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Genetically Modified Potato for Pest Resistance: Thrift or Threat?

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Abstract

Significant limitations in potato production are crop loss due to the damage made by insect pests, and the cost of enormous amount of chemicals, harmful to humans and environment, extensively used in their control. As an alternative, development of genetically modified potato offered possibility for pest management in a more sustainable, environmentally friendly way. Over the past 30 years introduction of pest resistance traits progressed from a single gene to multiple stacked events and from Bt-toxin expression to expression of proteins from non-Bt sources, dsRNA and their combination, while advances in molecular biology have brought “cleaner” gene manipulation technologies. However, together with benefits any new technology also bears its risks, and there are still a range of unanswered questions and concerns about long-term impact of genetically modified crops – that with knowledge and precautionary approaches can be avoided or mitigated. Sustainability of genetically modified crops for pest control largely depends on the willingness to gain and implement such knowledge.

Keywords: potato, *Solanum tuberosum* L., genetic engineering, pest resistance, environmental safety, Bt-toxins, protease inhibitors, RNAi

1. Introduction

Almost four decades after the initial success [1], production of genetically modified plants still takes a central place in the experimental studies and biotechnology of plants. Genetic engineering has made possible introducing beneficial traits from unrelated plants, bacteria, viruses, fungi, or animal species, to overcome the major limitations of conventional plant breeding. Introduction of one or more genes into commercial crop species has helped boost crop yields due to increased resistance of transgenic lines to abiotic stress, pests and pathogens, and manipulation of metabolic pathways resulted in improving the nutritional or industrial value of genetically modified plants. Also, plant “factories” have been designed to produce high amounts of various pharmacologically important compounds, nutrients or other useful substances.

Genetically modified (GM) crops have been cultivated for more than twenty years and in 2019, the global area under GM crops was 190.4 million hectares, a 112-fold increase since their first commercialization in 1996 [2]. Gains from increased yields and cost savings brought net economic benefits amounting to more than \$225 billion and added one hundred million tons to the global crop production without the need for using additional land for cultivation [3]. The development of insect

resistant GM crops resulted in reduction of insecticides by 775.4 million kg (8.3%) and decreased the environmental impact of these chemicals by 18.5%. By cutting fuel usage associated with the production of chemical spray runs and tillage, this technology also reduced carbon dioxide emissions equivalent to removing more than 15 million cars from the roads [4]. However, wider adoption of GM crops remains the subject of biosafety concerns due to potential risks such as gene flow, evolution of resistance in insects and weeds, adverse effects on beneficial non-target organisms, or toxicity and allergenicity to humans.

2. Incorporating insect resistance traits

A wide range of pests and pathogens (over 50 insect and about 10 nematode species, 11 viral, 6 bacterial and over 20 fungal pathogens) [5] threaten potato (*Solanum tuberosum* L.), causing at least 40% of production losses worldwide [6]. Among them, Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say) and Potato tuber moth (PTM; *Phthorimaea operculella* Zeller) are the most widespread insect pests of potato that, if not controlled, can cause total yield or storage losses [7, 8]. CPB, particularly capable of rapid build-up of resistance to toxins, is today resistant to 56 different compounds belonging to all major insecticide classes with different modes of action [9], and tens of millions of dollars are spent annually for its management [10]. The long history of failure in chemical control of CPB and other pests, dubbed “the 125 years of mismanagement” [11], gave way to alternative means of control, including genetic engineering as more pest-specific and less risky for the environment.

Potato is one of the few crops naturally susceptible to infection by agrobacteria, so the first report on the generation of transgenic potato plants using *Agrobacterium* [12] dates from the very beginning of the “era of plant genetic engineering”. Since then, many recombinant DNA delivery systems have been developed (biolistic, electroporation, PEG-mediated, etc), but, enabling high transformation frequency and efficiency, *Agrobacterium*-mediated transformation has remained the preferred method for heterologous gene integration into the potato genome, and became a routine technique in many laboratories. Over the past 30 years introduction of pest resistance traits progressed from a single gene to multiple stacked events (directed to the same or different pests), and from Bt-toxin expression to expression of proteins from non-Bt sources, dsRNA and their combination. Above all, recent advances in genome editing, with its nearly unlimited potentials, could bring about a new era in crop protection.

3. Constructing Bt-potato

Isolated in 1901 as the causative agent of silkworm disease, *Bacillus thuringiensis* (Bt) toxin became the first bioinsecticide commercially available since 1938, and remained for decades the most important microbial agent for insect control. Bt crystalline proteins (Cry toxins) appeared as an alternative to chemical insecticides, with molecular potency several hundred times greater than organophosphates and synthetic pyrethroids [13]. *Cry1Ab* was the first insecticidal gene introduced in tobacco [14], and since 1996, some Bt-plants such as maize, cotton, potato and rice, became commercialized. Now, more than 700 identified *cry* genes constitute a valuable “arsenal” with high and selective toxicity towards different insect taxa – and cloning, transfer and expression of these genes is a widely adopted strategy for incorporating resistance in commercially important crops. In the USA, for instance,

Bt-maize represents 82% of total maize production, while Bt-cotton accounted for 88% of all cotton grown in 2020 [15].

When insects feed on Bt-plants, ingested Cry protoxin is solubilized and proteolytically activated in the alkaline environment of the insect midgut to the active toxin. The activated toxin goes through complex sequential binding events with an array of receptors on the surface of midgut cells, beginning with binding to cadherin, that facilitates additional protease cleavage and assembly of oligomeric forms of the toxin. The oligomers have increased binding affinity to the secondary receptors, leading to membrane insertion and lytic pore formation [16]. Such midgut tissue disruption halts insect feeding and causes subsequent mortality.

Transgenic potato lines with introduced Cry3A delta-endotoxin from *B. thuringiensis* var. *tenebrionis*, that targets coleopteran pests, showed significantly increased resistance to the CPB. Constitutively expressed in potato, Cry3A toxin caused 100% mortality of neonate larvae within two days and 99% adult mortality within two weeks [17]. Bt-transgenic NewLeaf™ potato cultivars of Monsanto Corporation were commercialized in the USA starting in 1995, and potato became one of the first GM crops commonly used for human consumption. Next, CPB resistance was combined with virus resistance, and commercial potato cultivars NewLeafPlus™ and NewLeafY™ were launched in 1998. Additional virus resistance benefited seed producers, and commercial growers gained higher yields with reduced need for insecticides. Although commercially and agronomically successful, the NewLeaf™ varieties were withdrawn from the market in 2001, due to public concerns and competition with a new, highly effective insecticide imidacloprid [18].

Expression of several Bt-toxins of Cry1 or Cry9 classes, that target lepidopteran pests, conferred resistance to the potato tuber moth (PTM), a major potato pest in tropical and subtropical regions. Bt-lines with variable level of PTM resistance have been obtained after potato transformation with *cry1Aa* [19], *cry1Ab* [20], *cry1Ac* [21, 22], *cry1Ac9* [23, 24], *cry1Ia1* (previously known as *cry5*) [25, 26] or *cry9Aa2* [27]. Among them *cry1Ac* and *cry1Ia1* expressed in potato proved to be highly effective in PTM control, causing mortality of 80–97% of first-instar larvae fed on leaves and ~ 100% on tubers [21, 26], but none of these Bt-potato lines are available commercially. Additionally, *cry1Ac* or *cry1Ia1* expressing potato exhibit appreciable level of resistance to CPB - with up to 90% reduction of feeding, that correlates with increased first instar larvae mortality [22, 26].

Moreover *cry3A* [28], *cry1Ac9*, *cry9Aa2* [29] and *cry1Ab* [30] were independently expressed in potato under control of light-inducible promoters. Such spatial expression of *cry* genes enables high level of leaf protection against CPB or PTM, with minimal or no Cry toxin accumulation in the tubers, which represents a desirable feature for consumers.

