Chapter

Biotechnological Strategies for a Resilient Potato Crop

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Abstract

The aim of this chapter is to describe in a synthetic manner the most efficient biotechnological techniques which can be applied in potato breeding with emphasis on multiple resistance traits. To this end, most important results of all biotechnological techniques will be pointed out including new biotechnological tools of genome editing. The somatic hybridization will be the core of the presentation as the only non-GMO strategy with good results in transferring multiple resistances into potato gene pool. The chapter is presenting all data in a synthesized form and made comparisons between the existing techniques and their possible adoption in breeding in different parts of the world, depending on regulations and consumer choice. Moreover, the recently discovered value of potato as a healthy food and its possible applications in cancer treatment will be also discussed with new data on both potato and some of its wild relatives.

Keywords: advantages, genetic transformation, multiple resistance traits, new biotechnological techniques, potato breeding, somatic hybridization

1. Introduction

As a major food staple, the potato is contributing to the UN Millennium Development Goals of food security and poverty eradication. Today, potato is the most widly grown non-cereal crop [1] and important vegetable for human consumption [2]. The wide climatic adaptability and short growing time of potato facilitated its spread across diverse geographical regions. To date more than three thousand potato cultivars are cultivated in 165 countries with a production exceeding 350 million tonnes per year, particularly under temperate, subtropical and tropical regions, covering a major economic share in the global agricultural market [2]. For the last two decades, potato cultivation and utilization have also been notably increased in developing countries such as China, India and Bangladesh [3]. Although, classical breeding has developed thousands of new cultivars, potato is still sensitive to countless diseases and pests, which lead to 44.9% yield losses in every year [4]. Diseases such as late blight produced by the oomycete *Phytophthora infestans* (*Pi*), viruses like potato virus Y (PVY) and pests as Colorado potato beetle (CPB) are able to completely destroy a potato field if left uncontrolled. Even today the main way to combat diseases and pests is massive application of pesticides. Pesticides increase pollution of the environment, are toxic for non-target organisms including humans and exert selection pressure on the diseases and pests, which develop resistance. New sustainable and effective ways to combat diseases and pests of potato are required and biotechnological approaches have been lately developed

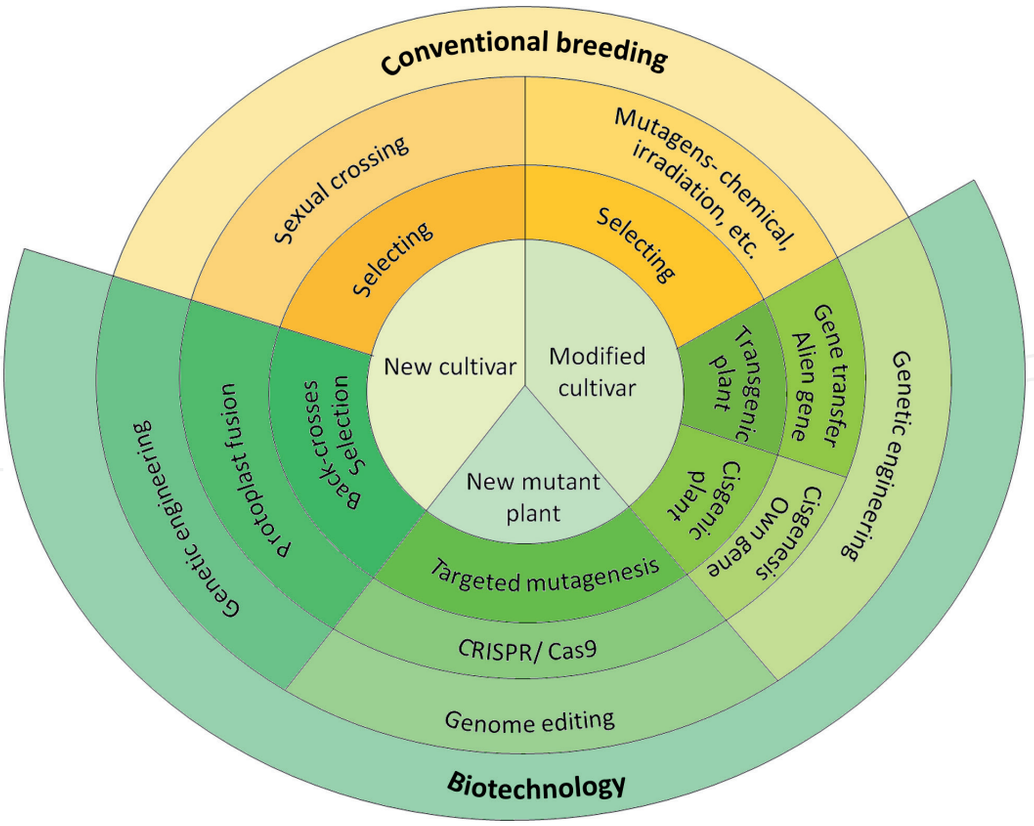

Figure 1.
Overview of classical breeding tools, as well as biotechnology and their applications for improving crops in general and potato resilience, in particular.

also to address this challenging issue (**Figure 1**). Moreover, climate change has challenged potato production worldwide in the last decades and new strategies to develop resilient potato to drought, high temperature, salt and other abiotic stresses or multiple stresses are an urgent need for potato cultivation. To achieve these goals, both classical breeding and biotechnology are aware of the resources of resistance genes in the crop wild relatives, as for example the project of International Potato Centre (CIP). There are published several books and reviews dealing with potato biotechnology and breeding [1, 2, 5, 6], but in this chapter we are going to overview, synthetize and point out those techniques that are included in potato genetic improvement for a resilient potato crop in order to develop a sustainable agriculture and reduce poverty.

2. Genetic engineering sustainability for a resilient potato crop

Modern biotechnology is defined as the technology which use living cells, microorganisms, or functional parts, such as enzymes, proteins, DNA or RNA molecules to develop basic research and deploy new useful products [7]. Genetic engineering, as part of plant biotechnology, covers techniques which change the genome of plants. In its larger sense, plant genetic engineering includes: (i) somaclonal variation, (ii) cell fusion and regeneration of somatic hybrid plants, (iii) gene transfer and (iv) genome editing. Since somaclonal variation has already been presented in detail and its results are currently not widely used in potato breeding [8], in this chapter we are presenting the other genetic engineering techniques and obtained results in developing resilient potato crop. Potato crop requires considerable inputs of: nutrients, pesticides, and water to maintain yield, tuber quality, and protection

from its pathogens, pests and extreme climate conditions. Genetic variations for the most important traits is low in commercial cultivars, but related wild relatives contain many unique, valuable traits missing from cultivars, which represent a rich genetic source for potato improvement [9]. Potato breeding efforts have historically focused primarily on yield, fresh market and processing quality, storability as well as disease resistance. Only after developing genetic transformation and/or other biotechnological approaches, a faster transfer of valuable traits like quality of tuber composition and resistance to biotic and abiotic stresses became possible. Moreover, with using classical breeding one new cultivar can be produced in 10 to 15 years from the initial cross to cultivar release, while with biotechnology, particularly gene transfer, shorter time is required, from some months (6–12 months) to a few years, ignoring the long regulatory clearances [6]. There are many attempts and results on the transfer and integration of economically important genes in potato crop and some previous reviews have presented the state of art in plants or in this tuberous crop [6, 8, 10].

2.1 Gene transfer to develop resilient potato to biotic and abiotic stresses

Genetic transformation of potato was first achieved in 1988 [11, 12], potato being the third plant to be successfully transformed. This technology uses *Agrobacterium tumefaciens* - mediated gene transfer, which is reported as the most efficient for potato crop and some of potato wild relatives [13]. The first commercially grown potato was introduced by Monsanto as New Leaf™ in 1995, the first released genetically modified crop of the company. Besides gene transfer from bacteria, fungi, animal or other plant species commonly called transgenesis, more recently wild species are considered as a rich reservoir of resistance genes. The transfer of genes from the same genus, i.e. from related species that can be crossed, is called cisgenesis. Because the genes can be also integrated into the recipient plant genome by classical breeding, cisgenesis was thought to be exempted from GMO low in Europe. Plant own genes can be also transferred in order to increase their expression, and this technique is called intragenesis [14, 15]. *Solanum* wild species, that evolved to resist in diverse climates in South and North America, are indeed a rich reservoir of genes which can be introgressed in potato genome. It is estimated that around 190 wild tuber-bearing relatives of potato, in the section Petota of the genus *Solanum*, are available for resistance breeding [16, 17]. Moreover, besides their rich genetic resources, potato and its wild relatives benefit from a good amenability to *in vitro* tissue and protoplast culture, making it possible to exploit this diversity through genetic engineering [8].

