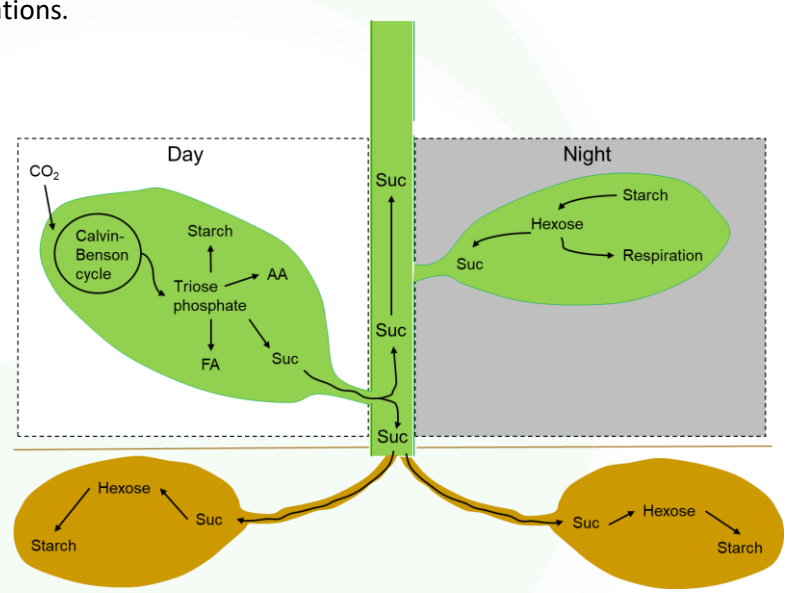
CRISPR/Cas in potato to improve agronomical traits

Potato belongs to the *Solanaceae* family, including related species like tomato, eggplant, and pepper. Potato is the world's fourth most important food crop for human consumption with a production of 368 million metric tons in 2018, fresh or processed for the use of industrial starch in several bio-based applications (e.g. foams, films, paper, textile, creams, fat replacer, soups, puddings, etc.). Potato is in the top three crops grown for starch production (2 million tons in EU). Traditional breeding to transfer desirable traits requires a long time due to the complex, tetraploid, and heterogenic genome of potato. In addition, the transfer of known interesting traits and mutations from closely related parental species is not always possible. CRISPR/Cas can be used as an efficient tool to simulate the transfer of interesting traits into elite cultivars. In GeneBEcon, CRISPR/Cas will be used to improve starch quality and to improve Potato Virus Y (PVY) resistance in selected elite cultivars.

Improving starch quality

Starch consists of two different types of molecules: amylose and amylopectin. By modifying different starch synthase genes, starch production can be redirected to amylopectin starch with short(er) chain length and can improve storage stability of starch pastes. A detailed bioinformatics study enables to find specific CRISPR-guide RNA molecule and reduces the risk for off-target mutations.

Principle of the formation of starch in potato plants: Starch is formed during photosynthesis as a temporary storage for energy distribution and in tubers as reserve starch for eventually fueling the next potato generation. A challenge in starch modification is to maintain biosynthetic power while changing specific enzymatic steps for desired starch quality.



For the modification of the starch quality, the CRISPR/Cas complex is built and delivered into isolated cells (= transfection) aiming to induce mutations disrupting and modulating the function of multiple enzymes to reduce or enhance the activity of specific steps in the formation of starch. Full mutations in four alleles of at least 4 genes/targets are possible in one round of transfection.

Improving Potato Virus Y (PVY) resistance

Symptoms of PVY infection in potato include yellow, light or dark green mosaic patterns on leaves, leaf drop, necrotic lesions on leaves and stems and tuber necrosis resulting in significant decrease in production yield. To prevent PVY infection, it is important to treat the aphids that are a vector for PVY. Once PVY infected the plants, no treatment is possible except removing diseased plants and tubers.