3.1 Resistance to Bt: a CPB case

Insect resistance has become a significant problem after WWII, when intensive agriculture with reliance on chemicals and uniform cultivation practices led to about 17,000 cases of insecticide resistance among 612 insect species by 2020 [9]. Since Bt-crops also provide strong and uniform selection pressure on insect populations it is hard to believe that pest problems can be solved with Bt-approach alone. By 2017, two decades after their commercialization, reduced efficacy of Bt-plants caused by field-evolved resistance has been reported in 16 out of 33 major crop pest populations, compared to only 3 reported in 2005 [31].

In Cry3A-potato, toxin was expressed at a very high level relative to the CPB susceptibility: at least 50 times as necessary to kill first instar, and at least twofold as necessary to stop third and fourth instar development or to arrest adult egg

laying [17]. Although effective in short term, this high-dose strategy represents an extremely high selection pressure for developing resistance in the insect populations, and without additional management practices, it has been predicted that CPB can develop resistance to Bt-potato within 6 generations [32]. CPB resistance potential has been demonstrated in the laboratory by repeated Cry3A toxin application, resulting in about 60-fold increase in resistance ratio after 12 generations [33], and about 300-fold increase after 35 generations [34].

Developing Bt-resistance is a complex and diverse process, and populations of the same insect species of different origins may exhibit different mechanisms of resistance to the same Cry toxin [35, 36]. Two major resistance mechanisms are: alteration of midgut proteases involved in processing of Cry proteins in the insect midgut; and modification of binding sites for Bt-toxins. Other resistance mechanisms may include retention of Bt-toxin by the midgut peritrophic membrane, aggregation of toxin proteins by the midgut esterase, elevated melanization activity of the hemolymph and midgut cells, increased rate of repair or replacement of affected epithelial cells, and increased antioxidant activity [37]. Bt-resistant CPB strains exhibit at least two levels of adaptive responses that render immunity to the Cry3A toxin: the first is lower toxin binding to the receptors, probably as a consequence of reduction of binding sites within the receptor or reduction in receptor numbers, while the second one are changes in digestive enzyme profiles and specific increase in aminopeptidase activity [38]. Although this alteration of CPB digestive profile is not connected with toxin processing or its inactivation, it can be involved in modulation and amplification of signals that activate specific innate immune responses such as melanization, coagulation and defense peptide synthesis [39] – mechanisms that have been confirmed in overcoming the exposure to Bt-toxin in other insect species [35, 40].

Moreover, plasticity of its life cycle, large pool of genetic variation in life history traits and capability to effectively cope with naturally occurring host plant toxins or almost every chemical insecticide, leave no doubt that CPB can develop resistance to Bt-potato, given sufficient time. This also brings concerns on whether CPB can be prevented from developing resistance to Bt-potato – since with only a single resistance gene expressed, the high dose/refuge strategy is the only resistance management option available [41]. Although such strategy can hinder accumulation of initially rare homozygous resistance genes in Bt-exposed insect populations by decreasing selection pressure, its effectiveness is questionable in the case of CPB. While the susceptible beetles are “arrested” on Bt-potato, in the resistant strains ingestion of Cry3A toxin significantly increased both CPB larval motility and adult flight activity, whereby more physiologically resistant individuals showed higher behavioral responsiveness. Such behavioral resistance can affect gene flow between susceptible and resistant beetles, increasing distribution of resistant homozygous CPB offsprings within and between Bt-potato fields [33, 42]. In addition, effectiveness of the refuge strategy will be compromised not only when expressed toxin genes do not kill all of the heterozygous progeny, but also if resistance is non-recessive. Evidence of both the laboratory-selected [43] and field-evolved [44] resistance to Cry toxins indicates that some populations of target pests evolve dominant resistance alleles, which can be hardly defeated with the refuge strategy.

3.2 Improving toxicity and preventing resistance

When exploring the functions of specific regions of Cry proteins, some of site-directed mutations resulted in increased binding affinity of Cry toxins to insect midgut receptors, conferring additional toxicity. For example, a triple Cry1Ab mutant protein showed up to 36-fold increase in toxicity [45], while multiple Cry3A

mutations conferred 2-fold higher toxicity against CPB [46] compared to wild-type Cry toxins. Deletion of small regions of the toxin can result in increased toxicity or in toxins that could counter insect resistance to native Cry toxins. Deletion of 42 residues of the amino-terminal region resulted in an up to 6.6-fold increase in Cry2A toxicity against a lepidopteran pest [47], while Cry1AMod toxins (that due to the lack of α -helix can form oligomers in the absence of cadherin receptor) are effective against Cry1A-resistant target pests with mutations in the cadherin gene [48]. Additionally, added cadherin receptor fragment showed significant synergistic effect with Cry toxins, including 3.7-fold and 6.4-fold enhanced toxicity of Cry3Aa and Cry3Bb, respectively, to CPB [49].

The specificity of Cry proteins allows targeting a single pest or closely related insect species within the same order, but such specificity does not provide a wide range of protection. Improving or broadening the range of protection (as well as minimizing secondary pest infestations upon primary pest control) can be achieved through combining multiple resistance factors – a strategy that at the same time prevents or delays the evolution of insect resistance. The construction of hybrid Cry toxins can confer a wider target spectrum or higher toxicity than each of the parental toxins from which they are derived. Examples include hybrid Cry1Aa/Cry1Ac and Cry1Ab/Cry1C toxins, that exhibited 30- and 10-fold higher toxicity against target pests [50, 51]. Furthermore, a *cry1Ba/cry1Ia* hybrid gene (*SN19*) driven by a light- or wound-inducible promoter protects potato leaves from attacks of coleopteran (CPB) and lepidopteran (PTM, European corn borer and tomato leaf miner) pests, causing 100% mortality of first instar larvae when fed on *SN19*-transformed potato [52, 53]. However, among all these strategies gene stacking appeared as most effective, and there are numerous examples of introducing multiple resistance or other agronomic enhancement factors in commercially grown plants, including potato where pyramided *cry3A* and *SN-19* genes can provide 100% control of CPB [54]. The first stacked-traits crop that gained regulatory approval in 1995 was cotton expressing *cry1Ab* and *epsps* (conferring resistance to the herbicide glyphosate), leading to the several hundred stacked events for increased pest resistance in commercial crops, approved to date. The recently released ten-gene maize under the name SmartStax™ Pro x Enlist™, combines three herbicide tolerance genes, six Bt-genes (targeting both lepidopteran and coleopteran pests) and *dvsnf7* dsRNA [55]. However, benefits of Bt-gene pyramiding can be compromised due to inappropriate management strategies, as well as insects capable of cross-resistance.

For instance, concurrent use of one-toxin and pyramided two-toxin crops will enhance resistance to pyramided Bt-plants if the two-gene plants produce a similar toxin as the single-gene plants (for example, this is the case for marketed maize and cotton where the additional Bt-gene was “added” to an already existing Bt-line). Target pests can evolve a single gene resistance that overcomes both Bt genes used in the pyramiding, even if expressed Bt-toxins have different binding sites. A clear example are *Helicoverpa zea* populations that exhibit increased survival on cotton with stacked *cry1Ac* and *cry2Ab* genes, as result of extensive exposure to Cry1Ac before two-toxin cotton was introduced [56]. Mechanisms that could cause cross-resistance in the target insects may include alteration in digestive proteases (if the same proteases activate or degrade both Bt-toxins) or changes affecting pore formation or pore function, a general step in the action mechanism of many Cry proteins [37]. Thus, the promising strategy for stacking varieties should be combining genes with different mechanisms of actions, such as a *cry* gene with host plant resistance or other heterologous factors (including Vip toxins, protease inhibitors or dsRNA, combined in some approved events) to minimize the possibility that random mutations in a single insect gene could confer resistance to both or more introduced traits.