2.1.1 Single or multiple resistance gene transfer to improve pathogen and pest resistance

Genetic engineering has the potential to transfer single genes to increase disease or pest resistance, if the selectable marker gene, which is necessary for transgenic plant selection is not considered. Such single genes can be introgressed in potato elite varieties to improve one resistance trait. The frequently used marker gene during potato gene transfer is *npt*II (bacterial neomycin phosphotransferase II gene), which renders transgenic cells resistant to aminoglycoside antibiotics, including kanamycin and G418 [18]. Selection based on kanamycin has been proven to generate escapes in potato crop [13]. In this study both genes: *npt*II and reporter *gfp* (green fluorescent protein), have been used to reveal the transgene transfer efficiency, which allowed to evaluate the escape events. In order to transfer single genes that increase host plant resistance to pathogens and pests, the researchers have

to identify and clone the genes of interest (GOI). At this stage, a good knowledge of mechanisms of host plant– pathogen interaction and gene characterization is necessary. In the last decades new insights into the complex molecular race between pathogens and/or pests and crop hosts were advanced and many genes are characterized and some cloned [19, 20]. With the advent of Potato Genome Sequencing Consortium [21] and completion of the first reference genome of potato [17], and later the release of genome data for some of its wild relatives i.e. *S. commersoni* [22], and *S. chacoense* [23], potato breeding and biotechnology entered into the genomic-based improvement era. Gene transfer is already taking advantage of genome sequencing data in first instance through the transfer of potato own resistance genes and secondly utilization of potato wild relative (PWR) genes. In **Table 1**, examples of the latest year's single and multiple gene transfer for improving potato resilience to biotic and abiotic stresses are given, as well as some results on insect resistance. Potato wild relatives have evolved defense mechanisms against pathogens and pests at multilayer level (**Figure 2**). The interaction between host potato species and its pathogens involves the following mechanisms: (1) physical and physiological barriers that prevent the pathogens to enter into the plant cells; (2) plasma membrane-bound and intracellular immune receptors that initiate defense responses upon the perception of pathogens; (3) interference RNA (RNAi) used by plants to detect invading viruses and fragment their RNA [20]. Pathogens as bacteria and fungi, respond to potato defense through: (1) production and release of cell-wall-degrading enzymes; (2) production and delivery into host cytoplasm of effector proteins, some of which suppress host defense and promote susceptibility; (3) viruses produce suppressors of host plant RNAi and/or hijack host RNAi to silence host genes and promote viral pathogenicity [20]. On the other hand, the interaction between herbivorous insect pests and plants also involves various mechanisms: (1) non-glandular and especially glandular trichomes that act as physical and physiological barrier to insect feeding; (2) toxins such as glycoalkaloids, which are well characterised in the *Solanum* genera; (3) enzyme inhibitors such as protease inhibitors; (4) use of bacterial insecticidal genes [61] (references herein) (**Figure 2**). All genes involved in host plant resistance to pathogens and pests as well as pathogenesis susceptibility genes can be transferred to produce resistant potato crop.

For instance, genes for pattern recognition receptors (PRRs), from other species can recognize pathogen associated molecular patters (PAMPs) and activate defense responses, as was demonstrated in *Arabidopsis thaliana* lectin receptor kinase LecRK1.9 transferred into potato that increased resistance to *Phytophthora infestans* (*Pi*) (**Table 1**) [31]. This first level of defense is known as pathogen targeted immunity (PTI). It is likely that there are different type of PRRs in potato but one was identified as ELR protein, which was capable to recognize the INFI elicitin from *Pi* [62]. Others are known from tomato and other species [6]. The tomato PRR *Ve1*, which recognize the Ave1 protein from *Verticillium dahliae*, when was expressed in potato was conferring resistance to this disease [63]. Gene transfer gave good results when R genes could be isolated and cloned. R proteins represent the second level of defense recognizing specific effector proteins of the pathogen, called effector targeted immunity (ETI) (**Figure 2**) [6]. Compared to PRR system, effectors use a similar defense response in the host plant, but effectors coupled with R genes elicit a stronger response which activates hypersensitive reaction (HR) in resistant plants. HR imply cell death surrounding the pathogen attack and represent a barrier for further pathogen spread. Pathogen effectors have high diversity but R genes have two conserved domains: nucleotide binding (NB) and leucine reach repeat (LRR), which makes their identification easier [6]. In the last two decades many R genes were cloned from potato wild relatives that induce resistance to *Pi* and transferred into potato varieties, either as single or multiple genes (**Table 1**). Some examples

Trait	Gene/s	Result/resistance to:	References
Resistance to bacteria	*5-UGT*	*Erwinia carotovora*	[24]
	ScSN1	*Erwinia carotovora Rhizoctonia solani*	[25]
	Overexpression of peptides with anti-fungal properties	*Rhizoctonia solani*	[26]
Resistance to late blight (*Pi*)	*Rpi-vnt1.1*	*Pi* in field trials	[27]
	Rpi-vnt1.1, Rpi-sto1	*Pi* cisgenic marker-free	[28]
	RB (*Rpi-blb*1)	Tolerance to *Pi*	[29]
	Rpi-vnt1.1, Rpi-sto1, Rpi-blb3	*Pi*, stacking three cisgenes	[30]
	LecRK1.9	*Pi*	[31]
	AtROP1	*Pi*	[32]
	hp-PiGPB1	Pi – (HIGS)	[33]
	Rpi-blb2, Rpi-blb1, Rpi-vnt1.1	Pi, stacking 3 Rpi genes in African varieties	[34, 35]
Resistance to diseases	*MsrA2*	Broad-spectrum fungi and bacteria	[36]
	MsrA3 with tissue specific promoter	Mitigates biotic and abiotic responses	[37]
	hLf	bacteria and fungi	[38]
	Accumulation of cystatin	Diseases, insects and fungi	[39]
Nematode resistance	*Gpa2*	*Globodera pallida*	[40]
	Gro1–4	*Globodera rostochinensis*	[41]
	Peptide disrupting chemoreception of nematodes	*Globodera pallida*–no off target side effects	[42]

Trait	Gene/s	Result/resistance to:	References
Virus resistances	dsRNA PVY coat protein (CP)	RNAi - PVY	[43]
	shRNA with *ipt* gene	PVY^{NTN} in a marker-free system	[44]
	CP gene	PVY in the field	[45]
	shRNA with I	PVY^{NTN} in a marker-free system	[46]
	shRNA	PVY	[47]
	eIF4E-1 variant *Eva1* (*S. chacoense*)	PVY	[48]
	eIF4E	PVY	[49]
	RNAi	PVY	[50]
	CRISPR-Cas13a	Durable resistance all strains PVY	[51]
	Overexpression of *StSAR1A*	PVY and PVA	[52]
Insect resistance	Hybrid *Bt* endotoxin	Both coleopteran and lepidopteran pests	[53]
	cry1Ac9	Tuber moth	[54]
	Cysteine *Pls*	Western flower thrips	[55]
	RNAi incapsulated in bacteria (*E. coli*)	CPB	[56]
	DsRNA for CPB control	CPB	[57]
	ACT-dsRNA expressed in chloroplasts	CPB	[58]
	RNAi of molting-associated EcR gene of CPB	CPB	[59]
	CRISPR-Cas9 site directed editing of *vest* gene in CPB	CPB	[60]

ACT – gene for βactin; AtROP1 – Arabidopsis thaliana gene for protein at the plasma membrane; CPB – Colorado potato beetle; hLf – human lactoferrin gene; MsrA2 – gene for frog antimicrobial peptide; LecRK1.9 - Arabidopsis lectin receptor kinase; Pi- Phytophthora infestans; Pls – protease inhibitors; ScSN1 - Snakin-1, a cysteine-rich peptide from Solanum chacoense; 5-UGT - anthocyanin 5-O-Glucosyltransferase; SAR1a - secretion-associated RAS super family 1 gene; vest – vestigial gene involved in wing development.

