PlantEd

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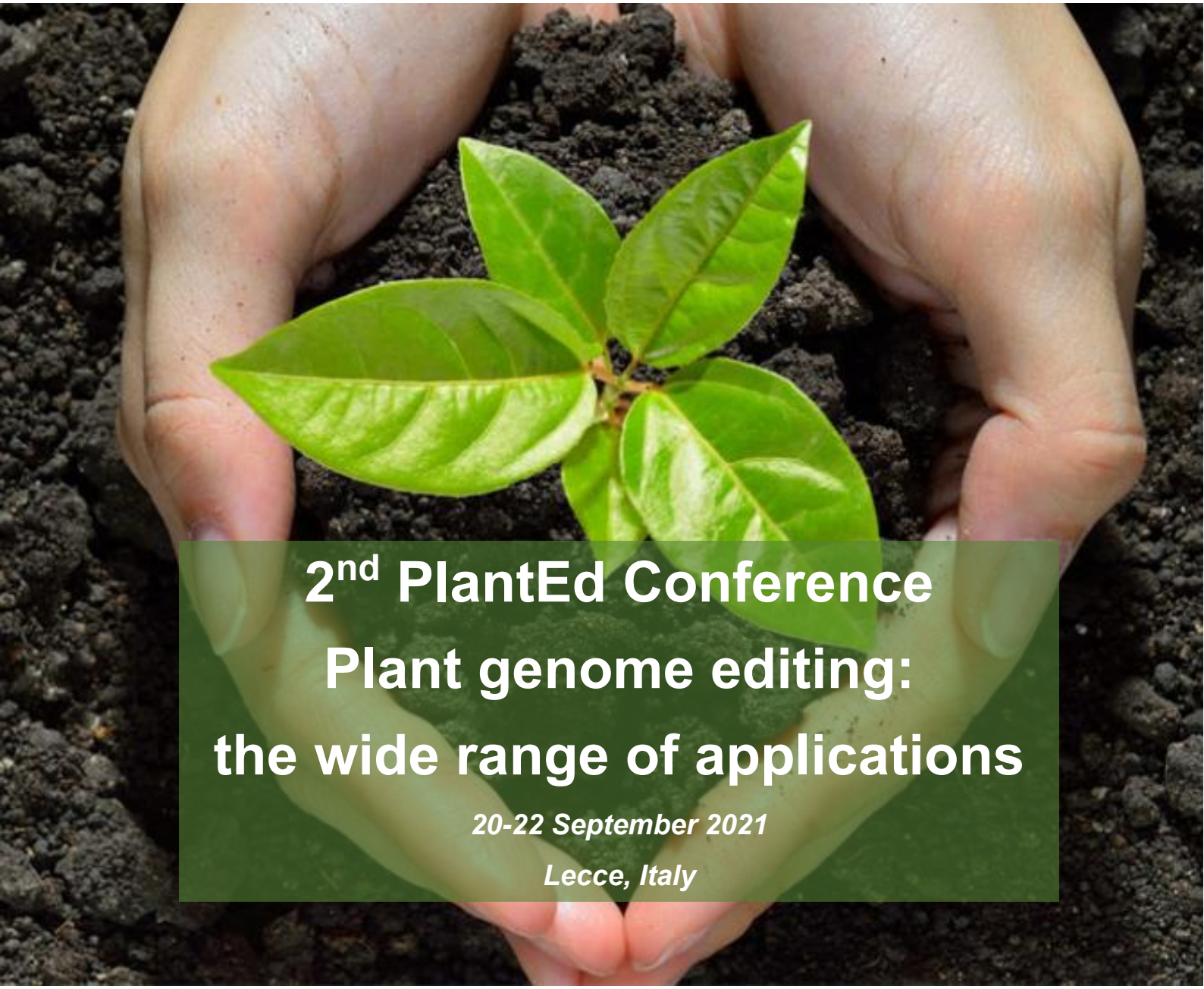