3.3 Bt-related concerns

In 1999, laboratory studies showed that Bt-maize pollen had deleterious effects on Monarch butterfly larvae [57], raising questions and concerns about Bt-crop impacts on non-target organisms. Additionally, since both target and non-target insect pests ingest toxin when feeding on Bt-plants, Bt-toxin may also affect beneficial predatory arthropods through consumption of target pests or by facultative feeding on transformed plants.

Riddick and Barbosa [58] showed no adverse effect on survival, fitness or predation potential of *Coleomegilla maculata*, an entomophagous and pollenophagous beetle, when fed on Cry3A-intoxicated CPB. Similarly, another beneficial carabid beetle, *Nebria brevicollis*, was not affected with Cry3A when fed with non-target potato pest *Lacanobia oleracea* larvae [59], indicating that, due to its high specificity, Cry3A toxin presents a very low risk to coleopterans other than the targeted CPB. In addition, EPA (Environmental Protection Agency) studies on impacts of Cry3A-potato found no adverse effects on non-target wildlife exposed to the crop, indicating that beneficial arthropods were generally more abundant in Bt-potato plots compared to those treated with synthetic insecticides. Natural enemies are sufficient for aphid control on Bt-potato, while high numbers of this secondary potato pest populations are present in plots where beneficial arthropods were eliminated by insecticide treatment and no chemical aphid control was applied [60]. For instance, ladybird beetles, that are abundant and valued predatory species, preferably feeding on aphids and occasionally pollen when prey is scarce, remain unaffected on Cry3A expressing potato [59]. It was shown that Bt-potato fields were inhabited with diverse populations of these aphidophagous coccinellids, whose numbers significantly decreased with application of chemical insecticides [61]. Also, Bt-potato is not a threat to other endangered coleopteran species, since their habitat does not overlap with potato fields and their larvae do not feed on potato [60]. In addition, 25 studies that assessed potential effects of Bt-toxins introduced in commercialized GM crops (lepidopteran-active Cry1, Cry2, or Cry9 and coleopteran-active Cry3 class) found no negative effect on survival of either honey bee larvae or adults [62]. However, it may be also expected that some CPB predators will be less abundant in Bt-potato fields due to low pest densities (rather than Cry3A toxicity), such as in the case of carabid *Lebia grandis* [63], or that complexity of interaction on tritrophic (plant-pest-natural enemy) level can be altered in an unexpected way. For instance, survival, weight gain and fecundity of the wasp *Aphidius nigripes*, parasitoid of the potato aphid (*Macrosiphum euphorbiae*), was negatively affected on Bt-potato, although Cry3A did not directly affect the aphid, nor should be toxic to parasitic wasps [64].

Furthermore, studies on commercialized SmartStax maize with six Bt-genes (*cry34Ab1*, *cry35Ab1*, *cry3Bb1*, *cry1F*, *cry1A.105* and *cry2Ab2*) provided evidence that the different Cry proteins do not interact in a way that poses a risk to the investigated non-target species under controlled laboratory conditions. [65, 66]. However, data available in the literature regarding the impact of Bt-crops on non-target arthropods are mostly incomplete and sometimes controversial. Most studies have focused on certain but not all aspects of non-target or beneficial insect fitness and most of the field trials were conducted on a small scale, over a relatively short period of time.

Although free Bt-toxin released in root exudates and from decaying plant residues is rapidly degraded by soil microbes, it can be stabilized by binding on clays or humic substances and stay unchanged for two weeks to 6 months [67], depending on soil composition and pH, or crop species [68]. However, studies on Bt-crops have generally revealed no or minor transient effect on earthworms, nematodes, protozoans, bacteria, and fungi in soil [68].

Due to the acidic environment of the mammalian digestive tract and the absence of specific receptors, it is generally accepted that Bt-toxins do not bear substantial risk for human health. Additionally, about 60 years of history of using Bt-products as biopesticides showed that risks of toxicity or allergenic reactions to the Cry proteins are minimal. Cry3A toxin does not exhibit acute oral toxicity to mammals in doses 10,000 times higher than its amount in potato tubers, and is rapidly digested *in vitro* [60, 69]. In simulated digestion models the protein is degraded within 30 s to polypeptides less than 2 kDa, suggesting that Cry3A will be even more efficiently degraded in robust gastrointestinal systems of humans and other mammals. Efficient degradation and lack of structural similarity to known allergenic proteins significantly minimize the potential for Cry3A to induce allergic reactions [69]. Likewise, similar findings on safety exist for other Cry toxins introduced in maize, cotton and soy, that are authorized for cultivation in one or more countries [70]. The only exception is Cry9c toxin, which due to its resistance to breakdown by digestive enzymes may be found in the bloodstream after oral feeding in the rat model, with potency to induce immunological responses [71]. In 1998, *cry9c*-expressing maize named 'Starlink' has been approved only for animal feed and industrial use, but recalled two years later in the USA, EU, Japan and South Korea, after detection of Cry protein residues through human food supply. This controversy indicated the need for a broader and properly managed assessment in monitoring and enforcement concerning potential health risks of toxicity, allergenicity and genetic hazards associated with Bt-crops, to ensure their greater acceptance. Although majority of studies indicate that Bt-crops would be as safe as parental lines – with few exceptions [72, 73] that were rather critiqued than accepted in scientific community – studies on the long-term health effects of Bt-plants will still be necessary [74]. Also, the potential of cumulative, combined or unexpected effects in the “next generations” Bt-crops with stacked *cry* genes, or combined with other resistance factors, clearly calls for revisions of “outdated” risk assessments made based on single Bt-gene expression.

4. Targeting digestive enzymes

As a reflection of more than one hundred million years of coevolutionary “arms race”, plants developed numerous mechanisms to resist the attacks of pathogens and herbivores. Here, being part of the plant “chemical warfare” arsenal, secondary metabolites take an important place, with more than 200,000 known compounds with defensive activity. Among that broad repertoire, protein antimetabolites such as lectins, α -amylase inhibitors and especially plant protease inhibitors (PIs) are the most used for engineering crop resistance against various pests.

The most important role of PIs in plants is protection from both biotic and abiotic stresses. They may also have other functions: from tissue-specific regulation of endogenous proteases – especially in storage organs such as seeds and tubers [75], to the regulation of programmed cell death [76]. About 500 plant PIs were described, and according to the protease type they inhibit, PIs are classified as cysteine, serine, aspartyl and metallo protease inhibitors [77]. Generally, the inhibition is based on PIs binding to or near the enzyme active site, forming a stable complex with a low dissociation constant. This complex is often additionally “locked” by disulphide bonds, so that upon eventual hydrolysis the inhibitor remains associated to the enzyme, effectively blocking access of the substrate [78]. The mechanism of PIs antimetabolic effect on insects has not been fully elucidated and, due to its high specificity, it is assumed that different types of PIs also have different modes of action. The simplest model implies a direct antidigestive effect due to inhibition of proteolysis [79]. The

second, more accepted model, is based on compensation for the loss of proteolytic activity – proteinase hyperproduction – which by redirecting amino acid utilization reduces their availability for insect growth and development [80] which, in addition to reduced performance, often increases insect mortality. PIs can also disrupt processes such as molting, neuropeptide synthesis, water balance, and enzyme regulation [81–83] or directly interfere with insect reproductive processes [84].

The early evidence on the protective role of PIs came in mid-20th century, when it was observed that soybean products negatively affect development of red flour beetle larvae [85]. In a pioneering research, Green and Ryan [86] reported on a rapid, both local and systemic, accumulation of PIs in potato and tomato leaves upon CPB attack, demonstrating the importance of PIs in plant defense against insects. Not long after, the first PI-transformed plant, tobacco expressing cowpea trypsin inhibitor, CpTI, conferred increased resistance to several lepidopteran, coleopteran and orthopteran insect pests [87]. This initial success triggered a generation of numerous transgenic plants expressing different PIs, more or less efficient in control of target pests. However, despite this promising development, none of PI-transgenic plants have been commercialized to date. One of the reasons is the conclusive “acute mortality” efficacy of Bt-plants, similar to the chemical insecticides. By contrast, PIs often cause decrease in insect fitness on a relative level, such as a reduction in growth and reproduction or extended development, that in a time scale can significantly reduce the size of pest population (for example, prolonged larval development brings longer exposition to predators, while the reduction in body mass decreases investment in reproduction). Secondly, a more important reason are adaptive capacities of insects that can compromise this approach, clearly demonstrated in some cases. These evolutionary, diet-induced strategies include overproduction of sensitive digestive enzymes that outnumber inhibitors, switching to digestive protease complements insensitive to PI or PI degradation with non-target proteases [88].