Table 1.
Synthesis of transgenesis and cisgenesis results presenting the transfer of single or multiple resistance genes in order to improve biotic stress resistance in potato.

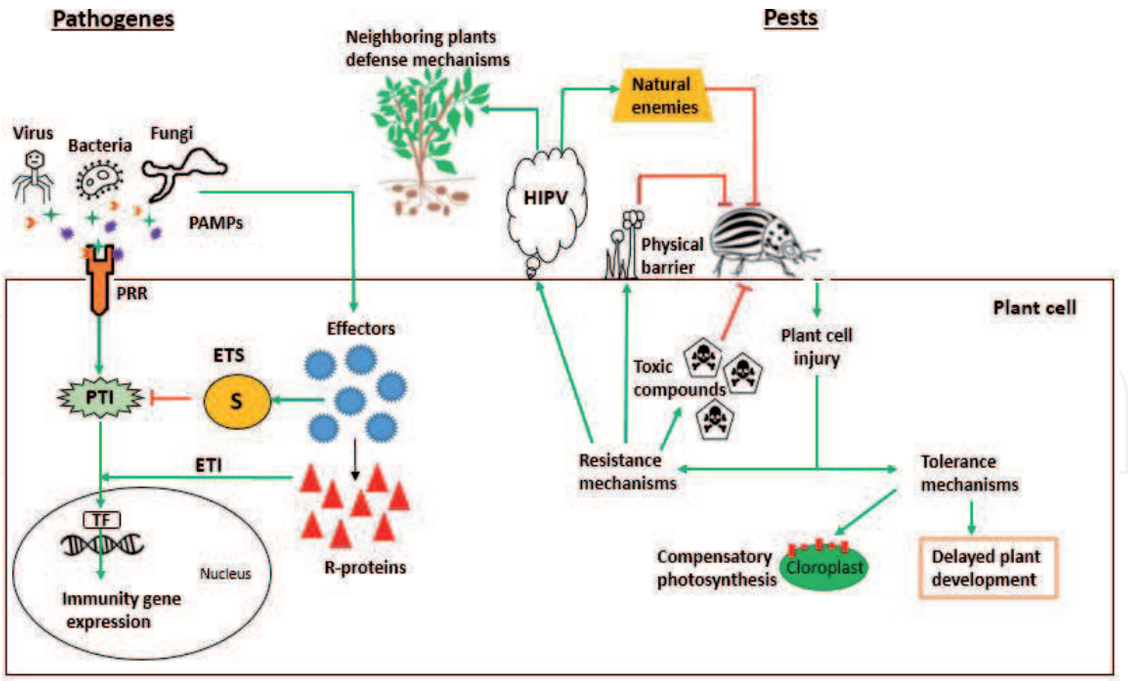

Figure 2.
The principal mechanisms of interaction between pathogens (bacteria, fungi and viruses) on the left and insect pests on the right with the potato host: the pathogens trigger two responses PTI (pathogen triggered immunity) and ETI (effector triggered immunity); in PTI the membrane proteins PRR recognize pathogen molecular patterns (PAMPs) and induce transcription factors (TFs) which activate immunity genes; in ETI effector molecules interact with specific resistance genes (R), but they can also interact with sensitivity genes (S) to inhibit PTI; insect pests interaction with its host is less understood but at first the pest interacts with leaf trichomes, glandular and/or non-glandular, mainly acting as a physical barrier; after wounding the leaf cells are inducing either tolerance responses like compensatory photosynthesis and delayed plant development, or resistance responses through synthesis of toxins like glycoalkaloids. Resistance mechanisms can activate HIPV (herbivore induced plant volatiles).

of R genes are: *R1*, *R2* and *R3a*, *R3b*, originally identified in *S. demissum*; *Rpi-blb1* (RB), *Rpi-blb2*, *Rpi-blb3* from *S. bulbocastanum*; *Rpi-vnt1.1* and *Rpi-vnt1.2* from *S. venturii*; *Rpi-mcq 1 from S. mochiquense* [6, 64], etc. R genes were also delivered into potato varieties as gene stacks. In Europe BASF Company petitioned for the release of potato Fortuna resistant to late blight (*Pi*) after stacking of two R genes: *Rpi-blb1* and *Rpi-blb2*, obtained after a long effort of breeding, but unfortunately, this cultivar was never marketed [6]. The Simplot's second generation Innate® potato which besides reduced browning and bruising, also carries R genes and hence is resistant to late blight (*Pi*), was approved for cultivation in USA [65], and for cultivation and consumption in Canada [66]. One important research project was developed in Netherland between 2006 and 2015 on Durable Resistance in potato against *Phytophthora* (DuRPh) at Wageningen University and Research Centre [64]. The aim of this project was to identify and clone new durable resistance genes from potato wild relatives and transfer them as single or stalked genes into varieties by cisgenesis using marker assisted selection (MAS). Through this project a great deal of data has been accumulated and cisgenic varieties resistant to late blight were produced but these will require some more backcrosses to be released as resistant and productive varieties [64]. Still cisgenesis is considered as GM in Europe. A successful cisgenic approach was applied in Africa, where highland varieties were transformed with an efficiency of 75% using three Rpi genes: *Rpi-blb1*, *Rpi-blb2* and *Rpi-vnt1.1* (**Table 1**) [34]. R genes that improve resistance to other pathogens were also discovered: *Rx1* and *Rx2* (from *S. tuberosum* ssp. *andigena* and *S. acaule*, respectively), that confer resistance to potato virus X (PVX) [67]; *Gro1–4* from *S. spegazzinii,* confer resistance to root cyst nematode *Globodera rostochinensis* (**Table 1**) [41]. Another strategy for resistance to

a broad spectrum of pathogens is overexpression of a single gene located upstream in signalling cascades and thus regulates large number of defense-responsive genes. There are many examples of successful engineered plants using different constructs to overexpress trans- and endogenous genes in crops, including potato. Overexpression of these upstream signalling genes and defense-related genes can lead to a constitutive expression of resistance phenotype. In plant disease resistance, a vital role is played by small G-proteins and subsequent cellular responses to pathogens such as bacteria, fungi and viruses [52]. A number of G-proteins have been transferred to different plant species including potato where stable overexpression of *AtRop1* (DN-*AtRop1*) increased resistance to *Pi* infection (**Table 1**) [32]. An important breakthrough is the continuous research identifying new molecular markers linked to resistance genes or more recently QTLs (quantitative trait loci) such are: AFLP, RFLP, SSR, RAPD and their maps available for potato breeding [68]. At International Potato Center a continuous effort, as mentioned above, aims to store genetic diversity and improve it for the benefit of the next generations and efficient alleviation of underdeveloped nations' poverty. Several other genes were also cloned and transferred into potato crop for improvement of resistance to: PVY (*eIF4E-1* variant *Eva1*) and *Pi* – host induced gene silencing (HIGS) (**Table 1**) [33, 48]. The aim of the latest strategy is to achieve more durable resistance than R genes, but this also uses gene constructs that fall under GM rules [6].

2.1.2 Insect resistant potato crop

Insects are also a plague for potato production but the most difficult to control is the voracious Colorado potato beetle (CPB). It is estimated that 75% of potato production can be lost by pests if left uncontrolled [69]. CPB develop on potato crop, larvae and adults eat leaves and are able to completely skeletonize the plants. During development, the three stages of instar larvae consume around 40 cm^2 of potato leaves [70]. Plant breeding and biotechnology were not able to release a variety resistant to CPB without GM technology. Wild potato relatives are a reservoir of resistance traits as it was discussed for pathogens. Two natural host plant resistances are known: glandular trichomes and specific glycoalkaloids, the leptines I and II [71]. Detailed knowledge on the interaction between potato and resistant relatives with the voracious beetle are still scarce (**Figure 2**). Another interesting mechanism of resistance was discovered [72], the hypersensitive reaction of plants to CPB egg masses and egg drop. Any breakthrough into the physical, physiological and molecular mechanisms of resistance will fasten the progress of resistance breeding using biotechnology. The main strategy of genetic engineering to induce resistance to CPB was based on bacterial toxin from *Bacillus thuringiensis* (*Bt*), a bacterium also used in integrated pest management by spraying bacterial suspensions in the field. The technology is very specific for a certain species of pest, because *Bt* not only has a large repertoire of the *cry* genes that produce the protoxins involved in pest induced mortality, but the toxin is formed only in the gut of feeding pests and would not affect non-targeted beneficial insects [71]. The first success was introducing by gene transfer the *cry3a* gene into potato cv. Russet Burbank to protect it from CPB attack [73]. The GM variety with resistance to CPB was approved for human consumption and was commercially available in USA between 1996 until 2001, proving to control the beetle in the field without any unwanted effects on the cultivar [74]. NewLeaf™ potato, developed by Monsanto, containing *cry3a* proved to supress CPB populations at greater extent as insecticides or sprays based on formulations from *Bt* bacteria containing CRY3A protein [71]. In the next years, after the first success with cry3a, other *cry* genes have been optimize and transferred into potato: *cry3Ca1*, *cry1*, *cry3Bb1* [71] (**Table 1**).

