Genetic Engineering Approach for Next-Generation of Bt-Based Agents

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Bacillus thuringiensis Berliner (*Bt*) and *B. cereus* sensu stricto Frankland and Frankland are closely related species of aerobic, spore-forming bacteria included in the *B. cereus* sensu lato group. This group is one of the most studied, but it remains also the most mysterious species of bacteria. Despite more than a century of research on the features of these ubiquitous bacteria, there are a lot of questionable issues related to their taxonomy, resistance to external influences, endophytic existence, their place in multidimensional relationships in the ecosystem, and many others.

endophytes insecticide nematicide Cry Vip Bt-crops

1. Introduction

Now, genetic modifications of *Bt* serve the purpose of dissolving two main problems of *Bt*-preparations, such as increase in bacteria tolerance to the impact of environmental factors and improving its insecticidal effects against different pests. The strategies of genetic engineering approaches to constructing strains with the required properties are as follows: (1) the up-regulation of the key enzyme gene involved in the target compound biosynthesis; (2) relieving the inhibition and/or repression of the key enzyme; and (3) the interruption of the pathways for synthesizing by-products [1]. The development of next-generation artificially improved *Bt* strains or strains heterologically producing *Bt*-toxins involves a broad spectrum of DNA reorganization, such as site-directed mutagenesis (SDM), the suppression and overexpression of genes, including RNA interference (RNAi) [2][3] Initially, RNAi was proposed as a suggestive strategy for the inhibition of viral infection. It is a post-transcriptional gene regulation mechanism characteristic of (possibly) all eukaryotes, including insect pests [4][5][6]. The mechanism is triggered by double-stranded RNA (dsRNA) precursors that are processed into short-interfering RNA (siRNA) duplexes, which then realize the recognition and repression of complementary dsRNAs, such as mRNAs or viral genomic RNAs [7].

2. Improvement of Insecticidal Properties of Bacterial Strains

Currently, genetic engineering approaches make it possible to transfer/supplement/modify genes encoding insectotoxin to other *Bt* strains or strains of another bacterial species using homologous recombination [8]. Current information on Cry- and non-Cry genes, which were used for the recombination of a broad spectrum of bacterial strains is assumed in [4][9]. An important tool for the recombination of *Bt* strains is site-specific recombination (SSR),

which is useful for engineering strains with original combinations of Cry toxins genes with improved insecticidal activity [2][3][10]. It seems interesting to create endophytic *Bt* or other bacterial species whose populations in the internal tissues of plants would be safe from the environment and have greater activity against pests. These investigations originated in the last decade of the 20th century when the *Bt* gene encoding Cry1Aa was expressed in root-associated *P. fluorescens* [11]. Thus, the introduction of the cry1Ia gene into the endophytic strain *B. subtilis* 26D does not lead to the loss of the endophytic status of *B. subtilis* 26DCryChS line and gives impetus to its insecticidal and aphicidal activity in vitro and in planta [12][13]. Endophytic *Burkholderia pyrrocinia* JKSH007 heterologically expressing the *Btcry218* gene showed an effectiveness against *Bombyx mori* L. [14]. The ability of *Pantoea agglomerans* 33.1:pJTT expressing *cry1Ac7* to inhabit *Saccharum officinarum* L. tissues was confirmed by re-isolation from the plant's rhizosphere, roots and shoots. Thus, the introduction of an exogenous gene did not affect the plant–host interaction but increased the mortality of *Diatraea saccharalis* Fabricius, 1794 fed on inoculated stems [15]. The transfer of "useful" insectotoxin genes from other economically important *Bt* strains to endophytic bacteria, as well as the maintenance of their consortiums, should contribute to the creation of new-generation biological agents based on them. At the same time, modern technologies for editing microbial genomes based on the CRISPRCas9 platform [7][16] can be proposed to disable the α-exotoxin and β-exotoxin synthesis of *Bt*.

3. Approaches to the Development of UV-Tolerant Bt

The problem of the UV-irradiation susceptibility of *Bt* seriously restricts its effective use. The exogenous addition of UV protective agents, such as rhodamine B or methyl green, can protect spores from the light [17]. Subsequently, for the same purpose, latex particles, ethanol, and olive oil have been used to encapsulate *Bt* in colloidosomes [18][19].