After evidence of deleterious effects of E-64, a broad spectrum thiole cysteine PI isolated from *Aspergillus japonicum*, on larval growth, survival, and adult fecundity of CPB [89], CPB cysteine proteinases (that account for most of CPB digestive proteolysis) have become target for heterologous cystatins expressed in potato plants. Two rice cystatins, oryzacystatins I and II (OCI and OCII), although exhibiting inhibition of CPB larvae cathepsin H-like proteases *in vitro* [90] proved ineffective in CPB control. With no increase in mortality, CPB larvae overcame initial digestive inhibition by hypertrophic behavior and restored cysteine proteinase activity by introducing isoforms insensitive to OCI [91] or OCII [92]. Contrary to expectations, some aspects of CPB larvae performance were actually enhanced by chronic ingestion of each of the two rice cystatins: faster growth and leaf consumption, shorter development time and even increase in body mass before pupation in case of OCI [91]. Slight reduction in insect growth rate was also observed with recombinant CDI (cathepsin D inhibitor from tomato), as a result of overproduction of inhibitor-sensitive proteases. However, after this initial response CPB larvae switched their digestion to the CDI insensitive protease complement, resuming normal growth and development despite ingestion of the inhibitor expressed in potato plants [93].

These results clearly demonstrate that, due to its exceptional adaptability to the different host plant protective compounds [88], CPB can hardly be controlled by a single, narrow spectrum PI. Thus, to achieve more efficient control and prevent compensatory insect responses, broadening the spectrum of inhibition by protein fusion, transgene stacking or using multidomain PIs appeared as a possible solution. However, only a slight reduction in CPB larvae performance was achieved in potato expressing stacked rice cystatins, OCI and OCII [94, 95] or with multidomain serine PI from locust (LIP), active against both trypsin and chymotrypsin [96].

In contrast to this, equistatin, a PI from the sea anemone, with one domain that inhibits cysteine and a second domain active against aspartic proteases, had detrimental effect on CPB larvae growth and significantly increased their mortality after ingestion of equistatin-coated potato leaves [97]. Unfortunately, with expression of this potent PI in potato very low resistance level against CPB was achieved: the amount of active inhibitor in leaves was considerably reduced due to its degradation by native potato proteinases [98]. The promising results came with a hybrid CDI-CCII inhibitor (fusion of CDI with maize cystatin II), also active against both aspartate and cysteine proteinases. When painted on potato leaves, CDI-CCII initially reduced CPB larvae growth and food consumption by about 50% [99], but its real effects still remain to be proved in long-term feeding assays. Finally, fungal cysteine PIs, macrocypin and clitocypin, emerged as more favorable. Exhibiting strong inhibition of CPB cysteine proteinases, these PIs, introduced in potato, reduced growth and increased development time of CPB larvae [100, 101]. Moreover, the most promising trait of macrocypin and clitocypin is the absence of CPB digestive compensatory responses [100, 101] observed for PIs derived from other sources. However, relatively low expression was achieved in transgenic potato and, since they act in dose dependent manner, it is necessary to improve macrocypin and clitocypin expression levels for more pronounced negative effects on CPB larvae.

Additionally, potato expressing serine PI (CpTI or Soybean Kunitz, C-II and PI-IV) exhibited enhanced resistance to the lepidopteran larvae with about 50% reduction in total insect biomass [82, 102].

Several approaches based on structure–function models have been used to improve the inhibitory potency of protease inhibitors against specific proteases, including site directed mutagenesis of specific amino acids, molecular phage display procedures involving random mutagenesis in specific regions of the inhibitor sequence, or activity-based functional proteomics approach. By single mutations at the positively selected amino acid sites of the tomato multicystatin SlCYS8, variants with improved inhibitory potency toward the CPB digestive proteases were generated [103], and functional proteomics approach was used for identifying variants that efficiently capture CPB digestive protease targets [104]. P2V10, the most potent variant of SlCYS8 PI, expressed in potato, significantly reduced growth of CPB larvae in a 72 h feeding assay [104]. Similarly, after 4 days of feeding on potato expressing a modified variant of cystatin from barley (HvCPI-1 C68 fi G), that targets the cathepsin B-like fraction of cysteine digestive proteolysis, CPB larvae had about 23% lower weight, probably due the metabolic cost associated with the hyperproduction of inhibited digestive proteases [105]. However, knowing the remarkable CPB larvae adaptability to adjusting their digestive profile to functionally distinct plant PIs, studies assessing the long-term detrimental effects of these engineered cystatins are needed.

Although the usefulness of recombinant PIs expressed alone still remains to be proved or improved, they can enhance Cry toxicity. Several serine protease inhibitors can increase the insecticidal activity of Cry toxins 2–20 fold [106] and delay the resistance evolution of the targeted pest [107]. Although it is not known how PIs enhance Bt-toxin activity, it is supposed that they may inhibit the inactivation of Bt-toxins by specific gut proteases, or prevent the degradation of membrane receptors, increasing binding ability of Cry toxins [108]. In such way, hybrid SN19 (*cry1Ba/cry1Ia*) combined with OCII in potato caused 100% mortality of all CPB larval stages within 6 days, and adults within 2 weeks [54]. However, as of today there are only three approved events with PI (all stacked with *cry1Ac*): cotton co-expressing CpTI, maize with *pinII* (from potato) and poplar with API (from *Sagittaria sagittifolia*) [55].

Due to the existence of targets in most organisms in nature, beside the toxic effect on the pest, recombinant PIs can directly affect the digestive proteolysis in pollinators, symbionts and/or indirectly, through prey feeding on transgenic plants, they can endanger the ecological function of predators. However, although artificial diet studies indicate that predatory insects may be susceptible to the PI, prey-mediated effects are usually not observed when cystatins or CpTI are expressed in transgenic potato. When *Podisus maculiventris* was fed with tomato moth (*L. oleracea*) caterpillars reared on CpTI-potato plants, no negative effects on the predator were observed [109]. Predation on neither CPB nor Egyptian cotton leafworm (*Spodoptera littoralis*) larvae reared on potato plants expressing barley cystatin had negative effects on survival and growth of the predatory bug *P. maculiventris* [105]. Also, no detrimental effects were observed on larvae and adults of the ladybird *Harmonia axyridis* upon consuming larvae of diamondback moth (*Plutella xylostella*) reared on OCI-expressing plants [110], or in *Diaeretiella rapae*, a parasitoid of potato-peach aphid (*Myzus persicae*) [111]. Stinkbug *Perillus bioculatus* feeding on CPB reared on OCI-potato compensated for the effects of this cystatin by introduction of serine-type proteases [112], while improved performance of secondary pest *Macrosiphum euphorbiae* on the same host plant also improved performance of the parasitoid wasp *Aphidius nigripes* [113].

On the other hand, although the effects of native plant PIs, such as CpTI or OCI, on non-target organisms have been well documented, there is little evidence of effects of new-generation inhibitors with stronger effects on pest proteinases, hybrid inhibitors or combined effects of several different insecticidal proteins. The challenge, of course, is to find or devise those variants of PIs that show increased activity against the target pest proteinases and decreased activity against proteinases of the host plant or of beneficial insects. Also, cystatins that occur naturally in seeds of rice and maize, present in potato tubers or in egg-white, are not novel in the human diet, and expressed in transgenic plants should not cause public concerns [114] – but the expression of strong broad-spectrum aspartate and serine PIs may raise many questions in the future.