Coombs *et al* [75] combined leptines, glycoalkaloids considered as toxic to CPB, with glandular trichomes and *Bt-cry3a* to obtain transgenic potato host plants resistant to CPB. In that way the main problem of *Bt* potato, the development of resistance, could be also managed [75]. To date, there are no *Bt* potato on the market, as discussed in public acceptance of GM potatoes. Recent studies have been focusing on RNAi technology, including direct spraying of dsRNA in the field [71]. The first success with dsRNA used in a transgenic approach [76], lead to long or short double stranded RNA used to target a specific gene at posttranscriptional level determining mRNA fragmentation and hence silencing the gene. This proof of concept brought about a growing interest for the use of RNAi technology for controlling the CPB pest [57]. Moreover, non-transgenic alternatives were developed including dsRNA spraying on the plants [59, 77], but in this year (2021), resistance development in CPB populations after dsRNA foliar-delivery in potato has been already observed [78]. Sequence of CPB transcriptome can assist in the identification of new target genes for RNAi that can be used to control this pest [79]. To date, 24 target genes with important roles in cellular functions were silenced using RNAi, as reviewed by Balaško e*t al* [71]. Knockdown of those genes affect insect morbidity and mortality. There were also different delivery methods of dsRNA into CPB, like the use of bacteria, liposomes and nanocarriers, all of them able to protect and deliver dsRNA [77]. Moreover, other improvements for CPB control were the xenobiotic transcription factor Cap 'n' collar isoform C (CncC) that regulates the expression of multiple cytochrome P450 genes, and plays crucial roles in CPB insecticide resistance. The suppression of CncC by RNAi reduced imidacloprid resistance of CPB [80]. Ochoa-Campuzano *et al* [81] identified prohibitin, an essential protein for CPB viability, as Cry3Aa binding protein. Combination of feeding prohibitin dsRNA and treatment with Cry3Aa enhanced the toxic effect by threefold and CPB was killed faster with 100% mortality in five days. The molecular mechanisms of synergism between prohibitin, RNAi and Cry3Aa toxin are not understood, but this study proposes an interesting method, combining toxins derived from bacteria or other organisms with RNAi in order to improve efficiency of dsRNA in pest control. Moreover, recently targeted mutagenesis using CRISPR-Cas9 technology in CPB was demonstrated [60], a technology which holds great promise for the future.

2.1.3 Gene transfer for resilience to abiotic stress

Abiotic stresses such as drought, salt, high temperatures and extreme weather also limit potato yield around the world. With global climate change, abiotic stress is expected to be less predictable in the years to come and also affect pathogen attacks and pest effects on potato and other crops. The response of the plants to abiotic stresses involve generally the expression of inducible resistance genes. In particular, transcription factors (TFs) that control resistance genes are a key in gene regulatory networks that control the expression of many genes involved in stress responses [82]. Transgenesis uses genes for such TFs like WRKY, MYB or DREB, the last also used in potato crop (**Table 2**). Other genes that were engineered in potato are related to response of the plants to abiotic stress, like *StProDH1,* which is a key player in potato response to drought stress [93]. Through the manipulation of abscisic acid signal transduction after loss of function of cap-binding protein (CBP) [71], in cv. Désirée, a higher tolerance to drought was reported [92]. Through transgenic approach, potato lines with increased betaine aldehyde dehydrogenase, an enzyme for glycine betaine biosynthesis, which has important role in drought stress, has been able to induce drought tolerance in potato [88]. Transcriptome analysis, comparing control with drought stressed potato plants, has indicated many genes that are overexpressed

Trait	Gene/s	Result/tolerance to:	Reference
Heat tolerance	*CaPF1*	High temperature	[83]
	AtCBF3	Heat tolerance	[84]
	Allelic variant *HSc70*	Moderately high temperatures in cv. Désirée	[85]
Freezing tolerance	*Atrd29A::DREB1A*	Freezing	[86]
Drought tolerance	*ScTPS*	Studies on water content and photosynthesis	[87]
	Glycin betaine aldehyde dehydro-genase	Drought	[88]
	TPS1	Drought - increased threhalose	[89]
	PaSOD	Increased photosynthesis under drought	[90]
	AtDREB1/CBF	Drought	[91]
	CPB80	Drought	[92]
	amiRNA silencing of *StProDH1*	Drought	[93]
Salt tolerance	*Δ1-pyrroline-5-carboxylate synthetase*	Salt - increased proline	[94]
	HvNHX2	Salt	[95]
	Overexpression of *AtHKT1*	Salt	[96]
	StCYS1	Salt	[97]
Two stresses	*BADH*	Drought and salt	[88]
	*StEREBP*1	Cold and salt	[98]
	SOD, APX	Oxidative stress and high temperature	[99]
	At DREB1B	Drought and freezing tolerance	[100]
	StDREB1, StDREB2	Salt or drought tolerance	[101, 102]
	ggpPS	Drought and salt/ tuber increased glucosyl - glycerol	[103]
	GB	Salt and cold	[104]
	StWRKY1	Resistance to Pi and improved tolerance to drought	[105]
	AtABF4	Salt and drought, increased yield and tuber quality	[106]
	AtHXK1 and *SP6A*	Drought and heat	[107]
Multiple stresses	*CodA*/chloroplast	Oxidative, salt, and drought stresses	[108]
	SOD, APX, CodA/ chloroplast	Oxidative, salt, and drought stresses	[109]
	*StnsLTP*1	Multiple tolerance to heat, salt and drought	[110]
	IbOr	Multiple tolerance to drought, oxidative stress and high salinity, increased carotenoid contents	[111]

amiRNA – artificial miRNA; APX - ascorbate peroxidase; BADH - betaine aldehyde dehydrogenase; CaPF1- pepper transcription factor belonging to the family of TFs ERF/AP2; CBF - C-repeat Binding Factor;DREB - dehydration responsive element binding protein; CodA - choline oxidase; GB – Glycinebetaine; HSc70 – heat shock cognate 70 gene; HvNHX2 – Hordeum vulgare vacuolar Na+/H+ antiporter; IbOr – Ipomeaea batata orange gene; ScTPS- Saccharomyces cerevisiae trehalose-6-phosphate synthase; SOD - superoxide dismutase; StEREBP1 – S. tuberosum ethylene responsive element binding protein 1; StnsLTP1 – S. tuberosum nonspecific lipid transfer protein 1; StProDH1 – S. tuberosum proline dehydrogenase 1; TPS1- yeast trehalose-6-phosphate synthase 1.

Table 2.
Examples of single or multiple resistance genes transfer to improve abiotic stress tolerance in potato.

or underexpressed during drought stress, with genes involved in processes like: intracellular water and ion homeostasis, membrane structural stability, and reconstruction of primary and secondary metabolism, and stress regulatory genes, as calcium ions, TFs and receptor protein kinases that are involved in stress response through signal transduction and metabolic pathways [112].

