4 Shaping the Sugar Beet of Tomorrow: Current Advances in Sugar Beet Biotechnology and New Breeding Techniques

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Abstract

Sugar beet genome was first published in 2014. Before this, there were GMO cultivars of sugar beet commercially available. The possibility of genetic transformation, together with the complete characterization of the genome facilitates the use of molecular techniques as well as new breeding techniques for sugar beet improvement. Another important aspect is the fact that sugar beet is a recently domesticated crop, and there is a huge wild genetic diversity, which constitute a great genetic pool which can be used as a source of useful genes for introgressions or for designing new GMO crops. In this chapter, we will review all the knowledge available in GMO sugar beet, the recent advances and applications of biotechnology and novel breeding techniques in sugar beet improvement, and the use of sugar beet genes in other crops as well as the future prospects.

Keywords

Abiotic stress · Beta vulgaris · CRISPR/Cas9 · Genetic transformation · GMO · Pest management · Sugar yield

Abbreviations

QTL	Quantitative Trait Locus
ALS	Acetolactate Synthase
AsA	Ascorbic Acid

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BCTV	Beet Curly Top Virus
BNYVV	Beet Necrotic Yellow Vein Virus
BWYV	Beet Western Yellows Virus
BYV	Beet Yellows Virus
D-DNA	Defective DNAs
DHA	Monodehydroascorbate
ds-DNA	Double-Stranded DNA
GA	Gibberellic Acid Biosynthetic
GMO	Genetically Modified Organism
ICPs	Insecticidal Crystal Proteins
MDHAR	Monodehydroascorbate Reductase
mtlD	Mannitol-1-Phosphate Dehydrogenase
PAT	Phosphinothricin N-Acetyltransferase
PEG	Polyethylene Glycol
PvPGIP2	Polygalacturonase-Inhibiting Protein 2
RNAi	Interference RNA
scFv	Single-Chain Variable Fragment
SPS	Sucrose-Phosphate Synthase
StGCS-GS	γ-Glutamylcysteine Synthetase-Glutathione Synthetase
TILLING	Targeting Induced Local Lesions In Genomes

4.1 Introduction

The world population is nowadays about 7.5 billion and it is increasing. Some models expect numbers of about nine billion in 2050. To feed this growing population, the agricultural yield must increase concomitantly. Sugar beet (*Beta vulgaris* L.) is one of the world's main suppliers of calories in the diet, together with sugarcane. Worldwide 80% of sugar comes from sugarcane and 20% comes from sugar beet. In the 2019/20 period, the production was about 166.18 million tons and is expected to be about 182 million tons in 2020/21, and this produces about 35 million tons of sugar per year. Over 120 countries produce sugar. While sugarcane cultivation is concentrated in the tropics, sugar beet is cultivated in temperate climates mainly in the northern hemisphere in regions of Europe, North America, and some countries in Asia. The Russian Federation, France, the United States, Germany, and Turkey represent the main producers in 2018 (FAOSTAT 2018). Besides its pivotal role as a source of sugar, sugar beet cultivation has other important outcomes. *Beta vulgaris* is used as an energetic crop (Zabed et al. 2014), for animal feed (Evans and Messerschmidt 2017) and some varieties (i.e., Cicla) are cultivated as green-leaf vegetables, commonly known as Swiss chard.

Environmental stress and several pests greatly threat affect yield. Sugar beet is grown in temperate areas of the northern hemisphere where the crop is usually sown in early spring. This allows improving the root production and avoids summer

drought and excessive heat at the plant maturity. In warmer climates or subtropical climates like southeast Asia, it is also possible to sow sugar beet in autumn (autumn sowing) or in other seasons, in order to anticipate harvest and escape drought and other environmental stresses. Sugar beet is also prone to pest of several kinds. Its cultivation is affected by weeds like the Redroot Pigweed (*Amaranthus retroflexus*), virus as beet necrotic yellow vein virus (BNYVV), bacteria such as *Pseudomonas aptata*, fungal disease as *Cercospora*, nematodes as *Ditylenchus dipsaci,* or insects like *Aphis fabae*; therefore, there is a great need to breed for more resistant *Beta vulgaris* cultivars.

There are pivotal achievements that have been obtained by classical breeding. Sugar beet is a recently domesticated plant created for the production of sugar (sucrose) from the beet used for animal feed in the 1800s. Classical breeding raised its sugar content from about 4-6% to the current 18% in 200 years (Mcgrath et al. 2018). Classical breeding has also made great achievements in traits of agronomical interest like the discovery and fixation of the monogerm gene-seed character (Savitsky 1950) which avoids multiple seeds germinating from a single fruit (utricle). Another major breeding achievement was the cytoplasmic male sterility for commercial varieties, which enabled the hybrid seed production, a trait that depends on a mitochondrial locus (Kitazaki et al. 2015). Hybrids present heterosis in root yield, among other traits (Ćurčić et al. 2017; Schwegler et al. 2014). Another achievement was the semitropical beet germplasm by increasing heat and bolting resistance which enables the cultivation in huge areas of India and southeast Asia (Srivastava 1995).