5. Lectins

Widely distributed in nature, lectins are a heterogeneous group of sugar-binding proteins with numerous biological functions. In plants they are involved in the transport and utilization of carbohydrates, cell organization, division and signaling, embryomorphogenesis, phagocytosis or as mediators of plant-microorganism symbiosis [115]. However, their most distinctive role is in plant defense mechanisms against pathogens and pests. Binding to a variety of glycoproteins, plant lectins can inhibit absorption of nutrients by disruption of insect gut epithelium structure or, by interacting with targets in insect hemolymph, fat tissue and ovaries, interfere with a number of physiological processes, such as growth, development and detoxification [116]. Although they can exhibit protective roles against insect pests from different orders, lectins are particularly useful for controlling Hemiptera, that are generally less sensitive to Bt or PIs.

Snowdrop mannose-binding lectin (*Galanthus nivalis* agglutinin, GNA) is the first lectin known for insecticidal activity. Expressed in potato, GNA can decrease growth and fecundity of potato-peach aphid (*M. persicae*) or glasshouse-potato aphid (*Aulacorthum solani*), reducing the rate of their population growth up to four times [117, 118]. Effects of GNA on *M. persicae* vary with its expression level in potato plants: at low level GNA reduces colonization of transformed potato, without significant impact on insect performance [119], while highly expressed, GNA can

reduce aphid survival and performance [120]. Besides, GNA can be effective in control of lepidopteran pests – tomato moth (*L. oleracea*) larvae exhibited about 50% reduction in biomass, prolonged development and 40% increased mortality rate when fed on transformed potato [121]. Concanavalin A (ConA), a glucose/mannose-binding lectin from jackbean (*Canavalia ensiformis*), can also be effective in control of both hemipteran and lepidopteran potato pests. Despite its relatively low expression level in potato plants, ConA decreased the fecundity of *M. persicae* (up to 45%) and reduced *L. oleracea* larval weight (about 50%) and retarded their development [122].

However, lectins can negatively impact beneficial non-target organisms, and for instance, preys that were fed on GNA potato were less favored or resulted in smaller, shorter-lived predators or parasitoids [123, 124]. Although they are present in most plants – especially abundant in cereal and legume seeds or potato tubers – lectins are generally considered toxic to animals and humans. So even though GNA did not show considerable toxicity in rat feeding studies [125], there is no doubt that food expressing such proteins requires long-term studies to evaluate its potentially harmful effects.

6. Silencing vital genes

After the Nobel prized discovery of RNA interference (RNAi) as a basic mechanism of post-transcriptional gene silencing by double-stranded RNA (dsRNA) [126] RNAi has become a powerful experimental tool for determining gene functions, had an immense impact on biomedical research and found its application in the management of insect pests. Evolutionarily conserved in all eukaryotes, the mechanism of RNAi is involved in different processes including internal gene regulation (micro RNA or miRNA pathway), genome protection against transposons (piwi-interacting RNA or piRNA pathway) and defence against viral infections (small interfering RNA or siRNA pathway) [127]. Although the siRNA pathway in insects mostly represents the first line of defense against viral RNA, it can be exploited for introduction of specific dsRNA that, through mechanism of RNAi, can initiate degradation of complementary endogenous insect mRNA. Thus, selection of any target gene and delivery of its sequence-specific dsRNA to cells can lead to functional knockout of that gene – affecting insect growth and development or increasing their mortality. A first proof-of-concept came in 2007, when transgenic maize expressing V-ATPase-specific dsRNA showed significant reduction in feeding damage caused by western corn rootworm (WCR) [128]. Maize with dsRNA transcript containing a 240 bp fragment of the WCR *Snf7* gene (encoding a membrane-remodeling protein) stacked with several *cry* genes (*cry3Bb1* and *cry34/35Ab*) was first such crop commercially approved in 2017, and five more events expressing *Snf7* dsRNA and different Cry proteins stacked in maize were approved to date [55].

However, various studies showed that different insect orders differently respond to orally delivered dsRNA – coleopterans are mostly sensitive, while RNAi efficiency is low for most lepidopterans. Multiple mechanisms contribute to this variability, including instability of dsRNA upon ingestion, insufficient dsRNA internalization, endosomal entrapment, deficient function of the RNAi machinery and reduced systemic spreading. Once consumed, the dsRNA first has to avoid degradation by dsRNases (dsRNA-specific ribonucleases) on their way through insect digestive tract. Level of dsRNA degradation by saliva or midgut nucleases varies among different insect orders and, for instance, midgut stability of dsRNA is greater in the CPB (Coleoptera) than in *Schistocerca gregaria* (Orthoptera) or budworm *Heliothis virescens* (Lepidoptera) [129, 130]. The next barrier is the internalization of the

dsRNA in the cell. Two mechanisms of cellular uptake of dsRNA have been identified in insects: SID-like (Systemic RNA Interference Deficient) transmembrane channels, and clathrin-dependent endocytosis. The latter mechanism seems to play the primary role in the uptake of dsRNA in many insect species, whereas SID-like genes have been identified in Hemiptera, Lepidoptera and Coleoptera but their additional role in mediating dsRNA uptake has only been confirmed for WCR and CPB [131, 132]. In clathrin-dependent endocytosis, after binding to the receptors and forming endosomes, the dsRNA is released into the cytoplasm before reaching the lysosomes. In CPB, such endosomal escapes occur easier than in most lepidopterans, where the dsRNA can enter the cells but remains trapped in the endosomes [130].

Once taken up in the cytoplasm, dsRNA is recognized by the core RNAi machinery and processed into 21–23 bp siRNA by the enzyme Dicer 2 (DCR-2). The siRNA are loaded onto Argonaute 2 (Ago-2) protein and incorporated into the RNA-induced Silencing Complex (RISC). Upon degradation of the passenger strand of siRNA, RNase active domain of Ago-2 cleaves the mRNA recognized by the siRNA guide strand, inducing gene silencing. One of the reasons for efficient RNAi in coleopterans is the duplication of core RNAi pathway genes, including DCR-2 and Ago-2 [133]. Additionally, in CPB, components of miRNA and piRNA pathways are also critical for effectiveness of gene silencing by the siRNA pathway, but their involvement in dsRNA-mediated RNAi needs to be further investigated in Coleoptera and other insects [134]. A particularly interesting aspect of the RNAi response in insects is its potential systemic character, whereby the silencing signal can spread from the midgut to other tissues, causing systemic RNAi. The exact nature of this signaling pathway still remains elusive, and efficient silencing of genes in midgut tissue was predominant, especially in more derived dipteran and lepidopteran species that appear to be more refractory to systemic RNAi [135].

Although there is a vast number of essential genes in insect genomes, the choice of the target gene can significantly affect the efficiency of RNAi – but the factors making one essential gene a better target than another one are not currently understood. Variation in transcriptional activity, mechanisms of expression regulation, mRNA stability and its accumulation level may play an important role in defining a particular gene susceptibility to dsRNA, and screening of a larger number of potential target genes for RNAi efficiency remains the only reliable method of choice.

6.1 Targeting CPB

The availability of the CPB transcriptome [136] allows specific targeting of CPB genes critical for normal physiological processes and numerous studies demonstrated successful knockdown of target genes in dsRNA-fed CPB. Silencing the expression of genes that are crucial for maintaining physiological functions, such as actin and V-ATPase genes, or genes coding components involved in protein transportation (*Sec23* and *COPβ*) can directly impair growth and induce mortality [137]. Knockdown of genes crucial for synthesis of 20-hydroxyecdysone and juvenile hormone, disrupts larval molting and pupal metamorphosis, decreasing the emergence of adults [138–141], while suppression of proline degradation (necessary for ATP production) reduces flight ability and increases mortality of CPB adults [142, 143]. In addition, RNAi can enhance the effectiveness of other control measures or resistance factors introduced in potato. For instance, suppression of CncC, a transcription factor regulating multiple cytochrome P450 genes, increased CPB susceptibility to insecticide imidacloprid [144], while silencing of a Cry3Aa-binding protein, prohibitin, enhanced the toxicity of Cry3Aa [145].