Salt stress caused by soil salinization is an increasing threat to agriculture worldwide [113]. Different factors lead to the continuous salinization of the soil, mainly different agricultural practices such as irrigation and some fertilization procedures. The mechanisms that are involved in salt stress response are cellular and physiological: e.g. different cellular signalling, various ion transport, water management and specific gene expression which are involved in growth, development and survival [113]. Researchers are working on halophytes, plants that are adapted to salty soil, to get new insights on plant responses to salt stress. In the case of potato, as presented in **Table 2**, there are transgenic strategies which proved their utility in obtaining salt tolerance, either alone or in combination with other stress factors. Potato plants adapt to salinity stress through different mechanisms like osmotic adjustment by accumulating compatible solutes in the cytosol, decrease leaf water potential leading to reduced cell turgidity and growth retardation and tuber yield loss. One of the most important compatible solute is proline, which was accumulated in cv. Désirée 3.5 fold and 11 fold at 100 and 200 mM NaCl, respectively [114]. However the proline effects on salt tolerance need additional studies because foliar application of proline has no effect on salt tolerance of plants [115]. Potato is adapted to cool weather mostly preferring temperate zone. The vegetative part of plants grow properly at 20–25°C temperature, while tubers develop better at 15–20°C. The response of potato plants to high temperature varies across the cultivars, one example being the commercial cv. Russet Burbank, which exhibit maximum rates of photosynthesis at 24 to 30°C and a reduction of photosynthetic activity only at or above 35°C [116]. Global warming and drought are expected to drastically reduce the potato productivity, but with biotechnology heat tolerant potato was successfully obtained (**Table 2**). Plants exhibit different strategies to cope with high temperature stress involving physiological, morphological and molecular levels. At molecular level heat stress increase the activity of heat stress TFs (HSFs), which trigger the accumulation of heat shock proteins (HSPs). HSPs are known to govern heat stress response (HSR) and acquired thermo-tolerance through their role as molecular chaperones [117]. In a genome wide study 27 *StHSFs* in the *Solanum tuberosum* genome were identified [118], which have diverse regulatory functions during stress. Underlining the molecular mechanism of how heat stress induces HSFs trimerization, their activation and synthesis of HSPs is still underway. Elucidation of the mechanisms of heat stress response may offer new insights that will be useful in breeding new heat resilient cultivars with sustained or even enhanced potato crop productivity and quality in response to climate change.

2.1.4 Multiple stress factors

In nature, generally multiple stresses act on crops at the same time and all of them contribute to noticeable losses in production. Nowadays, there is knowledge about various genes that contribute to both biotic and abiotic stress response and resistance/tolerance. The effects of abiotic stress on potato crop under climate change is detailed in a recent review [117]. Molecular and genomic analysis revealed transcriptionally regulatory pathways involved in modulation of stress responsive genes. As mentioned above TFs are playing a crucial role, particularly in multiple stress response of plants [119]. Examples of TFs that activate stress responsive genes

are AP2/ERF, containing AP2/ERF binding domain, a large superfamily that divides in AP2, ERF and RAV [120]. This family of genes participate in developmental processes. AP2 family is involved in regulation of development, together with ERF protein family. Based on the differences in DNA box-binding ability of the single AP2/ERF domain, the ERF family is divided in ERF and CBF/DREB (C-repeat Binding Factor/Dehydration Responsive Element-Binding) (**Table 2**). ERF proteins are mainly involved in inducing disease resistance in a negative or positive mode of action. Gangadhar *et al* [121] have identified 95 genes involved in heat tolerance in potato, eleven of them being associated with multiple stress tolerance, like drought, salt and heat. Prolamins are a group of plant storage proteins that represent useful factors implicated in controlling both abiotic and biotic stress-response in plants. The plant non-specific lipid transfer protein, nsLTP, is involved in phospholipid transfer but also various other biological functions as seed storage, lipid mobilization, cuticle synthesis, somatic embryogenesis and pollen tube adhesion [110]. Transgenic potato lines over-expressing *StnsLTP1* acquired improved tolerance to multiple abiotic stresses through enhanced activation of antioxidative defense mechanisms via cyclic scavenging of ROS and regulated expression of stress-related genes (**Table 2**) [110]. Another example is the use of TF *StWRKY1*, which successfully induced resistance to *Pi* and improved tolerance to water scarcity. This experiments prove the role of TFs and in particular WRKY in regulating both biotic and abiotic stress resistance thereby modulating plant basal defense networks and thus playing a significant role for potato crop improvement.

3. Use of cell fusion between potato crop and its wild relatives for resilient potato

Over the past fifty years the introgression of new traits from wild *Solanum* species have mainly achieved by using classical breeding methods. The number of wild species that could be integrated into potato breeding is quite limited because of sexual incompatibility and endosperm balance number (EBN), although there are techniques other than sexual crosses, such as manipulations of ploidy levels [122], breeding 2n gametes or using bridging species to integrate genes from wild *Solanum* species into modern cultivars [123]. Through sexual crosses the main source of resistance genes is still *S. demissum*, more than half of the modern cultivars contain introgressions from this species [123]. The main limitations of the potato classical breeding are tetraploidy and heterozygosity, which make breeding very complex and time-consuming [124]. Moreover, when genes from an incompatible wild species have to be exploited, as was in the case of *S. bulbocastanum*, the use of a bridging species was applied to produce new cultivars which took 49 years and then only one resistance gene (*Rpi-blb2*) against late blight was integrated into potato gene pool (cvs. Bionica and Toluca) [125]. Nowadays, somatic hybridization through protoplast fusion is a well refined and routinely used method in order to create *Solanum* hybrids with different useful properties [126, 127]. Plant protoplasts are naked somatic cells from which the cell wall has been removed by enzymatic digestion, therefore these cells can be used for gene transfer, somatic hybridization [128], and more recently for targeted mutagenesis and genome research. Protoplasts are still totipotent and they are able to regenerate new cell wall, divide to form new cell colonies, microcalluses, calluses and finally new plants. This protoplast technology proved to be very efficient in potato crop and is a reliable and useful way to regenerate large numbers of somatic hybrids (SHs) with distinct genetic backgrounds [129–131]. Among the agronomical important crops, potato was the first used in protoplast culture and somatic hybridization [132, 133], which opened

the way for free gene transfer from potato wild relatives into potato crop [134]. Leaf mesophyll cells of *in vitro*-grown plants were used to isolate protoplasts [135], then the obtained fused products were cultured in VKM medium [136], followed by shoot development on the MS13K medium [137]. Recently, selection of SHs (*S. tuberosum* + *S. chacoense*) based on callus growth tagged with *gfp* has been also observed [138]. Different methods are available for protoplast fusion, but only two are generally used: electrofusion and PEG (polyethylene glycol) induced fusion [128]. Electrofusion is the most widely used method since its discovery in 1979 [139], and it consists in first instance of protoplast agglutination induced by the use of an alternating current (AC) field, the so-called dielectrophoresis [140]. In the second phase the agglutinated aligned protoplasts are induced to fuse by using direct current (DC) square wave pulses with a high intensity (2000 V cm^{-1}) and very short duration (10–100 μs) [141]. PEG-induced fusion generally has a similar efficiency as electrofusion, especially after applying calcium solution washing step [128]. Immediately after fusion or after the plants have been regenerated, the obtained SHs are subject to different analysis, such as cytological (flow cytometry, chromosome counts, chloroplasts counts in guard cells, FISH - fluorescence in situ hybridization and GISH - genomic *in situ* hybridization), molecular: isozyme, molecular markers (e.g. RAPD, RFLP, ISSR - inter simple sequence repeat, SSR-simple sequence repeat, AFLP - amplified fragment length polymorphism, and DArT-diversity array technology) [8, 129], phenotypic changes (e.g. foliage, stem, leaf, flower and tuber traits) and pollen fertility. Due to their stability and universality SSR markers are the most widely used [129, 130]. Recently, the application of DaRT made it possible to find out the composition of the SHs genome between potato and *S. x michoacanum*, which demonstrated the presence of both parents genome in hybrid plants, and provided evidence for late blight resistance trait transfer from wild relatives into SHs [142]. SHs are also analysed for cytoplasm types (haplotype of chloroplast/mitochondria: W/α, T/β, W/γ, W/δ and S/ε) [143], based on organelle segregation after fusion and organellar genome-specific markers as described by Lössl *et al* [144]. Finally, SHs are examined for the presence of target traits under field or controlled conditions eventually being tested for phenotype and tuber qualities in the field [8, 145]. Somatic hybridization through protoplasts fusion, which circumvents pre- and post-zygotic crossing barriers, can be successfully used to insert resistance into potato (**Table 3**) [143]. It has a greater potential for self-generating biodiversity in numerous nuclear and cytoplasmic genome combinations than sexual hybridization [184]. It also provides an opportunity for initiating recombination events between parental genomes. Moreover, homeologous recombinations (recombination between similar but not identical DNA molecules), can also be increased, that might increase the integration of valuable traits, by inducing a DNA repair deficiency, for instance, mismatch repair deficiency (MMR) [145, 175, 185]. MMR was successfully induced by *Agrobacterium*-mediated transfer of *AtMSH2* gene in antisense orientation or a dominant negative gene into *S. chacoense* [185], followed by somatic hybridization with potato tetraploid variety Delikat through electrofusion. Resistance to Colorado potato beetle (CPB) was more common in MMR deficient somatic hybrid plants [175]; MMR was also responsible for greater diversity and a novel trait tolerance to drought stress [180]. Since 1980s, different wild *Solanum* species have been hybridized with potato using protoplast fusion, and many of them express various valuable traits, including resistance to viruses [186], bacteria [187], fungi [188], insect pests [175] or tolerance to abiotic stresses (**Table 3**) [181]. Furthermore, multiple resistance can be also transferred from wild relatives into the potato gene pool [130] and even SHs with multiple parent lines can be produced, as in the case of the tri-species somatic hybrids [178].