Homologous recombination technology was used for the insertion of the *yhfS* gene encoding acetyl-CoA acyltransferase in *Bt* LLP29 R-yhfS. The loss of the yhfS gene in the knockout strain *Bt* LLP29 Δ-yhfS led to the reduction in antioxidant ability and reduced UV resistance of the mutant [20]. The cell-surface exposure of chitinase Chi9602ΔSP was developed on the basis of *Bt* BMB171 using two repeat N-terminal regions of autolysin (Mbgn)2 as the anchoring motif. After continuous culturing for 120 h, the line of *Bt* expressing chitinase Chi9602ΔSP showed narrow pH tolerance and obviously enhanced UV radiation resistance capacity in addition to a high inhibitory effect towards phytopathogenic fungi, *F. oxysporum* FB012 and *Botryosphaeria berengeriana* FB016 [21]. CRISPR/Cas9 systems have been used to knock out the homogentisate-1,2-dioxygenase (*hmgA*) gene and obtain a melanin-producing mutant *Bt* HD-1-1 hmgA. The anti-UV test shows that melanin arranges protection to both *Bt* cells and Cry toxin crystals. After UV-irradiation the strain *Bt* HD-1-1 hmgA still had an 80% insecticidal activity against *H. armigera*, while the wild line only had about 20% [22].

Cry genes have been expressed in *P. fluorescens* and *Anabaena* sp. to increase the damage to crystals from UV light [23][24], as well as in *E. coli*; *B. megaterium* [25]; *B. subtilis* [26]; *Clavibacter xyli* Davis et al. 1984; *Herbaspirillum seropedicae* Baldani et al. 1986; *R. leguminosarum* [9]; *Beauveria bassiana* (Bals.-Criv.) Vuill., 1912 [27]; etc. The recombinant strain *P. fluorescens* is the base of biopesticide "CellCapTM" (Mycogen Corp.; Indianapolis, IN, USA),

contains encapsulated Cry toxins [28]. The increase in the amount of *Bt*-plants can be partially attributed to the means of the protection of insectotoxins from UV rays [29].

4. Bt Crops Prospects

Since 1996, genetically engineered *Bt* crops have been planted in the fields, which led to a “gene revolution” in agricultural production [30][31]. By the early 21th century, *Bt*-potato, *Bt*-cotton, *Bt*-maize, *Bt*-eggplant, etc. were actively distributed worldwide, which allowed for a significant reduction in the amount of chemical insecticides used in a number of countries [32][33]. However, this approach lead to a fairly rapid spread of resistant pest populations [34][35][36]. To overcome insect resistance, it is possible to introduce *Bt* crops containing more than two genes encoding insecticidal proteins [35][36]. The Bollgard cotton variety bearing *Cry1Ac* gene decreased the viability of pink bollworm *Pectinophora gossypiella* (Saunders, 1844) and corn earworm *Helicoverpa zea* Boddie, 1850. Plants of the Bollgard II variety, expressing two *Bt* endotoxins, expand the spectrum of protective features against lepidopteran pests [37]. *Bt*-cotton with cassettes of protective genes (1Ac/Cry2Ab/Vip3A), (Cry1Ab/Cry2Ac/Vip3Aa19) or (Cry1Ac/Cry1F/Vip3A) was cultivated in the 2016–2017 season on more than 90% of the arable lands of Australia [38].

Currently, the creation of plants containing not only *Cry* or *Vip* genes but also containing other gene sequences is of interest in order to increase the effectiveness of biological plant protection against pests. Recently, the US EPA approved a transgenic corn, SmartStaxPRO, expressing the Cry3Bb1 protein, and a dsRNA complementing the RNA of the vacuolar protein DvSnf7 of *Diabrotica virgifera* LeConte, 1858 [39][40]. A vector containing information about dsRNA targeting the acid methyltransferase gene of the juvenile hormone biosynthesis of *H. armigera* was inserted into the genome of *Bt*-cotton and impaired the resistance of pest compared to plants expressing only insectotoxic proteins [41]. The RNAi-mediated knockdown of *H. armigera* acetylcholinesterase, the ecdysone receptor, and v-ATPase-A genes by producing dsRNAs homologous to genetic targets in potato plants led to mortality and abnormal development in the larva of this insect (recorded ten days post feeding) [3].

Apparently, the application of single *Bt* genes to modify plant genomes will be gradually replaced by multiple *Bt* toxin genes or *Bt* with other nucleotide sequences.