Reduction in CPB juvenile hormone (JH) titer, that regulates metamorphosis and reproduction in insects, was achieved by knockdown of JHAMT (JH acid methyltransferase), the last rate-limiting enzyme in JH biosynthesis. Feeding on transgenic potato plants expressing *dsJHAMT* had negative impact on CPB larvae growth and development, increased larval mortality (about 25%) and reduced pupation rate by 50%. Moreover, emerged CPB adults had lower weight and females lay fewer or no eggs, which was confirmed in field trials [146]. Additionally, feeding CPB larvae on transgenic potato expressing *EcR* (molting-associated Ecdysone receptor) gene dsRNA resulted in 15–80% mortality, reduction in body weight and disturbed metamorphosis [147]. However, the success of the RNAi gene silencing is limited by the level of dsRNA expression and dsRNA stability in transgenic plants. Since insects lack RNA-dependent RNA polymerase, the RNAi signal cannot be amplified in their cells, and efficiency of target gene knockout mostly depends on the amount of ingested dsRNA. Also, insects are more responsive to longer dsRNA – but dsRNAs produced in plant cytoplasm are usually processed into siRNAs by native plant RNAi machinery. For example, dsRNAs longer than 60 bp can trigger *DvSnf7* gene silencing in WCR, while 21 bp siRNAs were not efficient [148].

On the other hand, transformation of chloroplast DNA has potential for overcoming the constraints of nuclear transformation in dsRNA-mediated pest control. First advantage of transplastomic plants are markedly high gene expression levels, that due to tissue specificity, occur predominantly where functional plastids are present. An example is expression of *Cry2Aa2* protoxin in tobacco chloroplasts in 20- to 30-fold higher levels than current commercial nuclear transgenic plants, which is lethal for both susceptible and Bt-resistant target insects [149]. Secondly, a great advantage of plastid transformation is the stability of dsRNA in plastids, as chloroplasts do not have the RNAi machinery. Among about 130 genes encoded by the chloroplast genome, none is Dicer-like or Argonaute-like, and there is no evidence of import of these nuclear-encoded proteins in chloroplasts [150]. Three recent studies demonstrated that when expressed from chloroplast genome, hp/dsRNA can confer a high level of protection against either lepidopteran (*Helicoverpa armigera*) [151, 152] or coleopteran (CPB) pests [153], compared to their nuclear transgenic counterparts [152, 153]. Transplastomic potato expressing β -actin (*ACT*) or *SHRUB* (analog to *Snf7*) dsRNA, or both, produced large amounts of unprocessed dsRNA in leaves (but not in tubers) with detrimental effect on CPB growth and development. All first-instar larvae fed on transplastomic *ACT* dsRNA-expressing plants died within 5 days, while 40% of larvae survived on *SHRUB* dsRNA-expressing leaves. Nuclear-transformed plants produced much less dsRNA but more siRNAs, exhibiting a weaker suppression of target mRNA and almost no mortality was observed in CPB fed with leaves from nuclear transgenic potato [153].

Furthermore, chloroplast genome transformation also offers other advantages over nuclear transformation, including introduction of multiple genes in a single transformation event and lack of gene silencing, position or pleiotropic effects. Additionally, maternal inheritance excludes plastid genes and therefore reduces dispersion of the transgene by pollen transmission, increasing the biosafety of transgenic plants. However, plastid transformation is still much more challenging than nuclear transformation and limited by the methods of DNA delivery, homologous recombination efficiency and the methods for efficient selection and regeneration of transformants [154].

6.2 RNAi-related concerns

Numerous studies have shown that under long-term pressure of control strategies such as chemical insecticides or Bt-toxin, insects can rapidly evolve

resistance, and there is no reason to believe that it would be differently with RNAi. Theoretically, there are three possible sources of resistance: mutations in the sequence of the target gene, mutations inactivating the RNAi machinery and mutations that affect the stability and/or uptake of ingested dsRNAs in the insect digestive tract. First two mechanisms are unlikely to become source of resistance. For instance, in CPB the mismatch rate of β -Actin dsRNA and a target mRNA lower than 3% does not reduce the RNAi efficiency [155], while drastic sequence changes in target (essential) genes or those that inactivate the highly conserved genes of the RNAi machinery can easily jeopardize insect fitness and survival. However, the third scenario is quite possible and a first insect population, WCR with developed resistance to RNAi was reported in a transgenic maize field. Moreover, *DvSnf7*-dsRNA resistance in WCR is not sequence-specific, and cross-resistance to other dsRNAs is connected with dsRNA uptake rather than degradation [156]. Similarly, cross resistance to dsRNAs was achieved in a laboratory population of CPB, where foliar application of V-ATPaseA dsRNA resulted in >11,100-fold resistance after nine generations of selection [157]. Again, reduced uptake of dsRNA in midgut cells was responsible for the evolution of RNAi resistance.

With perfect sequence homology between dsRNA and mRNA only target gene suppression is expected, but it appears that siRNAs operate within cells with a certain level of “freedom” among targets. Mutation analyses showed that RNAi can be efficiently triggered with >80% sequence identity between siRNA and mRNA [158] but this mismatching tolerance can vary with insect species, target gene and dsRNA concentration [159, 160]. Moreover, dsRNA can provoke responses independently of its sequence, affecting insect antiviral immunity, gene expression and performance [158, 160]. Although not fully understood, these effects are particularly pronounced for dsRNA administered at high concentrations, supposing that high levels of siRNA may saturate the core RNAi machinery [161]. Given the small sizes of siRNAs, off-target effects that can appear in RNAi are probably quite common [162] and not considered as a concern in target organisms, but off-target binding in non-target organisms can represent a hazard if they are sufficiently exposed to the RNAi. To date, question how dsRNAs affect target and off-target genes in non-target organisms has received little attention, and existing studies indicate that the insecticidal effects of *V-ATPase*, *DvSnf7* or *NUC* (nuclease) dsRNAs are narrow, presuming adverse effects on non-target arthropods to be very low [163–165]. Additionally, in crops expressing dsRNA non-target insects can be only affected by feeding on plant. In the case of transplastomic potato expressing β -actin dsRNA [153], non-target insects had to consume potato leaves to be affected by RNAi – but by doing so they were considered pests. At the same time, pollinators and pollen-eating insects are exposed to minimal amounts of dsRNA, since chloroplasts are excluded from pollen due to maternal inheritance. Thus, careful design of the dsRNA and bioinformatic analyses can minimize non-target or off-target effects, but they cannot be completely excluded, since siRNAs do not need to share perfect sequence identity with target mRNAs to inhibit their translation in both predictive and unpredictable ways.

dsRNAs exhibit low persistence in environment and microbial degradation of nucleic acids has been shown to be a key driver for such lack of stability. Biological activity of *DvSnf7* dsRNA expressed in maize was undetectable within approximately 2 days after application to soil [166], and within 7 days in the aquatic environment [167]. In addition, biodegradation kinetics of dsRNA were independent of the dsRNA concentration, sequence length and secondary structure (hairpin or linear) [166].

Vertebrates are exposed to dietary intake of a number of various dsRNAs from animal, plant or microbial origin. Some are completely complementary to human or

animal genes [168] and capable of initiating the RNAi pathway if they reach a target cell. However, there are numerous biological barriers, including nucleases along the digestive tract, and in bloodstream, series of cellular membrane barriers and endosomes significantly reduce dsRNAs to the levels insufficient for mediating RNAi. In 28-day repeat oral toxicity study in mice with *DvSnf7* dsRNA or with siRNAs and a long *V-ATPase* dsRNA (effective in WCR control and with 100% sequence complementarity to mouse vacuolar *ATPase*) no adverse effect was observed, even with doses billions time higher than anticipated human dietary exposure [169]. Thus, according to available data, it is likely that consumption of plants expressing dsRNA will not present a safety issue. However, whether ingestion of dsRNA can affect the immune systems of humans and animals, both directly or through impacting the gut microbiota, is currently unclear [170].