Traits of interest	Somatic hybrid St + wild relative:	Tools for characterization and/or selection	Reference
Biotic factors			
Resistance to bacterial diseases			
Clavibacter	*S. acaule*	Glycoalkaloid aglicones	[146]
Erwinia carotovora	*S. brevidens (S. palustrae)*	RFLP, GISH, FISH	[147]
Ralstonia solanacearum	*S. chacoense*	SSR, cytoplasm type, MAS, BC_1	[148, 149]
	S. melongena	SSR, *smPGH1* gene	[150]
	S. stenotonum	Isoenzymes, SSR, PEPC/ RUBISCO ratio	[151]
Streptomyces spp.	*S. brevidens* (*S. palustrae*)	Laboratory and field resistance tests	[152]
Resistance to fungal diseases			
Alternaria tomatophila	*S. brevidens (S. palustrae)*	RFLP, GISH, FISH	[147]
Phytophthora erythroseptica	*S. berthaultii* (+) *S. etuberosum*	NS	[153]
Phytophthora infestans	*S. bulbocastanum*	MAS for RB gene (Rpi-blb1) GISH, cytoplasmic DNA	[154, 155]
		SSR, cytogenetics, *Rpi-blb1*; *Rpi-blb3* gene	[131, 145]
	S. cardiophyllum	RAPD	[156]
		SSR, AFLP, MFLP, ploidy	[130]
		RAPD, SSR, ISSR, AFLP, cytoplasmic type molecular markers, FC	[157]
	S. circaeifolium	Morphology, RAPD, chromosomes	[158]
	S. chacoense	RAPD, morphology	[156]
	S. x michoacanum	Ploidy, RAPD	[159]
		DaRT	[142, 160]
	S. nigrum	Morphology, ploidy, RAPD	[161]
	S. pinnatisectum	RAPD, morphology	[156]
		Ploidy, cytoplasm type	[162]
		RAPD, SSR, cytoplasm type, FC	[163, 164]
		ISSR, BC_1 characterization, *Rpi-blb2* gene, field resistance tests	[165, 166]
	S. tarnii	SSR, AFLP	[129]
	S. verrucosum	RAPD	[167]
	S. villosum	RAPD, GISH, ROS	[168]

Traits of interest	Somatic hybrid St + wild relative:	Tools for characterization and/or selection	Reference
Pythium spp.	*S. berthaultii* (+) *S. etuberosum*	NS	[153]
	S. tuberosum cvs. Aminca (+) Cardinal Cardinal (+) Nicola	Isoenzymes, SSR, ISSR	[169]
Verticillium spp.	*S. commersonii*	Southern analysis of organelles	[170]
Resistance to viral diseases			
PRLV	*S. etuberosum*	Characterization of BC populations	[171]
	S. tuberosum x *S. berthaultii* (+) *S. etuberosum*	NS	[172]
PVX	*S. tuberosum* x *S. berthaultii* (+) *S. etuberosum*	NS	[172]
PVY	*S. cardiophyllum*	SSR, AFLP, MFLP, ploidy	[130]
	S. etuberosum	RAPD, SSR, GISH, cytoplasm type	[173]
		Cytoplasm type, FC, RAPD, SSR	[174]
	S. tuberosum x *S. berthaultii* (+) *S. etuberosum*	NS	[172]
	S. tarnii	SSR, AFLP	[129]
	S. tuberosum cvs. Aminca (+) Cardinal Cardinal (+) Nicola	Isoenzymes, SSR, ISSR	[169]
Resistance to insects			
Colorado potato beetle	*S. tuberosum* (+) *S. cardiophyllum*	RAPD	[156]
		SSR, AFLP, MFLP, ploidy	[130]
	S. tuberosum (+) *S. chacoense*	MMR deficiency, SSR, RAPD marker for leptines	[175]
	S. tuberosum x *S. berthaultii* (+) *S. etuberosum*	NS	[172]
	S. tuberosum (+) *S. pinnatisectum*	RAPD, morphology	[156]
Meloidogyne chitwoodi	*S. tuberosum* (+) *S. bulbocastanum*	Laboratory and field resistance tests	[176]
		MAS *RMc1(blb)*	[177]
Green peach and potato aphids, wireworm	*S. tuberosum* x *S. berthaultii* (+) *S. etuberosum*	NS	[172, 178]

Traits of interest	Somatic hybrid St + wild relative:	Tools for characterization and/or selection	Reference
Abiotic factors			
Drought tolerance	*S. tuberosum cvs.* Aminca (+) Cardinal Cardinal (+) Nicola	Greenhouse tolerance test	[179]
	S. chacoense	Laboratory and phenotyping	[180], Molnar *et al.* (under publication)
Frost tolerance	*S. malmeanum*	SSR, ploidy, BC_1 characterization, laboratory tolerance tests	[181]
Salt tolerance	*S. berthaultii*	ISSR, cytoplasmic DNA, FC	[182]
		Oxidative stress responses	[183]

NS – not specified.

Table 3.
The most important somatic hybrids with proved resistance to pathogens, pests and tolerant to abiotic stresses and the methods applied for their analysis.

One of the most economically valuable SH was obtained by fusion between the incompatible *S. bulbocastanum* species and cultivated tetraploid potato [189], which highlighted the advantages of somatic hybridization in potato genome improvement, because the SHs were highly resistance to *Pi* in the laboratory and a field under intense disease pressure. After back-crossing of these SHs with potato cultivars the resistance to this disease was not lost. Subsequently, RB gene involved in durable resistance was isolated, which is located on chromosome VIII [190]. Transgenic plants with RB, were regenerated after *Agrobacterium*–mediated gene transfer and proved durable resistant [191]. Since then, *S. bulbocastanum* demonstrated several times its value as a resource of durable resistance genes against late blight, therefore it has been an increasing interest in transferring the resistance traits of this species to cultivated potato [154, 192]. RB gene was the first durable resistance gene described for late blight, but soon many other genes were discovered both in *S. bulbocastanum* and other wild species. To date, there are four characterized resistance genes in *S. bulbocastanum*: *Rpi-blb1* (formerly RB), *Rpiblb2*, *Rpi-blb3* and *Rpi-bt1* [190, 193–196]. In addition, late blight resistance from other sources was also accessed by generation of interspecific SHs with the wild species *S. pinnatisectum* [163], *S. tarnii* [129], *S. cardiophyllum* [130] and more recently *S. x microachanum*, a wild diploid derived from a spontaneous cross between *S. bulbocastanum* and *S. pinnatisectum* [160]. These newly produced SHs were also tested in the field and were resistant after two or three years of assessment, therefore they are suitable for introducing in breeding. *S. stenotonum* is an exquisite source of resistance to bacterial wilt caused by *Ralstonia solanacearum*, and all of the SHs obtained by fusion of potato protoplasts with this wild species were as resistant as the wild parent line [197]. Similarly, *S. chacoense* was explored for molecular markers associated with bacterial wilt resistance, and for introgression of resistance into the potato gene pool [148]. A very successful approach involved the transgenic induction of MMR deficiency in a high leptine-producing accession of *S. chacoense*, followed by somatic hybridization, because large number of generated plants exhibited both antixenosis and antibiosis against CPB [175]. Recently, by using gene specific markers four *Pi* resistance genes: *Rpi-blb1, Rpi-blb3, R3a* and *R3b* were identified in *S. bulbocastanum* and derived SHs with potato cvs. Delikat and Rasant. The genes were present also in BC1 and BC2 progenies and resistance to late blight