5. Bt as a Means of dsRNAs Deliverance

The important problem of interference methods is dsRNA degradation by nucleases in the gut lumen and tissues of insects. Retaining dsRNA molecules in the gut or hemocoel of pest insects is the key aspect of an effective dsRNA delivery [2]. Thus, dsRNase catalyzing the specific cleavage of dsRNA has been found in the saliva of *Lygus lineolaris* Palisot de Beauvois, 1818 [42]; pea aphid *Acyrthosiphon pisum* Harris, 1776 [43]; *Schistocerca gregaria* Forskal, 1775 [44]; *H. armigera* [3]; etc. A dsRNA cassette targeting the multiple genes of *H. armigera* revealed more rapid cleavage in midgut juice compared to the hemolymph [3], and for this reason, the *Bt*-mediated appearance of pores in digestion membranes, can improve the efficacy of dsRNAs along with dsRNAs targeting dsRNases [45].

The stability of mRNA provided by, for example, the Shine–Dalgarno sequence (GAAAG-GAGG), is a promising factor of the high-level expression of Cry genes in *Bt* [3][46][47]. The binding of the 30S ribosomal subunit to this sequence might prevent mRNA cleavage by RNAses of pest. The use of a sporulation-dependent promoter of Cry genes of *Bt* for the transcription of the target dsRNA sequence, leads to the fact that the dsRNA will be spontaneously produced during the sporulation phase [47][48]. It has been demonstrated that the incorporation of plasmid pBtdsSBV-VP1, which carries out dsRNA complemental to the VP1 sequence of the sacbrood virus (SBV) in *Bt* 4Q7 and the subsequent appliance of exogenous total RNA, leads to a decrease in SVB severance in *Apis cerana* (Fabricius, 1793) families [48]. Then, the plasmid p*Bt*dsSBV-VP1 was inserted into the *Bt* NT0423, which expresses Cry1 protein, resulting in SBV replication being repressed in *A. cerana* bees as well as the viability of the *A. cerana* parasite *Galleria mellonella* L. [46]. These results demonstrated that dsRNA-expressing *Bt* products could be efficiently exploited for the control of both viral diseases and insect pests simultaneously.

And furthermore, it is possible to enhance the toxicity of Cry toxins using dsRNA cassettes. Thus, *Bt* strains 8010AKi and BMB171AKi expressing the dsRNA of the arginine kinase gene (PxAK) of *P. xylostella*, flanking two ends with the promoter Pro3α, effectively decreased PxAK expression in ones treated with the composition with wild Cry-producing *Bt* 8010 and caused a higher lewel of mortality of the pest [49]. Separately, *E. coli* HT115 dsINT expressing the dsRNA of integrin β1 subunit gene (SeINT) cause a less than 50% mortality rate against *Spodoptera exigua* Hubner, 1808 larvae, and *E. coli* expressing Cry1Ca led to a maximal 58% mortality rate of the pest. When *S. exigua* larvae were treated with the Cry1Ca-expressing bacteria (*E. coli* or *Bt* subsp. *aizawai* from commercial Xentari insecticide) after treatment with *E. coli* HT115 dsINT, the insecticidal activity of the Cry1Ca was significantly enhanced up to about 80% [47]. The nuclease gene HaREase characteristic for Lepidoptera is up-regulated by dsRNA and affects RNAi in *H. armigera*. When this gene was knocked out using the CRISPR/Cas9 system, the midgut epithelium structure was not affected in the ΔHaREase mutant, but when larvae were fed an artificial diet with sublethal doses (2.5 or 4 μg/g) of Cry1Ac, the growth rate of the ΔHaREase line was repressed significantly [50]. The insecticidal activity of the *Bt*-based biopesticide Xentari™ (Valent BioSciences) against larvae of *S. littoralis* was significantly enhanced by pre-treatment with dsRNA-Bac targeted against the *Sl* 102 gene, which is responsible for insect cell aggregation and encapsulation to protect against *Bt* infection [51]. Likewise, the efficacy of biological preparations based on live *Bt* cells was enhanced when used together with dsRNA-Bac specific to sequences of the *P. xylostella* Pxf gene [52], which caused insect resistance to Cry1Ac toxin. The RNAi-mediated suppression of the Cat L-like gene encoding the lysosomal cathepsin L-like cysteine protease of *Bombyx mori* led to an increase in larvae mortality under the influence of *Bt* subsp. *kurstaki* strain ABTS-351 (Dipel®, Valent BioSciences, Libertyville, IL, USA) [53]. It is probably that the increase in insect resistance to biocontrol agents based on *Bt* strains producing toxins and the use of this bacterium as a platform for the expression of dsRNA can help in pest control using the Cry + RNAi strategy [51][52][53].

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