Abiotic stress is also a main limiting factor for yield. Soil salinity is a great constraint for productivity in many areas of cultivation (i.e., the Mediterranean basin, Iran, India, Southeast Asia, or the western United States). On the other hand, the advantage is that sugar beet is phylogenetically related to halophytic wood plants, being the most important the sea beet (*Beta vulgaris* ssp. *Maritima)* (Munns and Tester 2008). Sugar beet can stand concentrations up to 250 mM of sodium chloride in soil, which induces a 50% reduction in yield. In other major crops like beans, a 60 mM concentration of NaCl produces a similar reduction (Taïbi et al. 2021). Also, the defense system against salt stress is quite effective, for instance, in response to 300 mM sodium chloride solutions, beets are reported to suppress the generation of reactive oxygen species by transcriptional regulation (Hossain et al. 2017). The problem faced by farmers is that sugar beet is sensitive to salt stress at the seedling stage, and therefore, getting early vigor for salt stress tolerance is a great objective (Khayamim et al. 2014). The genotype "EL56" (PI 663211) is able to germinate in soils in up to 150 mM sodium chloride (McGrath 2011). Drought is also a problem faced in many cultivation areas. There are at least three loci described by quantitative trait locus (QTL) analysis suggested to be important for drought tolerance, but so far, the advances in sugar beet tolerance to drought stress are very limited (Rajabi and Borchardt 2015).

Also, classical breeding has focused on introgression disease and stress tolerance into novel germplasm and genotypes to offer solutions to farmers (Doney 1995; Panella et al. 2015). Thanks to that there are cultivars available with resistance or tolerance to predominant seedling diseases such as *Pythium*, *Aphanomyces*, and

Rhizoctonia. There are also some cultivars resistant to the main root disease Rhizomania "crazy root," due to the viral pathogen BNYVV (beet necrotic yellow vein virus). The main source of disease resistance genes for classical breeding, again, is the sea beet although some resistance has been found in wild beets, in fact, four different resistant genes (Rz1-5) isolated from different sources have been introgressed into commercial cultivars (Pavli et al. 2011b) although there are some strains that have broken the resistance (Biancardi and Tamada 2016). There have been some advances in resistance to nematodes and insects by classical genetic means (Zhang et al. 2008b). Therefore yield, pest resistance, and abiotic stress tolerance are the main objectives for the biotechnological improvement of sugar beet.

4.2 Sugar Beet Transformation

As we have seen classical plant breeding has improved the effectiveness of sugar beet for yield, pest, and to a very minor extent abiotic stress tolerance. It becomes clear that there is a need for biotechnological improvement. The first genetically engineered plant is considered to be tobacco and was reported in 1983 (Bevan et al. 1983). Attempts to transform sugar beet started almost immediately. The first was not successful as plants prove to be difficult to regenerate from transformed calluses (Harpster et al. 1988; Krens et al. 1988; Jacq et al. 1993) or cells (Wozniak and Owens 1989; Kallerhoff et al. 1990). Although transgene was incorporated in the plant genome, in most of these early reports stable transformation and reproducibility were not confirmed (Gurel et al. 2008).

Lindsey and Gallois (1990) described the first successful regeneration of a transformed sugar beet. Authors co-cultivated shoot-base tissues with *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) strain LBA4404 transformed with the gene *nptII* which confers resistance to the antibiotic kanamycin and either the chloramphenicol acetyltransferase (*cat*) gene (antibiotic resistance) or the β-glucuronidase (*gusA*) gene (enables staining). Shortly after the first genetically modified organism (GMO) sugar beet expressing an agronomically important trait (Herbicide tolerance) was described by D'Halluin (D'Halluin et al. 1992). In this case, the authors used callus derived from several organs transformed with *R. radiobacter*. As a selectable marker, they used (*bar*) (herbicide and bialaphos resistance) or a mutated acetolactate synthase (*ALS*) gene (herbicide resistance). These protocols were lengthy (2 years to obtain shoots) and showed a great genotype dependency. In fact, several years later it was described that easiness to transformation is a heritable trait (Kagami et al. 2016). The first genotype-independent protocol was described by (Fry et al. 1991) using cotyledonary explants inoculated with *R. radiobacter* containing different genes for herbicide or antibiotic tolerance. In this report, they confirmed the presence of transgenes, their mendelian segregation, and their functionality and the phenotype conferred (tolerance to herbicide). But the specific protocol was not published. Since then, other protocols based on callus transformation have been described (Kagami et al. 2015). Transformation with other

techniques has been described, specifically, particle bombardment (Saunders et al. 1992; Hashimoto and Shimamoto 1999); polyethylene glycol (PEG)-mediated transformation of protoplasts (Hall et al. 1996), petiole explants of haploid and diploid sugar beet genotypes using *Rhizobium rhizogenes* and sonication (Klimek-Chodacka and Baranski 2014). Most of these techniques took advantage of the advances in somatic embryogenesis (Zhang et al. 2008a) and regeneration (Ninković et al. 2010). Transformation techniques are so advanced that there are also several molecular tools available to the community, like root-specific promoters (Padmanaban et al. 2016), a promoter from Swiss chard that directs petioles and roots preferential expression (Yu et al. 2015) and a promoter that drives expression specific to nematode-feeding sites (Thurau et al. 2003). There are other tools to modulate gene expression such as the lox gene (Wang et al. 2003), and there is also a description of a set of transgenic plants and cell lines of sugar beet transformed with the maize transposable elements Spm/dSpm which allows gene-tagging (Kishchenko et al. 2010). Moreover, heterologous expression of the *Arabidopsis thaliana* GRF5 (AtGRF5) in sugar beet callus cells greatly increases transformation efficiency (Kong et al. 2020).