was maintained along good tuber traits [146]. The resistance gene pool of wild *Solanum* species can also be used to combat abiotic stresses like salt, drought and frost. For example SHs originating from fusion between potato and *S. bertaultii* are tolerant to salt stress [182]. Freezing is another abiotic factor, which decrease the yield of potato and SHs of *S. tuberosum* (+) *S. malmeanum* proved to be tolerant to frost [181]. Furthermore, SHs of potato and *S. chacoense* show different level of drought and salt tolerance beside resistance to CPB [184]. Interspecific somatic hybridization gave good results but the intraspecific somatic hybridization proved to be also suitable for potato improvement. Starting in the 1990s, somatic hybridization was used to study different dihaploid lines of potato generated by crossing with *S. phureja* [198] or pollen and anther *in vitro* culture. The results of the protoplast fusion of two dihaploid potato lines were at first not very promising, but the restoration of tetraploids from two dihaploid lines with valuable yield and resistance traits soon proved to be a valuable approach for potato breeding. Furthermore, resistance to nematodes, viruses (PVY) and *Phytium* bacterial diseases were achieved by intraspecific protoplast fusion [169, 199]. The intraspecific hybridization has a finite repository, but as long as this area is not exploited, it is worth considering. During interspecific somatic hybridization two obstacles may occur: (1) transfer of too much exotic, wild genetic material along with the desirable gene(s) from the wild species; and (2) genetic imbalance which lead to somatic incompatibility. These limitations result either in abnormal growth and development of the SHs, and/or regeneration of infertile plants. In order to reduce the wild imprint, the introgressive hybridization is followed by one or multiple back-crosses of the somatic hybrids with cultivars. The purpose of these cross-hybridization processes is on the one hand to eliminate the undesirable part of the wild genome, on the other to retain the target traits inherited from the wild parents and to restore the agronomic valuable cultivars, with high yield and adequate tuber quality [129, 130, 139]. Several experiments proved that, the above mentioned disadvantages could be eliminated through multiple back-crosses. Somatic hybrids of cultivated potato and *S. tarnii* were resistant to late blight and PVY, and these valuable traits were successfully transferred to BC1 progenies, which also presented good tuber yield and quality [129]. Multiple years of field evaluations of *S. etuberosum* + *S. tuberosum* and progenies showed stable transmission and expression of PLRV and PVY resistances in three BC1, BC2 and BC3 and two BC1 and BC2 generations, respectively [171]. Furthermore, late blight resistance can be transferred through breeding from tetraploid somatic hybrids (*S.* × *michoacanum* + *S. tuberosum* and autofused *S.* × *michoacanum*) to common varieties [142]. Bacterial wilt resistance was transferred to advanced progenies of somatic hybrids between *S. commersonii* and cultivated potato, and three highly resistant clones (BC1 and BC2) were selected as breeding materials [170]. In the case of potato there are many reports of symmetric interspecific somatic hybridization between diploid wild species and potato dihaploid lines [127]. The main problem with the majority of these hybrids was the infertility, which made difficult the restoration of valuable cultivar. For this reason symmetric somatic hybridization between tetraploid potato cultivars and diploid wild species became more popular [200]. The expected results after tetraploid with diploid protoplast fusion are hexaploid SHs, but among them aneuploid or mixoploid hybrids are often regenerated [131]. Genetically, the hybrids may be unstable and usually eliminate chromosomes from the wild species during the next stage of tissue culture, as occurred in the case of potato and *S. bulbocastanum* hybrids, but, after two back-crosses with cultivated potato, many of them re-stabilize at tetraploid level [131, 145]. Theoretically hexaploid or near hexaploid SHs of potato will tend to eliminate the wild species chromosomes and maintain only a few alien chromosomes or introgress some genes from the wild

parent. Chromosome elimination in some interspecific somatic hybrids of potato largely depends on the phylogenetic relationship, type of genome: A, B, C, D and P [201], cell cycle synchronization after fusion and the two parent chromosomes interaction during mitosis [202]. Asymmetric somatic hybrids can be a result of the ordinary symmetric fusion or can be induced by fragmenting the donor species DNA by using the donor-recipient method [203]. Production of asymmetric somatic hybrid plants aroused interest of breeders, because with controlled chromosome transfer the restoration process of cultivars is faster and easier [204]. Usually, the donor protoplasts are treated with sub-lethal doses of ionizing irradiation, such as gamma, X rays [205, 206] or UV irradiation [207], in order to induce double-strand breaks and hence partial genome elimination [208]. In addition to irradiation, chemical agents can be used to induce chromosome elimination, such as restriction endonucleases, spindle toxin or chromosome condensation agents [209]. With applying these methods, asymmetric potato hybrids with some wild *Solanum* species [210] and intergeneric somatic hybrids were successfully produced [211, 212]. Another possible limitation of somatic hybridization is the production of somatic hybrids with resistant traits, but with decreased tuber yield and/or quality (mis-shaped tubers). Various solutions exists to overcome these disadvantages: use of haploidization and intra-specific hybridization of dihaploid potato lines [198], or the use of somatic fusion in which tetraploid potato cultivars are fused with sexually incompatible diploid wild species, when the resulted hexaploids are most of the time fertile and are crossable with other tetraploid cultivars [129–131]. Somatic hybridization produced a large number of somatic hybrids in potato some of them being integrated into pre-breeding and then breeding programs. The advantage of somatic hybridization is the transfer of multiple resistance genes, although it is difficult to control the genes transferred into the crop from its wild relative. It was thought that asymmetric fusion will allow better control on the genetic material to be transferred but soon it was demonstrated that only a low amount of donor DNA is eliminated and there is no correlation between the dose of radiation and DNA fragmentation. Nowadays, new strategies can be applied to better control the genetic fate of the SHs. Molecular markers can be used to select the traits or genes of interest [145], selection pressure like pathotoxins can be applied to increase the number of resistant SHs to a certain pathogen, *etc*. Moreover, there is a new opportunity to use all the genomic tools to get more insights into the complexity of the SHs and better understand the complex interaction between six genome forced together by artificial fusion in one cell. The main advantages of this biotechnological tool is its status as non-transgenic in Europe (directive 2001/18/EC, annex 1B) and its acceptance by consumers.

4. The new biotechnological techniques (NBT) and their success in improving potato crop

In the last decade, new plant breeding technologies (NPBT) have been developed to address plant breeding for important traits of current days. Those technologies were refinements of transgenesis and ended up with such advancements as leaving no foreign DNA in the new modified plants. In a review published by Lusser *et al* [213] the techniques used for NPBT were zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs). In the same year a new NPBT was discovered and became the preferred alternative for plant genome editing, the clustered regulatory interspaced palindromic repeats or CRISPR [214]. CRISPR, a natural system used by bacteria and archaea to fight bacteriophages and foreign genetic fragments, has emerged as one of the most powerful and promising

genome editing techniques shaping the future of biotechnology [215]. CRISPR-Cas method is based on a short single-guide RNA (sgRNA), with a 20 bp guide sequence complementary to a target region in recipient genome, a promoter and a sgRNA scaffold, which in combination with a Cas9 nuclease [214], can induce mutations in a target region of choice. Cas9 or an alternative nuclease induce double strand breaks (DSBs), that are repaired by the cell's own repair mechanism, either through non-homologous end joining (NHEJ) or homologous recombination (HR) [214]. NHEJ is an error prone and often leads to random-sized inserts or deletions (indels), which may cause a knockout of gene function. In potato the first results using CRISPR-Cas9 have shown mutations by using *Agrobacterium*-mediated stable transformation. The targeted genes were: gene encoding an Aux/IAA protein, the *StIAA2*, in a double haploid potato cultivar [216] and the *ALS* gene in both diploid and tetraploid potato [217]. More recently, TALEN and CRISPR-Cas9 were stably introduced targeting *ALS* and using a geminivirus-mediated guide, to facilitate designed mutations [218]. Because of its simplicity and cost efficiency CRISPR-Cas9 was adopted for many plant species [219], including potato as a tetraploid where targeted multialleles mutagenesis was achieved [220]. Traits such as: improved resistance to cold-induced sweetening, herbicide tolerance, processing efficiency, modified starch quality and self-incompatibility have been targeted in potato using CRISPR/Cas9 and TALEN editing technologies in diploid and tetraploid clones [221]. Potato varieties with knockout mutations in all alleles of the *VInv* (vacuolar invertase gene) through precise genome engineering were also produced [222]. This was accomplished by transiently expressing transcription activator-like effector nucleases (TALENs) designed to bind and cleave specific DNA sequences in the *VInv* locus. The double-stranded breaks (DSBs) created by the TALENs were repaired by NHEJ, which introduced indel (insertion/deletion) mutations that compromised *VInv* gene function. Due to the high levels of heterozygosity in the potato genome, the task of simultaneously targeting multiple alleles required careful TALEN design and optimization [223]. In contrast to previous RNAi work, TALENs achieved complete knockout lines without incorporating foreign DNA. As a result, the new potato lines have significantly lower levels of reducing sugars and acrylamide in heat-processed products [224]. In another attempt CRISPR-Cas9 was successfully applied to reduce browning of potato silencing *PPO* gene [225]. Increase resistance to late blight was obtained by mutating S (sensitivity gene) genes *StDND1* and *StCHL1* [226]. CRISPR-Cas13a was used to increase resistance to PVY, and it induced resistance to all strains of the virus [51], while RNAi confronted with many drawbacks because of the virus genetic evolution (**Table 1**) [50]. Although, the successfully edited plants by using CRISPR-Cas are deposited in Plant Genome Editing Database (PGED) [227], to date (2021-04-31) there is no registry for potato.