A detailed bioinformatic and translational biology study will be performed to identify the genes and enzymes involved in PVY resistance and in the biosynthetic starch pathway. Plant viruses as PVY recruit translation factors (= factors that facilitate the translation from DNA to RNA to enzymes) of the plant to translate their own viral RNA. Therefore, modifying plant translation factors, e.g. eIF4E, can induce resistance to viruses. A bioinformatics study

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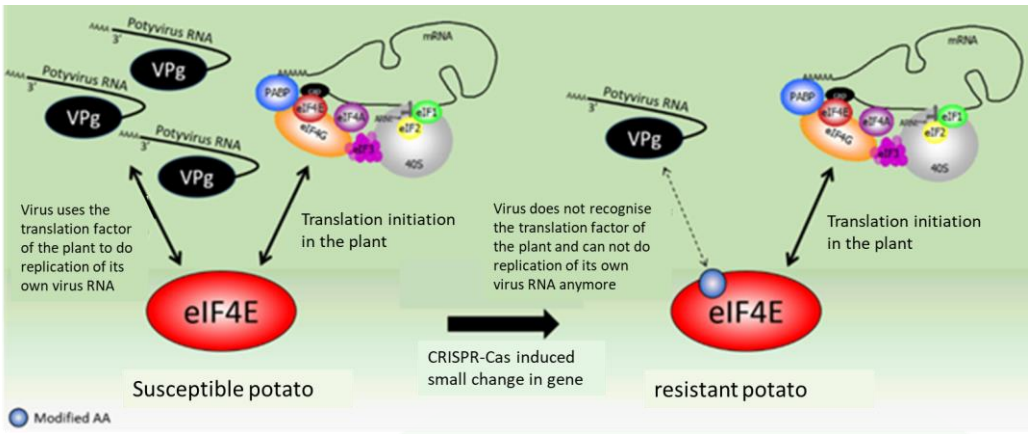
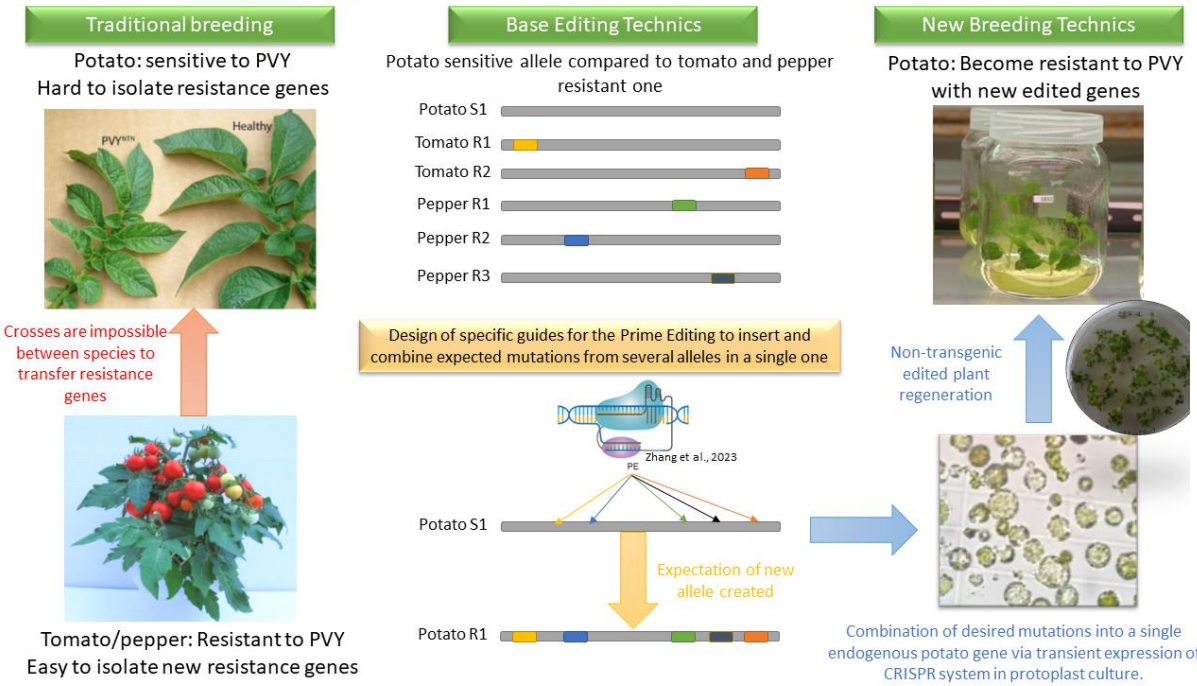
will enable to determine the best CRISPR-guide RNA molecule to target the translation factors. For this, existing sequence knowledge from tomato and pepper can be used too.