4.3 Plastid Transformation

Another point of interest has been transforming plastids into sugar beet. This technique has many advantages, among them, the fact that pollen does not have chloroplast, and therefore, gene flow is prevented. The first description dates back to 2009 although a previous communication can be found in the literature (De Marchis et al. 2007) and was achieved from petioles of the line Z025 by biolistic bombardment. Chloroplast was transformed with a vector that directs genes to the rrn16/rps12 intergenic region, employing the *aadA* and the *GFP* genes as markers (De Marchis et al. 2009). The technique has been improved but is not a routine technique yet (De Marchis and Bellucci 2021), and all the available literature comes from the same laboratory.

4.4 Transient Expression

Although the transient expression is not a routine technique in agronomy, it is very useful for investigation purposes, mostly related to basic science. There is a recent description of a transient expression in sugar beet using *Rhizobium radiobacter* (Moazami et al. 2018). One of the most common strategies for transient expression is agroinfiltration in leaves with bacterial cultures of *Rhizobium* transformed with the transgene. This is the election technique, for instance, for subcellular localization of a given protein using confocal microscopy (Locascio et al. 2019b). In our laboratory, we have transiently transformed sugar beet leaves using the published protocol for lettuce and tomato (Wroblewski et al. 2005) with positive results (our unpublished observations) (Fig. 4.1).

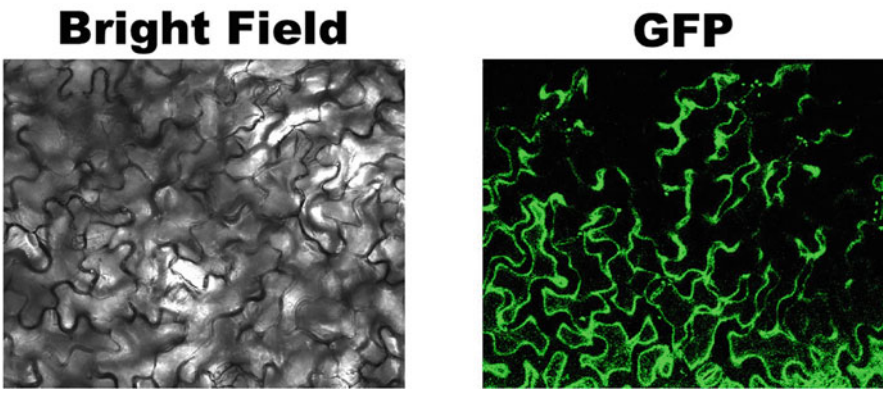

Fig. 4.1 Transient expression of GFP protein in sugar beet leaves

4.5 Heterologous Genes Transformed in Sugar Beet

We have just reviewed the different methods for sugar beet transformation. It becomes clear that inserting exogenous genes in the sugar beet genome poses no technical problem. Transgenic sugar beets have existed for almost three decades with a wide variety of traits with different objectives.

4.5.1 GMO Sugar Beet for Improvement of Biotic Stress

4.5.1.1 Herbicide Tolerance

Beta vulgaris culture is strongly affected by weeds (May and Wilson 2006). Standard control techniques require herbicide spraying at different periods, so controlling weeds in sugar beet cultivation is difficult, expensive and increases the carbon and the water footprint of the cultivation, this reducing sustainability (Klenk et al. 2012; Gerbens-Leenes and Hoekstra 2012).

As mentioned, the first stable transformed GMO sugar beet was resistant to herbicide (D'Halluin et al. 1992). There is only three commercial sugar beet approved and all contain genes conferring tolerance to herbicides. Liberty Link™ sugar beet (name: T120-7; code: ACS-BVØØ1-3) by Bayer crop science contains the gene *pat* encoding the phosphinothricin N-acetyltransferase (PAT) enzyme from *Streptomyces viridochromogenes* (resistance to glufosinate/phosphinothricin) and the *nptII* from *Escherichia coli* Tn5 transposon which confers tolerance to neomycin and kanamycin antibiotics. This variety was authorized in the United States in 1998, in Canada in 2000, and in Japan in 2001 (James 2011). The Roundup Ready™ sugar beet by Monsanto (name: H7-1; code: KM-ØØØH71-4) contains a single transgene, the cp4 epsps (aroA:CP4) from *Rhizobium radiobacter* strain CP4 conferring tolerance to glyphosate. This variety was approved in many countries worldwide for

food, specifically Australia, Canada, China, Colombia, European Union, Japan, Mexico, New Zealand, Philippines, Russia, Singapore, South Korea, Taiwan, and the United States, but for cultivation only Canada and the United States (since 2005) and Japan (since 2007). Finally, the InVigor™ sugar beet (name: GTSB77 (T9100152); code: SY-GTSB77-8), by Novartis and Monsanto which contains three different genes, the mentioned cp4 epsps (aroA:CP4), the glyphosate oxidase goxv247 from *Ochrobactrum anthropi* strain LBAA that confers tolerance to glyphosate and the marker gene uidA (GUS) gene from *Escherichia coli*. This was authorized in the United States in 1998, New Zealand, and Australia in 2002 and Japan in 2003. From an economic and environmental perspective, the use of these varieties (mostly the Roundup Ready) has saved millions of tons of herbicide. Usually, a glyphosate-resistant GMO sugar beet requires about 2 kg ha^{-1}, while non-GMO cultivars require about 6 kg ha^{-1} or higher, depending on the amount of weeds (Märländer 2005).

