may affect the Cry1Ab protein content in transgenic plants. This factor is the most common reason of differences in observed quantitative Cry1Ab analyzes results. Furthermore, differences in Cry1Ab content in plant tissues occur even in plants growing next to each other. This may be due to natural variability in plant metabolism and genetic or epigenetic based differences. The content of transgenic protein varies in different parts of the plant and may change during the growing season (Nguyen and Jehle, 2007). The concentration of Cry1Ab measured at different stages of plant growth ranged from 0.1 to 4.89 mg/g in the stem, from 0.17 to 6.7 mg/g in pollen, from 0.2 to 5.32 mg/g in root and from 0.1 to 0.9 mg/g in seed, while the highest content was observed in leaves from 0.1 to 36.69 mg/g (US EPA, 2001; Nguyen and Jehle, 2007; Kamath *et al.*, 2010; Szèkács and al., 2010; Habuštová *et al.*, 2012). The highest diversity in Cry1Ab protein content was also observed in leaves, this may be due to the fact that protein synthesis is correlated with the chlorophyll and total nitrogen content in leaves (Abel and Adamczyk, 2004; Dutton *et al.*, 2004). According to Abel and Adamczyk (2004) the concentration of transgenic protein is related to photosynthesis rate and plant ability to produce amino acids through the photosynthesis. Related values for other tissues could indicate that in tissues which do not contain chlorophyll or possess a low level thereof, the concentration of the protein is more stable and less dependent

on the climate and soil conditions. Due to the fact that Cry1Ab protein content corresponds to insecticidal effectiveness of GM plants against target organisms (Adamczyk *et al.*, 2001; Olsen *et al.*, 2005) it is important that transgenic protein content remained stable, on sufficiently high level in tissues that are susceptible to pest attack throughout the growing season. The comparison between bacterial and Cry1Ab protein synthesized in MON810 is shown in Table 1.

Table 1

Comparison of Cry1Ab protein in the form of a biological insecticide (Dipel WG), and in GM maize (MON810).

	Dipel WG	MON810
Protein sequence	as in *B. thuringensis*	as in *B. thuringensis*
Size of the protein	131 kDa	92 kDa
Form of the protein	protoxin	protoxin
Protein size after enzymatic activation	60-70 kDa	60-70 kDa
Plant protection	depends on the weather conditions, time of application and pest monitoring	constant through plant vegetation
Protein residues	during application of insecticide	through plant vegetation
Risk of insect resistance	yes, requires rotation of active ingredient	yes, requires refugees

CONCLUSIONS

The insecticidal properties of Cry proteins have been used in plant protection for nearly 100 years. The most widely used agents are biological insecticides containing Cry1Ab protein isolated from the soil bacterium *B. thuringiensis*, which is toxic to lepidopteran insects. Biological insecticides currently used are the mixture of bacterial endospores, but due to restrictions on its use, low stability and lack of systemic effect, an effective alternative in the protection of plants was demonstrated by genetically modified varieties. Since 1998 good example are MON810 maize varieties allowed to be grown in the EU, expressing the *cry1Ab* gene responsible for Cry1Ab protein synthesis in all tissues, throughout the entire plant growth and development. The effect of both biological insecticides and GM plants proved its effectiveness in pro-ecological pest control. However substantial differences exist in the availability of these solutions for farmers in some countries. Biological insecticides containing Cry1Ab protein are approved for use in Poland and other EU countries also in organic farming, but require registration for relevant crop species. Although GM crops are one of the technologies approved for use in EU agriculture, in spite of proved lack of their negative effects on non-target organisms and agroecosystem, GM crops are not accepted in the organic production systems in the

EU. Additionally the Directive of the European Parliament and of the Council (EU) 2015/412 of 11 March 2015 gives the possibility for the Member States to restrict or prohibit the cultivation of genetically modified organisms in their territory. This restriction was applied in 19 of the 28 Member States in regard to the cultivation of MON810 maize and two other maize genetic modifications (DAS 1507 Bt11) that are in the process of authorization for cultivation in the EU.

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