Principle of inducing resistance to the PVY-virus: PVY uses the eIF4E translation factor to induce its own viral RNA multiplication. A small modification (change of only one amino acid) of the translation initiation factor results in resistance of the plant, since the PVY-virus cannot use the translation factor of the plant to multiply its own RNA anymore.

The CRISPR/Cas complex is delivered into isolated potato cells to induce small modifications in the translation initiation factor, since larger mutations would result in translation problems in the plants. To induce small mutations, base editing or prime editing will be used. Base editing is a modification of the CRISPR/Cas system. A modified Cas enzyme (dCas) is used that does not cut the DNA but still can bind to the DNA on a specific place guided by the target CRISPR-RNA molecule. The dCas complex is also fused with a base editor which allows to change 1 nucleotide into another nucleotide (e.g. C into T, A into G). In prime editing a modified Cas enzyme is used inducing a single strand DNA break (instead of a double strand DNA break) at a specific place in the genome. At that place a mutation can be inserted.



Concept of New Breeding techniques for transferring improved resistance in potato. The transfer and combination of several mutations inducing resistance to PVY virus from Tomato and Pepper into potato is done by Prime Editing.

Screening for mutated plants

Isolated and transfected cells are regenerated into new potato plants. Via molecular DNA techniques the insertion of desired mutations is screened. A good bioinformatics study prior to performing CRISPR enables to screen for off-target mutations. If present, the number of off-target changes will not differ from the number of spontaneous mutations. Interesting mutant plants are further grown and the improvement of both PVY resistance and starch quality is evaluated. Mutations will be inherited in next generation plants.

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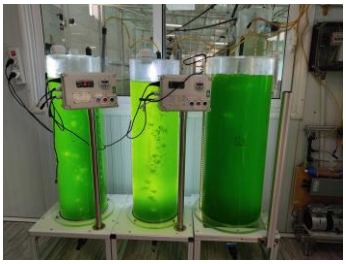
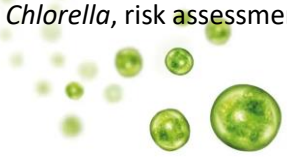
CRISPR/Cas in microalgae to modulate the production of high-value compounds