In 2012, there was a partial ban on the use of GMO sugar beet in the United States motivated by the case of Center for Food Safety v. Vilsack (CFS v. Vilsack), set in the U.S. District Court for the Northern District of California (CA District Court). The point was the presence of species like *Beta macrocarpa* and *Beta vulgaris ssp. maritima* in areas of California where RR sugar beet was planted, raising concerns on the possibility of transfer of the genes conferring tolerance to herbicides (McGinnis et al. 2010). Finally, following the different scientific reports, the USDA deregulated RR Sugar beet (Khan 2014). GMO sugar beets account for about 95% of the sugar beet cultivated in the United States (United States Department of Agriculture 2019). Given that the US imports 30% of sugar and produces 70%, and that sugar beet production is mainly for the sugar industry, which means that 66.5% of the sugar consumed in the United States comes from glyphosate-resistant GMO sugar beet. Similar numbers apply in Canada (Dillen et al. 2013). There are no reports of any adverse effects on human or animal health related to the use of GMO sugar beet. Studies show that after 2 weeks of the herbicide application only trace amounts of glyphosate are detected in roots or shoots, and in the crystalline sugar glyphosate is below the levels of detection (Barker and Dayan 2019).

Although not commercially available, there are also reports of transgenic sugar beet resistance to phosphinothricin with the *bar* gene (D'Halluin et al. 1992; Mishutkina et al. 2010), to imidazolinone by transformation with the *als* gene from *Arabidopsis thaliana* (a mutated acetolactate synthase). This mutation confers tolerance to some commercial herbicides such as imazethapyr (Pursuit®, BASF) (Kishchenko et al. 2011). There are also reports of crop trials of novel sugar beet varieties with tolerance to a specific acetolactate synthase (ALS) inhibitor herbicide bred by KWS using standard breeding techniques and branded Conviso Smart, but these varieties have not been marketed yet, and there are no published reports of the results of the assays (https://www.fwi.co.uk/arable/herbicide-tolerant-sugar-beet-trialled-uk). The herbicide-resistant sugar beet has been a great advance for sugar beet farmers but is the only one available in the market. Even though, there is a lot of investigation being performed in other traits.

4.5.1.2 Insect Resistance

Transgenic insect-resistant plants are in the market for a long time with great success in crops such as maize, cotton, soy (Koch et al. 2015), or brassicas (Poveda et al. 2020). These varieties have been developed mainly by transformation with the *Bacillus thuringiensis* insecticidal crystal proteins (ICPs) encoded by the cry gene family. Sugar beet plants transformed with cry1Ab (Shimamoto and Domae 2000), proved to be very effective protection against lepidopterans (Jafari et al. 2008) and the Egyptian leafworm (*Spodoptera littoralis*) (Sedighi et al. 2011). Sugar beet has also been transformed with cry1C (Kimoto and Shimamoto 2001), or with cry1C and cry2A (Lytvyn et al. 2014). A joint transformation with Cry1Ab and Cry1C protected against cabbage armyworm (*Mamestra brassicae*) (Kimoto and Shimamoto 2001).

4.5.1.3 Nematode Resistance

Sugar beet is very sensitive to nematode attack, among them *Heterodera schachtii*, the sugar beet cyst nematode (SBCN) is the one causing more damage to farmers (Villarías Moradillo 1999). The standard treatment depends on the use of chemicals with nematicide activity, but most of them are being banned or have undergone a strict regulation in many countries due to their environmental toxicity. Other strategies rely on resistant cultivars obtain by breeding, crop rotations (Joersbo 2007), or trap crops (Lathouwers et al. 2005). Another problem is that nematode pests are recalcitrant, as eggs can stay latent for years, even in adverse environments or in the absence of the host crop.

There was some success using classical breed to introduce the $Hs1^{pro-1}$ gene from *Beta procumbens* in commercial sugar beet cultivars (Jung 1998), but this causes a severe yield penalty, perhaps due to linked genes that correlated with decreased yield (Heller et al. 1996). Using genetic engineering is a way to override the problem with the linked gene (Ali et al. 2017). The $Hs1^{pro-1}$ gene from *Beta procumbens* was transformed in sugar beet, and the observed resistance was higher than in resistant cultivars developed by conventional breeding. GMO sugar beet expressing the SpTI-1 gene (a Kunitz-type trypsin inhibitor) exhibited similar phenotypes (Cai et al. 2003).

4.5.1.4 Fungal Resistance

The most important fungal diseases in sugar beet are *Cercospora* leaf spot (*Cercospora beticola*), root and crown rot (*Rhizoctonia solani*) and powdery mildew (*Erysiphe betae*, syn. E. polygoni) (Villarías Moradillo 1999). So far traditional control is based on integrated approaches, using both culture techniques, chemicals, or resistant varieties. There is not any GMO variety in the market with resistance to any fungal disease, indicating that from the biotechnological point of view there is still a need for improvement. There are several strategies which target the increase of the systemic acquired resistance by increasing the salicylic acid production or the hypersensitive response (with elicitors like the ones encodes by the *avr* genes). There are also attempts of transforming sugar beet with different defense proteins. The expression of a chitinase gene from pumpkin was able to increase chitinase activity