7. Transgene flow

Gene flow is the transfer of genetic material from one organism to another, including inheritance (vertical gene transfer) or transfer between unrelated species (horizontal gene transfer). Although horizontal gene transfer can contribute in “shaping” genomes of both prokaryotes and eukaryotes, there are almost no evolutionary examples of gene transfer from eukaryotes to bacteria [171]. Transfer of plant DNA to bacteria has been demonstrated at a very low frequency under artificial conditions, and the only genes from GM plants that are likely to be successfully transferred are other bacterial genes, commonly used for selection in transformation [172]. More than 90% of transgenic plants that have been generated in different laboratories carry one of the three genes used for selection (resistance to antibiotics kanamycin or hygromycin, or herbicide phosphinothricin) [173], all of bacterial origin. Antibiotics are the most effective selection system for potato transformation, increasing its efficiency from 0.2%–4.5% under non-selective conditions to over 80% [174, 175]. However, they generally have no use after the selection phase of transformation, and can be completely removed or excised by different approaches, including segregation from the gene of interest after co-transformation, and different site-specific or homologous recombination systems [176]. In this way, using self-crossing segregation or inducible self-excision by the Cre-loxP system, selectable marker-free transgenic potato lines with increased resistance to pest or pathogens were created [177, 178], alleviating possibility of horizontal gene transfer.

On the other hand, vertical gene transfer, especially mediated by pollen, raises more concern. Transgene escapes have been documented for cotton, maize, soybean, oilseed rape, rice and wheat, indicating global dimensions of this problem [179]. In the case of Bt-plants, crop-to-crop gene flow can cause seed contamination, decrease efficiency of refuge strategies, or interfere with conventional or organic crop production. For instance, in Mexico where GM maize was not allowed for commercial cultivation, transgene escapes (*Bt-cry9C*, *Bt-cry-1Ab/1Ac* and *CP4 EPSPS* herbicide resistance transgene) have been found in traditional maize varieties [180]. An additional concern is the risk that pharmaceutical proteins, industrial enzymes, and vaccines produced by transgenic crops considered unsuitable for human consumption, can enter the food supply by outcrossing [181]. Transgenes can also move from GM crops to their wild relatives and alter their fitness, so that wild or weedy populations become more competitive and/or invasive, especially with introgression of insect-resistance or herbicide-tolerance genes. Although this invasiveness is more hypothesized than proven, GM crops or their volunteers often grow in vicinity of their wild variants, and hybridization with these plants

has frequently occurred. Examples include cotton and oilseed rape, where traits of insect and herbicide resistance, even stacked in combinations that do not exist in commercially available crops, were found in their wild relatives [179].

Cross-pollination between GM and non-GM potato should be less worrying, since vegetative propagation by tubers (rather than true seeds) is the dominant reproduction strategy of potato, and tubers are not affected by the plant fertilization with “foreign” pollen. Outcrossing has been observed to occur only between adjacent potato fields, with rapid decreasing rate with distance, and no cross-pollination detection when the pollen-receiving plants were separated by more than 20 meters from the GM plants [182]. Additionally, majority of modern cultivars that evolved from complex hybridizations among several diploid and polyploid potato species, suffer from different types of male sterility and produce little or no viable pollen. Also, *S. tuberosum* is not able to hybridize with any of the non-tuber bearing *Solanum* species outside of the section *Petota* [183], and in most parts of the world, crosses with wild or cultivated relatives are highly unlikely, due to geographical isolation from potential crossing partners with a suitable endosperm balance number [184]. In contrast, from Southwestern USA to Southern Chile, in areas of potato diversity, natural hybridization occurs between wild and cultivated *Solanum* species [185], bearing risk of the gene flow from transgenic potato to neighboring plants of related species. Nevertheless, with measures such as increased isolation distance and development of transgenic lines from male sterile potato varieties [186], undesirable introgression in these wild species can be prevented or minimized. Besides, other biological means of confinement, including chloroplast transformation, apomixis, cleistogamy and diverse genetic barriers [179], can further minimize risks of transgene escapes.

8. Unintended traits

Crop improvement by genetic engineering requires obtaining transgenic lines with adequate expression of the heterologous gene and simultaneous preservation of all elite parental genetic attributes. One of the main limitations in achieving these requirements is the emergence of atypical plants – most often as a result of insertional mutagenesis or somaclonal variations that may occur in the tissue culture itself and/or during transformation.

In many plant species, including potato [187], the frequency of heterologous DNA insertions within coding or regulatory gene sequences exceeds 50% upon genetic transformation. Additionally, insertion-site mutations can alter the expression patterns of neighboring genes, especially if the heterologous gene is under the control of a strong promoter [188]. Another type of mutation, related to the transformation process itself, can occur in any part of the plant genome (genome-wide mutations) and is reflected in DNA polymorphism between transgenic and non-transgenic plants [189]. These latter changes are of epigenetic nature: the transformation process can activate transposon elements (TEs – whose activity is normally prevented by DNA hypermethylation), which then increase mutation rates and genomic rearrangements [190]. It is assumed that the same mechanism – activation of TEs – underlies somaclonal variations, a phenomenon associated with *in vitro* tissue culture and particularly pronounced during the callus phase which is characterized by a general reduction in cytosine methylation levels [191].

Insertional mutagenesis is not expected to be manifested in potato, being autotetraploid and possessing three other alleles that can potentially compensate for the insertional effect of a gene functional deletion. Even when insertional mutagenesis produces visible phenotypic changes due to the high heterozygosity

of commercial potato cultivars, such phenomena are considered an extremely rare event [192]. On the other hand potato is quite susceptible to somaclonal variations in tissue culture even in the absence of transformation [193]. The incidence of atypical plants attributed to somaclonal variations, ranges between 15% and 80% in the population of transgenic potato lines, depending on cultivar [192, 194]. These are often manifested as reduced growth, deformed leaf shape, lower yield and other changes in development, clearly visible in changing field conditions, rather than in uniform ones such as greenhouses or *in vitro* cultures [192, 195]. Elimination of these variations by sexual hybridization is impossible without the simultaneous loss of the genetic integrity of the initial line, while asexual reproduction permanently fixes the status of the transgene within potato genetic background. Thus, the emergence of atypical plants is most often overcome by creating a large population of transgenic lines and selection of several lines with the desired phenotype and high transgene expression.

Beside insertional mutagenesis or somaclonal variations, the unexpected changes in transgenic lines may be a consequence of the transgene expression itself. It is especially expected with PIs, that may interact with plant endogenous protease targets structurally and functionally related to insect digestive proteases, bringing both positive and negative pleiotropic effects *in planta* [196]. For example, metabolic interference of introduced resistance factors in potato can impact protein levels in leaves, positively or negatively [197, 198], reduce glycoalkaloid levels naturally involved in host-plant resistance [199] or, on the contrary, trigger constitutive expression of naturally abiotic or biotic stress-responsive proteins, unexpectedly providing wider protection than the transgene itself [200].