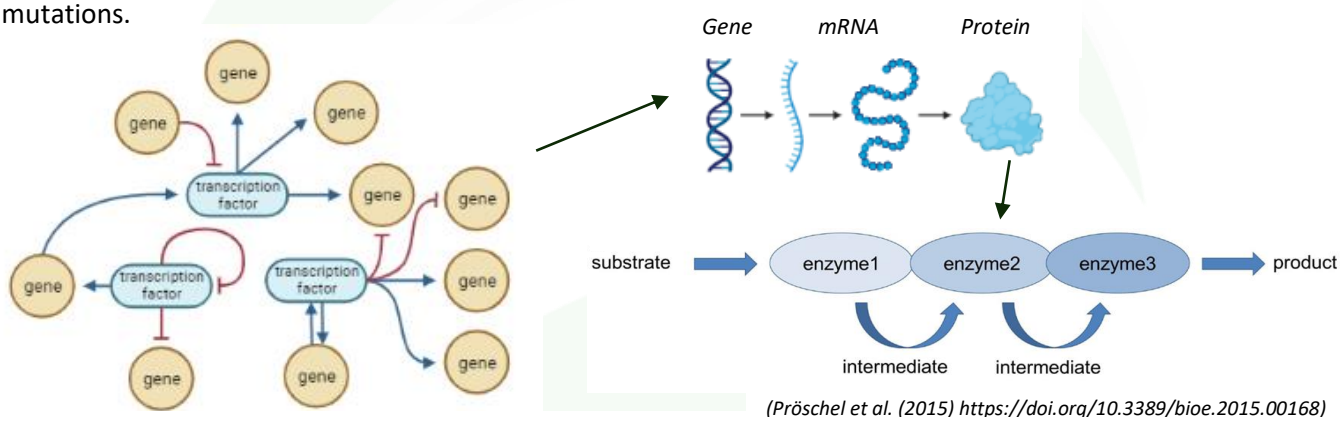


Chlorella spp. are interesting microalgae because they are approved for use in food and feed. *Chlorella* is grown in photoreactors (feeding on light) or in bioreactors and fermentors (feeding on nutrients). Microalgae generally produce a range of high-value compounds, e.g., mycosporine-like amino acids (MAAs), that are used in several cosmetical, pharmaceutical and industrial applications (sunscreens, anti-oxidants, coatings, anti-weathering, etc.). To make *Chlorella* an interesting production vehicle for these high-value compounds, CRISPR/Cas can be used to increase the production of MAAs. Because of the closed contained production system of *Chlorella*, risk assessment of CRISPR/cas applications may be different compared to open field crops.



To perform CRISPR/Cas, a 3-step-workflow will be followed:

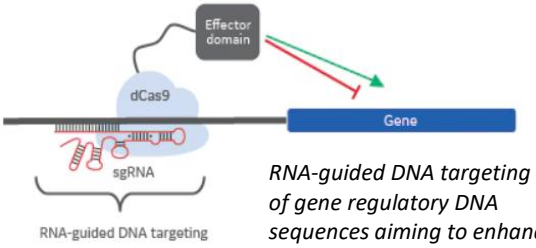
(1) A detailed **bioinformatic study** will be done to identify the components and enzymes involved in the biosynthetic pathway (= formation) of MAAs in *Chlorella* and to select the genes responsible for the activity of the pathway. Based on that information **the best CRISPR-guide RNA molecule** will be designed that will specifically guide the Cas enzyme to the desired place in the genome. A detailed and proper bioinformatics study enables to design a very specific guide RNA molecule and therefore minimizes the risk of off-target mutations.



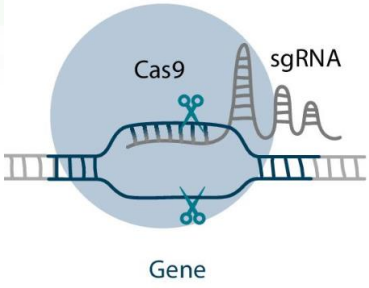
(Pröschel et al. (2015) https://doi.org/10.3389/bioe.2015.00168)

The formation of MAAs is accomplished by a complex network of genes that interact with each other and with gene regulatory factors (= transcription factors). The expression of genes give rise to proteins/enzymes that can perform metabolic and chemical reactions. A sequence of chemical reaction (= a pathway) results in the transformation of a substrate (large complex molecule) into specific compounds, in our case MAAs. By a detailed bioinformatic study the genes that are responsible for the enzymes in the MAA pathway will be determined.

(2) The CRISPR-RNA guide molecule and the Cas-enzyme are combined and **delivered into the *Chlorella* cell**. There are **two optional uses of the repair mechanism after the break**: (1) disrupt the function of compounds that reduce the activity of the pathway (SDN-1) or (2) add extra components to insert DNA where required (SDN-2), e.g. activation elements in the gene promotor that increase the activity of the promotor and hence increase activity of the pathway.



RNA-guided DNA targeting of gene regulatory DNA sequences aiming to enhance activity of the pathway.



SDN-1 resulting in knock-out of genes that are involved in inhibition of the MAA pathway. If the MAA pathway is no longer inhibited, more MAAs will be produced.

(3) Via **DNA sequencing techniques** it is then checked which cells contain a mutation induced by the CRISPR/Cas system. Cells with a desired mutation are selected and **regenerated** and further grown. The mutations are inherited in the next generation.

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