and in some cases even suppressed some symptoms of fungal infection, as this protein is able to degrade the fungus cell wall (Hashimoto and Shimamoto 2001). Following a similar strategy, the polygalacturonase-inhibiting protein 2 *(PvPGIP2)* of common bean (*Phaseolus vulgaris*) protects GMO sugar beet against *Rhizoctonia solani* and *Alternaria alternata* (Goudarzi et al. 2015). This protein inhibits fungal polygalacturonase and therefore slows downs the ability of the fungus to degrade the plant cell wall (Mohammadzadeh et al. 2012). A recent report describes that overexpressing the native enzyme of *Beta vulgaris* (*BvPGIP2*) in hairy roots confers resistance to *Fusarium oxysporum* (Li and Smigocki 2019). The transformation with the chloroplastic and the cytosolic superoxide dismutase from tomato also increased resistance to *C. beticola* (Tertivanidis et al. 2004). Even a gene from a different species of this fungus, the cercosporin toxin export (*CFP*) gene from *Cercospora kikuchii*, has been transformed into sugar beet, but to date, there are no reports of whether transformed plants exhibited any phenotype (Kuykendall and Upchurch 2004). Another strategy consisted of the transformation of sugar beet with the Mannitol-1-Phosphate Dehydrogenase (mtlD) from *E. coli*. This increased the resistance to *Alternaria alternata*, *Botrytis cinerea* and *Cercospora beticola* although it depended on the transgenic line and the conditions of the assay (Goudarzi et al. 2016).

4.5.1.5 Viral Resistance

There are GMO commercial crops in the market whose trait is viral resistance (Fuchs and Gonsalves 2007), but this is not the case for sugar beet yet. Sugar beet is prone to viral diseases that cause great yield losses like rhizomania, caused by beet necrotic yellow vein virus (BNYVV), or can be infected by viruses such as beet western yellows virus (BWYV), beet yellows virus (BYV), or beet curly top virus (BCTV). There are descriptions of viral-resistant varieties using classical means (Scholten and Lange 2000). Genetic engineering has been used either expressing transgenes or using double-stranded DNA (ds-DNA) or interference RNA (RNAi) to interfere with the viral infection and dissemination, and therefore gain resistance (Lennefors et al. 2006). The expression of viral coat protein proved to be effective against BNYVV as early as in 1990 (Kallerhoff et al. 1990) and in hairy roots cultures (Ehlers et al. 1991), in protoplasts from transformed suspension cells (Mannerlöf et al. 1996) or in protoplast derived from guard cells (Lathouwers et al. 1997). Expression of the hrpZ gene of *Pseudomonas syringae pv. Phaseolicola* also conferred rhizomania resistance (Pavli et al. 2011a). In some cases, the virus resistance does not correlate with the level of expression of the transgene (Kerr 2005).

Another method to induce resistance is to disrupt the viral cell-to-cell movement. The usual strategy is to disrupt the P42, P13, and P15, triple gene cluster from BNYVV (Lauber et al. 1998; Erhardt et al. 2000). This has been done by designing an RNAi with a three-intron hairpin construct carrying parts of the BNYVV replicase gene (Pavli 2010) or a dsRNA derived from the replicase gene. This construct was able to confer protection to the roots of transformed plants (Pavli et al. 2010). Constructions using a similar strategy, but against other sites of the virus induced stronger resistance (Zare et al. 2015). Gene silencing against rhizomania has proved

to be effective even in field tests (Safar et al. 2021). There is also a report describing the use of a single-chain variable fragment (scFv) specific to a major coat protein of virus, p21, with a construct that targeted the antibody to different organelles. After mechanical infection with BNYVV, the cytoplasmic construct showed the greatest efficiency in preventing the infection (Jafarzade et al. 2019). A different strategy has been found against BCTV. This infection provokes the generation of defective DNAs (D-DNA) which is suspected to be a plant response to attenuate symptoms. Transgenic plants expressing synthetic D-DNA presented attenuated symptoms (Horn et al. 2011).

4.6 Abiotic Stress

4.6.1 Drought Tolerance

In the current context of anthropogenic global warming, and the concomitant change in the precipitation regime the global aridity is increasing and affecting many sugar beet producing areas. Sugar beet is more tolerant to abiotic stress than most major crops, but under suboptimal water concentrations yield decreases drastically (Rajabi et al. 2007). There is no drought-resistant phenotypes of sugar beet identified so the use of classical breeding is very limited (Ebmeyer et al. 2021). A strategy to generate drought tolerance in crops is the transformation with genes able to increase the cellular content of osmolytes or osmoprotectans (small hydrophilic molecules that plants and other organism accumulates under drought stress conditions in order to avoid turgor loss) although in some cases with a severe yield penalty that compromises its market value and the utility for farmers (Van Camp 2005). There are only two cultivars in the market whose trait conferred by the transgene is drought tolerance: the DroughtGard® maize (Wang et al. 2015) and very recently the soybean expressing the HB4 transcription factor from sunflower (Ribichich et al. 2020). GMO plants transformed with the *Bacillus subtilis* gene, *SacB* accumulated fructans to low levels (0.5% of dry weight) but plants performed better under drought stress (dry weight +25–35% than control plants) and with no yield penalty under control conditions (Pilon-Smits et al. 1999).

4.6.2 Salt Tolerance

There are very few reports on GMO sugar beet resistant to salt stress. The first was authored by Yang et al. (2005). They introduced they introduced the *AtNHX1* gene (a sodium proton vacuolar exchanger from *Arabidopsis thaliana*) and reported salt tolerance. Sugar beet plants transformed with a paralogue of the same gene (*AtNHX3*) accumulate more soluble sugar and less salt in storage roots (Liu et al. 2008). There is also a report in which authors describe the transformation of sugar beet with *AVP1*, the vacuolar H^+-pyrophosphatase of *Arabidopsis thaliana* (Wang et al. 2012). And also, the co-expression of the sodium proton vacuolar antiporter

(*ZxNHX*) and the vacuolar proton pyrophosphatase (*ZxVP1-1*) from the xerophyte plant *Zygophyllum xanthoxylum* increased the osmoregulatory mechanism and diminished sodium toxicity, and also accumulated a higher content of sugars (Wu et al. 2015). And although not directly related to salt stress, overexpression of the γ-glutamylcysteine synthetase-glutathione synthetase (StGCS-GS) from *Streptococcus thermophilus*, and enzyme of the glutathione biosynthetic pathway increased the tolerance sugar beet to grow under high cadmium, copper, and zinc (alone or in combination) and also the ability to accumulate these heavy metals, providing a useful tool for bioremediation (Liu et al. 2015).