Unintended traits have been identified in commercial GM crops, including insect or herbicide resistant maize, cotton, soybean and oilseed rape – that can exhibit different agronomic and compositional changes relative to their non-GM parental lines [201]. For example, Mon810 maize, carrying *cry1Ab*, exhibits compositional differences such as increased lignin, altered sugar and protein content, and a slight but significant delay in seed and plant maturation, connected with differential expression of 140 genes compared to its near-isogenic variety [202, 203]. On the other hand, plants protected by introduced insect or pathogen resistance are expected to reduce upregulation of self-defense proteins and metabolites compared to less protected near-isoline plants [204] since they experience a different level of biotic or abiotic stress related to transgenic traits. Furthermore, occurrence of unintended effects is not unique to the introduction of recombinant DNA. Traditional breeding is also confronted with undesired changes that result from hybridization, natural genetic recombination and chromosomal rearrangements or activity of transposable elements in plant genomes. There are a number of examples where conventional methods resulted in undesired effects, including potato cultivars with high level of glycoalkaloids [205], that were withdrawn from the market. Thus, for a safety assessment, it is necessary to ensure that transformation does not introduce new compounds, or cause changes in the levels or characteristics of endogenous compounds that may negatively impact human health [206]. Whether the transgenic line is as safe as its conventional variety is the fundamental safety issue to be addressed, rather than how much different they are.

9. Beyond transgenesis

Owing to public concern and reserved acceptance of transgenic crops in many parts of the world, two approaches, cisgenesis and intragenesis, are designed as an alternative to “old” transgene technology. Both concepts include introduction of

genetic material derived from the species itself (intragenesis) or closely related, cross compatible species (cisgenesis). Although they use a genetic transformation step, the modified crop genome is designed to not contain any foreign gene, including selectable markers. Therefore, crops developed using these techniques correspond to plants generated through conventional breeding, but without unintentional introduction of undesired genetic elements. Intragenesis has been successfully used for developing potato with high amylopectin content by silencing of the granule-bound starch synthase gene, *GBSS* [207] or for potato with improved processing qualities, by specific tuber-silencing of several genes, *StAst*, *PhL* and *R1* (for low acrylamide) and *ppo* (reduction in black spot bruise development) [208, 209]. On the other hand, cisgenesis has been used for late blight resistance, with introduction of *Rpi-vnt1* gene from *Solanum venturii* in potato [210]. These intragenesis-generated potato varieties have been approved under different commercial names, including traits of modified tuber quality stacked with cisgenic late blight resistance (for instance Innate® Hibernata or Innate® Acclimate) [55].

Genome editing is the latest and most potent molecular technology. Using programmable endonucleases (Zincfinger, TALENs or CRISPR-Cas), alterations can be made at precise locations in the genome, including targeted insertion, replacement or disruption of genes in plants. Because of their precision, these techniques can produce fewer unintended effects, and therefore “edited” crops are considered potentially safer than those generated by random mutagenesis or insertion. In case of potato, both TALENs and CRISPR/Cas9 technologies have been mainly used to improve tuber quality (glycoalkaloids reduction, low acrylamide content and altered starch metabolism) or for herbicide resistance [211], but despite unlimited potential in genetic engineering, no pest-resistance gene incorporation has been reported yet. Importantly, CRISPR-based gene drives could be implemented to spread desirable genetic elements through pest populations themselves. For CPB, there is only one such report to date, where CRISPR/Cas9 was used for *vest* gene knockout, which resulted in a wingless phenotype [212]. However, this potential of gene editing for pest control or even pest eradication is currently highly controversial.

10. Looking into the future

For the growing world population that is expected to reach 10 billion by 2050, food production should be increased by 25–70% and, at the same time, it is necessary to reduce nutritional losses, greenhouse gas emissions from agriculture, pesticide overuse and address other environmental concerns [213].

Potato is now the world’s third most important crop for human food consumption, after wheat and rice, but its production in the last 10 years stopped between 360 and 370 million tons annually [214]. Additionally, yield potential of potato has remained relatively unchanged, despite intensive breeding efforts [215], and century-old varieties (i.e., Russet Burbank and Bintje) are still cultivated due to lack of significant genetic improvements in potato. Narrow genetic base as a result of clonal propagation, multiple constraints such as inbreeding depression, self-incompatibility and incorporation of undesirable traits, limit the progress in conventional development of inbred potato lines [216]. On the other hand, genetic engineering has shown potential for fast, feasible, economic and environment-friendly introduction of resistance (and other beneficial) traits in commercially grown crops. However, to make full use of that potential it is necessary to improve existing and bring about new, more sustainable and cleaner gene manipulation technologies. By optimization of transgene expression level, its temporal or spatial programming (i.e., by use of wound-inducible or tissue-specific promoters), generating

marker-free modified plants and exploiting new approaches such as cisgenesis/intragenesis or genome editing – it is possible to both decrease unintended effects and increase efficiency and public acceptability of transgenic crops.

For potato, there are no GM varieties with insect resistance traits in the markets, and strategies that rely on insecticides cannot be avoided – as well as their failure in pest control. For instance, imidacloprid, a neonicotinoid successfully used for almost 10 years in CPB control, started being ineffective at the beginning of this century [217]. On the other hand, as global population continues to expand, food production, including potato, has to increase by many folds and with wild potato varieties as only source of resistance traits and their introduction by breeding, that seems unattainable. Also it is questionable whether all potato pests, CPB especially, could be stopped by resistance factors existing in *Solanum* species [218] while these resistance traits combined with heterologous sources such as Cry-toxins can offer more extensive and durable protection [219]. Moreover, there are other Bt-toxins, such as Vip, Cyt and Sip [220], or toxins from other bacteria, waiting to prove their usefulness in pest control. So far only Vip3A has been commercialized in Bt cotton and maize [55]. Additionally, RNAi and even PIs can efficiently supplement and strengthen such protection. However, insects are exceptionally adaptable and evolution of resistance to any of these control measures, including combinations of different traits, is inevitable – but the rate of resistance evolution can be slowed down by efficient management strategies.

As a crucial concept of insect resistance management, refuges are essential for durability of both stacked and single-toxin crops, and where resistance is rare, 20% (or at least 10% for stacked traits) of a pest host plant refuge may be sufficient to delay resistance by a decade or more [31]. Smaller refuges are insecure even under highly effective toxins (or other traits) and all cases of field-evolved resistance are associated with low refuge presence, as one of the main factors [221]. Additionally, within IPM context, refuges also provide better support to populations of natural enemies, that are not only important in target pest control, but to prevent non-target secondary pest outbreaks that can seriously reduce benefits from introduced traits and bring production back to running on the insecticide treadmill [222]. Adding pheromone disruption, mass trapping or intercropping arrangements – integrated into scientifically supported management and adapted to the pest biology – can efficiently reduce pest population size, keeping damage below the economical threshold. Experiences with combination of the simplest practices in potato fields in some parts of the USA, such as rational use of chemical insecticides, trap rows and crop rotation [223] proved a potential of well-structured IPM approach to balance one technology with other complementary strategies. Such avoidance of relying on only one means of control would require complex pest adaptations that are less likely to happen compared to the occurrence and fixation of random single gene mutations that can render resistance to insecticides, Bt-toxin, PIs, RNAi or any other measure that may be implemented in the future.

The benefits of pest-resistant GM crops, incorporated in well-balanced IPM strategies, are clear – but it is also necessary to define and understand their limitations and risks. Heavy dependence and overuse of insecticides undoubtedly had many consequences: food poisoning, reduction in biodiversity, negative effects on non-target species and other formidable impacts on environment – and genetic engineering provides a chance to not repeat all those mistakes. However, we cannot expect that Bt or other pest-resistant modified crops will not have long-term ecological or evolutionary consequences, as well as that small or substantial compositional changes, as intended (or unintended) quantitative or qualitative alterations of metabolites, nutrients or toxins, cannot impact ecological interactions and/or food or feed safety. Such risks are present and inevitable, can vary depending on

traits introduced and strategy used for its introduction – and with a precautionary approach, at least some of them can be avoided or mitigated. Additionally, every generated crop line is created in a unique event and should be evaluated for risks, benefits and sustainability only on a case-by-case basis.

So, taking all together, is genetic modification of plants a thrift or a threat? It only depends on how carefully and advisedly we use that tool in our hands.

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Conflict of interest

The authors declare that they have no conflict of interest.

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