5. Acceptance by consumer and combinatorial biotechnology

Potato biotechnology has developed potato varieties with one or multiple genes, which resist one or multiple biotic and/or abiotic stresses. Unfortunately, the continuous debate and consumer lack of trust affect the GM cultivation in the field and specifically the adoption of GM plants in the food chain. There were success stories about genetically engineered potato crop, some of them were deregulated and had a short time of field cultivation. One of the examples that presents the fate of GM potato is the Monsanto potato story. Monsanto has developed GM potatoes with insect resistance (IR) and virus resistance (VR). In 1995, Monsanto received US government approval for Cry3A *Bt* potato, resistant to CPB. 600 ha were planted with this transgenic potato in USA. Another GM potato with resistance to potato

leaf roll virus (PRLV) was approved in 1998 and a variety resistant to PVY in 1999. Moreover the *Bt* trait was stacked with PRLV and/or PVY resistance. From 1995 to 1998 the area with GE (genetically engineered) potato increased to 20,000 ha representing 3.5% of total area of potato crop in USA. But, in 2000 the area planted with GE potato declined sharply, a decline attributed to lack of acceptance by some consumers, the fast-food chain refusal of GE potato use and the incapacity of potato industry to test and segregate GE from non-GE potato. In these conditions, growers were concerned that their GE potato will no more be purchased by their buyers. The farmers, on the other hand, were purchasing a new insecticide for CPB and other pests rather than using GE varieties. In 2001 Monsanto decided to close its potato division [69, 228, 229]. Another story is about Amflora potato in Europe. After authorization procedure and favourable scientific opinions the European Commission approved the cultivation of BASF Amflora starch potato in 2010. This was the first GE plant approved for cultivation in EU in 12 years. The Amflora potato was not intended to be authorised for food, only for industrial use in starch production and its by-products as feed. Many member states were reacting against the GE potato authorization. In 2013, the EU General Court annulled the authorization of Amflora potato. In 2012 the BASF Company decided to move it's headquarter in USA (North Carolina) and halted the production of Amflora potato from EU market. Although, new breeding technologies and particularly CRISPR-Cas technology does not leave any foreign DNA into targeted mutagenized crops, EU has decided to consider edited crops under GM low in 2018, but there is hope that these modified crops will be accepted for cultivation and commercialization in the near future. The acceptance of modified crops by consumers varies from one country to another, depending on culture, history, environmental pressure *etc*., but it seems that the benefits of transgenic and editing methods will at the end extend at scientific level, because cost benefits, CO_2 reduction and reduction on pesticides use will override the consumers unscientific doubts. But until then there are other effective biotechnological tools that are not considered as GMOs, for instance, somatic fusion and production of somatic hybrids as presented above can also address many resistance traits and be included in breeding. We have proposed a new strategy of biotechnological results integration in potato breeding called combinatorial biotechnology and already gave some good examples for the SHs of potato varieties (4x) with the diploid wild species *S. bulbocastanum* and *S. chacoense* [145, 180]. For instance in the case of somatic hybrids potato + *S. cahacoense*, presented above, transgenesis using *AtMSH2* gene, somatic hybridization, molecular analysis and stress selection were combined. For further integration in breeding somatic hybrids have to be back-crossed with cultivars and embryo rescue will be applied for BCs regeneration. Moreover, to remove the transgenes another strategy has to be applied as: gene segregation, RNAi or CRISPR-Cas. Finally, these genotypes with very interesting traits: resistance to CPB (antibiosis and antixenosis), tolerance to drought and salt would be integrated in breeding. The adoption of these biotechnological tools coupled with new knowledge on potato genomics and phenome's will most probably change the ways how the biotechnology is integrated in potato breeding for resilient potato, which is indispensable in today's challenging agriculture.

6. Conclusions

There are many tools in potato biotechnology which could be applied to improve potato resistance to biotic and abiotic stresses and to increase the quality of potato tubers for different application as food, feed, industrial use of even medicinal applications (**Figure 3**). These tools coupled with the new knowledge of genomics

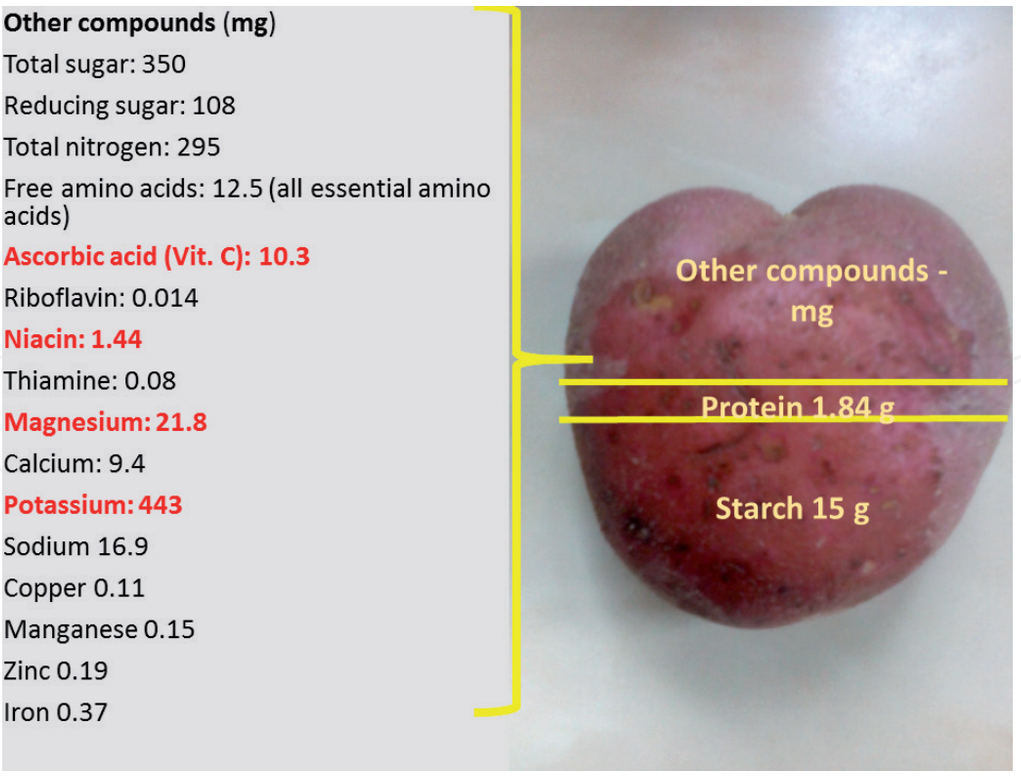

Figure 3.
Starch, protein and other valuable compound content of a potato raw tuber (detailed on the left (mg)) (modified data for cv. Russet Burbank https://www.researchgate.net/publication/265480176_27_).

and phenomics will be more and more accepted in improving potato crop for actual and future agriculture. Combinatorial biotechnology that in our opinion will use all advantages of potato genome manipulation, tissue culture techniques, and next generation biotechnologies along with genome, transcriptome and metabolome research will contribute to resilient potato crop.

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