4.7 Development and Metabolism

4.7.1 Bolting Resistance

Bolting resistance is a prime objective for breeders as in some areas such as California and the Mediterranean sugar beet is a winter crop, but as they do not suffer freezing temperatures, bolting is a serious problem. This is also a problem in subtropical or warm areas of India and southeast Asia. Bolting depends on the expression of flowering genes, most of them regulated or part of the gibberellic acid biosynthetic (GA) pathway. The use of GA inhibitors like paclobutrazol is a standard strategy to avoid bolting (Sadeghi-Shoae et al. 2014). Therefore, there is a huge interest in modulating GA response and production in sugar beet to avoid bolting, as this will expand the growing season and permit plants to accumulate higher yields. There have been some studies coping with the modulation of bolting-related genes, and some important QTL have been identified (Pfeiffer et al. 2014). The first attempts to develop bolting resist sugar beet by biotechnology consisted of the transformation with the repressor of gibberellin biosynthesis from *Arabidopsis thaliana gai* or by or deactivation of gibberellic acid by heterologous expression of the scarlet runner bean (*Phaseolus coccineus*) gene *GA2ox1* (Mutasa-Gottgens et al. 2009).

B. vulgaris genome holds a vernalization-responsive FLC homolog (*BvFL1*), but opposite to what happens in other plants, its role is not important in bolting regulation, as plants transformed with iRNA to knock down *BvFL1* did not eliminate the requirement for vernalization of biennial beets and did not have a major effect on bolting time after vernalization. Overexpression of *BvFL1* only had a minor effect delaying the bolting after vernalization for about 1 week. Genes *BvFT1* and *BvFT2* are targets of regulation of BvFL1, and their protein product forms a module of regulation. These genes have antagonistic roles in the control of flowering (Pin et al. 2010). When these modules are downregulated, the phenotype is a several weeks delay in bolting, indicating some kind of redundancy in the BvFL1 function (Vogt et al. 2014). But so far, genetic engineering has not succeeded in creating a bolting-resistant genotype.

4.7.2 High Sucrose Yield

As sugar is the main product from sugar beet, increasing the yield is an obvious objective. This has been sought for long. First attempts consisted of knocking down the homolog of the SNF1 gene, a negative regulator of sucrose biosynthesis (Monger et al. 1995) or a maize sucrose-phosphate synthase (SPS) gene (Hashimoto and Shimamoto 1999), but the desired sugar yield increase was not obtained. Elliott et al. (1996) hypothesized that increasing the levels of cytokinin, a hormone related to development and mass accumulation, may increase sugar yield. They expressed a bacterial cytokinin biosynthesis gene, the isopentenyl transferase (ipt) (Snyder et al. 1999; Ivic et al. 2001). There was an effective increase in cytokinin accumulation, but also an inhibition of the taproot development and a minor sucrose accumulation.

4.7.3 Fructan Production

Sugar beet can be a source of molecules of industrial interest. For instance, fructans a fructose polysaccharide with many industrial applications (Turk and Smeekens 1999; Sévenier et al. 2002). There is a great interest in finding a cheap source of fructans, which includes the possibility to produce them from GMO sugar beet. First attempts consisted of using a gene from the traditional source of fructans, the Jerusalem artichoke (*Helianthus tuberosus*). The 1-sst gene of this crop, encoding a l-sucrose:sucrose fructosyl transferase was able to convert native sugar beet fructose to low molecular weight fructans in tap root cells and to a minor extent in leaves (Sévenier et al. 1998). Similar results were obtained when sugar beet was transformed with the 1-sst orthologue of onion (*Allium cepa*) and an additional gene of the fructan biosynthetic pathway (6g-fft, a fructan:fructan 6G-fructosyl transferase) (Weyens et al. 2004). Pilon-Smits et al. (1999) also tried to produce fructans in sugar beet by expressing the sacB bacterial gene, but as mentioned in a previous section, with very low yield and the drought tolerance phenotype was more interesting. There is also a report from transgenic beet transformed with PpFT1 and PpFT2, (two homologous sucrose:fructan 6-fructosyltransferases from timothy (*Phleum pratense*)) to produce levan, a molecule of the fructan family. Levans were successfully produced in GMO sugar beet, but the polymerization was much shorter than the polymerization obtained when levan was obtained from microorganisms (Matsuhira et al. 2014).

Finally, red beet has been used as a biofactory to develop test vaccines against type-I diabetes (Santoni et al. 2019), and there are also some reports of GMO crops designed for basic science, like the gene silencing of CYP76AD1, that blocks the red pigmentation of beets and induces a yellow coloration due to the accumulation of a betaxanthin pigment (Hatlestad et al. 2012) also the maize Ac transposase was expressed in sugar beet to confirm its alternative splicing in an heterologous system (Lisson et al. 2010).

4.8 Sugar Beet as a Source of Genes for Biotechnological Applications

Sugar beet genome was completed in 2014 (Dohm et al. 2014). This facilitates the identification of sugar beet genes useful for biotechnological applications, which has been a field of research interest in the last decades with some remarkable results.

4.8.1 Biotic Stress

There are some sugar beet genotypes that are resistant to pests. It means that could be a source of genes to transfer the resistance to plants in which interbred is not possible. This strategy has been used with the genes for nematode resistance *BvcZR3* and *BvHs1*$^{pro\text{-}1}$ that have been transformed into canola (*Brassica napus*) (Zhong et al. 2019) and tobacco obtaining resistance to nematodes (Sönmez et al. 2014). There are some sugar beet genes that have been transferred to other plants in order to get insect resistance. The serine proteinase inhibitor gene (*BvSTI*) has been expressed in *Nicotiana benthamiana* (Smigocki et al. 2008). In another report, Nicotiana transgenic plants overexpressing this gene were bioassayed against five lepidopteran pests with disparate effectiveness (Smigocki et al. 2013). There are also woody plants transformed with sugar beet genes. The silver birch (*Betula pendula*) has been transformed with the chitinase IV gene. The observed phenotype has been an increase in the fungal (Pappinen et al. 2002; Pasonen et al. 2004; Vihervuori et al. 2013) although in field trials transgenic trees suffered higher attacks from aphids than control plants (Vihervuori et al. 2008).

The gene codifying a germin-like protein (*BvGLP-1*), involved in nematode resistance, was transformed in *Arabidopsis thaliana* and conferred resistance to several pathogenic fungi (*Verticillium longisporum* and *Rhizoctonia solani*), without affecting beneficial fungus such as *Piriformospora indica* (Knecht et al. 2010). Rhizoctonia is a major problem of fungal origin for sugar beet cultivation. The overexpression of BvMLP1 and 3 major latex proteins in *Arabidopsis thaliana* resulted in less infectivity (Holmquist et al. 2021). Another strategy has been the overexpression of *BvPGIP1* and *BvPGIP2* Polygalacturonase-inhibiting proteins (PGIPs) that contain 11 leucine-rich repeat domains, contrary to most of the plants that only have 10 leucine-rich repeats. This construction conferred resistance in *Nicotiana benthamiana* (Li and Smigocki 2019).

4.8.2 Abiotic Stress

Sugar beet is a semi-domesticated crop that belongs to the Amaranthaceae family. Its closest cultivated relatives are spinach (*Spinacia oleracea*) and quinoa (*Chenopodium quinoa*). All three crops are suitable for poor soils or are able to resist environmental stress. It means that may be a source of useful genes to develop novel crops resistant to abiotic stress. One classical strategy is to test genes that have

demonstrated the ability to confer salt stress in other organisms or which have some previous evidence that relates this gene to abiotic stress. For instance, S-adenosylmethionine decarboxylase is an enzyme involved in the biosynthesis of polyamines, and its overexpression increases the amounts of spermine and spermidine. This enzyme was identified in a proteomic study for M14, a salt-tolerant monosomic addition line obtained from the intercross between *Beta vulgaris L.* and *Beta corolliflora Zoss* proteins with a differential expression upon a salt treatment (Yang et al. 2013). The overexpression of this enzyme from conferred salt stress tolerance to *Arabidopsis thaliana* (Ji et al. 2019). Similarly, the overexpression of another enzyme of the same pathway, S-Adenosyl-l-Methionine Synthetase 2 increased abiotic stress tolerance (salt and oxidation) (Ma et al. 2017). Some other genes cloned from this germplasm have been assayed for their biotechnological potential against abiotic stress. The cysteine protease inhibitor, cystatin, increased salt tolerance when overexpressed in *Arabidopsis* (Wang et al. 2012) and also a monodehydroascorbate reductase (MDHAR), an enzyme that reduces monodehydroascorbate (DHA) to ascorbic acid (AsA), when overexpressed in *Arabidopsis thaliana* confers salt stress, longer roots, higher chlorophyll content, and higher AsA/DHA ratios (Li et al. 2020).

The overexpression of the glyoxalase I gene from the same cultivar increased tolerance to pleiotropic abiotic stresses (salt, drought, oxidation) in *E. coli* and tobacco (Wu et al. 2013). A proteinase inhibitor BvSTI has been expressed in the forage legume *Lotus corniculatus L.* increasing the resistance to salt stress salt and altering plant architecture (Savić et al. 2019).

There is a strategy that has been very useful to identify sugar beet genes, the screening in a heterologous system such as yeast (Locascio et al. 2019a). There are several reports of such screenings which have been performed using *Beta vulgaris* cDNA libraries (Serrano et al. 2003). This is a fast and straightforward methodology that can unveil novel genes to identify limiting factors for abiotic stress tolerance. This strategy allowed the cloning of *BvCK2*, the catalytic subunit of the casein kinase which conferred salt tolerance upon overexpression in yeast (Kanhonou et al. 2001), the translation initiation factor BveIF1A which conferred salt tolerance by overexpression in yeast and Arabidopsis thaliana (Rausell et al. 2003) and *BvSATO1* (RNA-binding protein with RGG and RE/D motifs), *BvSATO2* (paralogous to *BvSATO1*), *BvSATO4* (RNA-binding protein), *BvSATO5* (RNA-binding protein), and *BvU2AF* (U2snRNP AF protein) (Téllez et al. 2020), all of them conferred tolerance to salt stress when overexpressed in yeast.

The same library was overexpressed in an osmosensitive yeast strain and screened for osmotic stress tolerance. The results were a serine acetyl-transferase, an enzyme involved in the biosynthesis of cysteine form serine (Mulet et al. 2004) and class 2 non-symbiotic plant hemoglobin (BvHb2) (Salort et al. 2010). *BvHb2* conferred tolerance to drought stress in yeast and *Arabidopsis thaliana*. When transformed in a horticultural crop, tomato (*Solanum lycopersicum* L.) also conferred resistance to drought induced withering. Another interesting aspect is that it also altered iron content by overexpression, increasing it in leaves and decreasing in fruit (Gisbert et al. 2020). *BvHb2* has also been expressed in wild

field cress (*Lepidium campestre*) and increased the seed oil content without altering its composition (Ivarson et al. 2017).

A different screening of the mentioned *Beta vulgaris* cDNA library for genes able to improve growth under cold conditions (10 °C) identified (*BvCOLD1*), a novel aquaporin gene not conserved in *Arabidopsis thaliana* and other model plants, which could only be found in evolutionarily related crops such as spinach or chinoa. Overexpression of *BvCOLD1* conferred pleiotropic abiotic stress tolerance and increased growth under poor boron medium (Porcel et al. 2018). In the same screening, several genes related to endosomal vesicle transport (Salort and Salom 2009), a ring finger protein (Sanz Molinero et al. 2009), a protein from the *PATELLIN* family (Molinero et al. 2014), and a growth-regulating factor (Reuzeau et al. 2017) were also identified.

In some cases, transformation has been performed to investigate the role of a given gene. This has allowed knowing that *BvCMO* the gene encoding a choline monooxygenase is required for salt stress tolerance, as the transformation of sugar beet with an antisense construction to block the expression of this gene conferred a phenotype of salt sensitivity (Yamada et al. 2015). Similarly, *Arabidopsis* transformed with the tonoplast glucose exporter *BvIMP* exhibit decreased freezing tolerance and germination (Klemens et al. 2014).

4.8.3 Development and Metabolism

Most of the studies found in the literature are not interested in the biotechnological improvement of sugar beet but in gaining knowledge on the molecular mechanisms in sugar beet. For instance, the role of *BvCOL1* as a photoperiod regulator was determined by its ability to complement *Arabidopsis* AtCOL1 and AtCOL2 mutants (Chia et al. 2008). Genetic engineering has been used as a tool to investigate sugar accumulation in sugar beets. *BvSUT1*, the gene that encodes the protein responsible for sucrose loading to the phloem, was transformed into yeast and expressed in oocytes of *Xenopus laevis* to investigate its mechanism (Nieberl et al. 2017). To investigate the subcellular localization of the betalain biosynthetic enzymes, those were expressed in tobacco (Chen et al. 2017). The sugar beet enzyme CYP716A was expressed in yeast to evaluate its ability to oxidize triterpenoids, molecules with several pharmacological and industrial applications (Suzuki et al. 2018).

The line M14 has also been a source of genes for studying the molecular biology of *Beta vulgaris*. For instance, the BvM14-MADSBOX was overexpressed in tobacco and led to severe phenotypic changes (Ma et al. 2011). The male sterility of the phenotype of some *Beta vulgaris* cultivars was mimicked in tobacco by expressing the mitochondrial ORF129 in tobacco with a mitochondrial targeting pre-sequence and a promoter for expression in flowers (Yamamoto et al. 2008). Transgenic sugar beet overexpressing bvORF20, a nuclear factor known as a restorer of fertility, was able to partially restore pollen fertility when overexpressed in cytoplasmic male sterile plants (Matsuhira et al. 2012) and has a complex, post-

translational regulation which includes the interaction with the preSATP6 protein (Kitazaki et al. 2015).

Another strategy is that during harvesting the plant suffers wounds and this induces the expression of invertase genes which degrades sucrose, and therefore reducing yield. Overexpression of sugar beet invertase inhibitor BvC/VIF to block this postharvest decrease proved to be ineffective (Jansen 2009).

4.9 New Breeding Techniques in Sugar Beet

New breeding techniques are the hyperonym for a set of techniques that go beyond classical breeding (hybridization, mutagenesis, grafting) but cannot be considered GMO as do not involve the transformation of a gene into another species. Among these techniques, the most popular is CRISPR/Cas9 that consists of using a bacterial defense system to edit the genome of the host species in a specific site of the genome, defined by the guide RNA. Although there should be intense research in this field, there are not many descriptions published yet the use of CRISPR in sugar beet, nor there is information on a field trial with CRISPR sugar beet. There are descriptions of the design of a CRISPR system based in the BNYVV that allows transient expression of four different proteins in different tissues of the plant. This system has been used to transform *Nicotiana benthamiana*, *Beta macrocarpa*, and *Beta vulgaris* (Jiang et al. 2019). Also, considered a new breeding technique the TILLING (Targeting Induced Local Lesions in Genomes), the platform is a reverse genetics technology that is being used in *Beta vulgaris* to increase its agro-diversity (Kornienko and Butorina 2013).

4.10 Future Prospects

We have summarized in this book chapter the main advances in genetic engineering in sugar beet. It is clear that new breeding techniques are still starting and there is not much development yet. CRISPR, TILLING, and other strategies may be implemented. In a similar manner, we have described a lot of advances on developing GMO sugar beet for avoiding bolting, sugar yield increase, abiotic stress tolerance, or pest management, but farmers currently only have access to herbicide-resistant cultivars. There is a long way ahead, not only for scientists, breeders, and agro-bio companies but also for politicians to enable the use of these varieties that are already in hand. Let's hope that in the close future some of the advances described in this chapter or some unexpected could help produce more and better food.

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