2nd PlantEd Conference
Plant genome editing: the wide range of applications

20-22 September 2021

Lecce, Italy

BOOK of ABSTRACTS

DiSBA

UNIVERSITÀ DEL SALENTO

PlantEd
COST Action CA18111

2nd PlantEd Conference

Plant genome editing:

the wide range of applications

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COST Action CA18111 (PlantEd)
"GENOME EDITING IN PLANTS - A TECHNOLOGY WITH TRANSFORMATIVE POTENTIAL"

2nd PlantEd Conference

Plant genome editing: the wide range of applications

20-22 September, 2021

Lecce, Italy

The 2nd conference of PlantEd (COST Action 18111) will take place over three days, with open scientific sessions dedicated to genome editing technology in plants, followed by PlantEd Working Group (WG) meetings as well as a Management Committee (MC) meeting. With PlantEd being a network for research on plant genome editing across Europe and beyond, this conference is an excellent platform for dissemination, discussions and connections, and to stay updated on the latest research and innovation forefront. The conference will be carried out as a hybrid event, with physical presence of a limited number of participants as well as live streaming, through the Gotowebinar platform). The conference will be promoted by the scientific journal Plants (IF 2.76).
The conference will host sessions on the application of genome editing in various types of economically important plants (cereals, oilcrops, roots and tubers, legumes, fruits and vegetables, trees, algae), as well as present the latest technological advancements for genome editing in plants. We will also host joint sessions with other relevant COST Actions to explore mutually beneficial interactions.
This conference takes place towards the end of the second grant period, which marks the half time of the duration of the Action. The outcome of the conference, including the WG meetings and the MC meeting, will help shaping the activities of PlantEd over the second half. The conference will be followed immediately by a WG2 Training School on impact.

Dear Conference participant,

I am very much looking forward to this conference, which for me will be the first on-site academic event in more than a year and half. The previous PlantEd conference took place in Novi Sad, Serbia, on 5-7 November 2019. Since then we have experienced very challenging times indeed with the global Covid-19 pandemic. The world of plant genome editing has nevertheless continued to develop fast. To mention but a few advances: the Cas protein PAM range and specificity has improved[1], carbon-based nanoparticles promise a more efficient delivery into cells[2], editing through de novo induction of meristems has bypassed the need for extended tissue culture[3], prime editing has been applied in cereals[4], multiplexing has allowed six genes to be edited simultaneously[5], and CRISPR/Cas is being applied widely for epigenetic editing[6]. In addition, companies are getting started to facilitate gene editing through outsourcing[7], and the Nobel Prize of chemistry was – finally! – awarded in 2020 to two outstanding women who have paved the way for the field of genome editing as we know it today. On the regulatory front, it is noteworthy that the European Commission has now opened up for discussion on how to change the EU GMO law.[8] The implications of this for plant genome editing research and innovation in Europe remain to be seen, but hopefully it means a step towards a regulatory system that enables safe and sustainable applications of genome editing for an environmentally-friendly plant-based production that sustain humanity´s needs.

I am looking forward to meet you in Lecce!

Yours sincerely,

Dennis Eriksson

Action Chair PlantEd

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PROGRAMME 2nd PlantEd Conference

Monday 20 Sept

08:30-09:10	**REGISTRATION**
09:10-09:30	***OPENING of the CONFERENCE***
09:30-12:00	**Genome editing in cereals** ***Moderator: Roberto Defez, IBBR-CNR, Italy***
09:30-10:00	*Raffaella Battaglia, CREA, Italy* **Modulating yield components in barley**
10:00-10:20	*Goetz Hensel, Heinrich-Heine-University, Germany* Precise gene editing of barley using ribonucleoprotein complexes
10:20-10:40	*Pouneh Pouramini, Leibniz Institute of Plant Genetics and Crop Plant Research, Germany* Targeted knock out of barley endosperm-specific storage proteins as a prerequisite for molecular farming purposes
10:40-11:20	*Coffee break- Posters display*
11:20-11:40	*Stefania Masci, University of Tuscia, Italy* CRISPR-Cas9 genome editing for the development of wheat lines with improved nutritional properties
11:40-12:00	*Sadiye Hayta, John Innes Centre, UK* Extending genome editing into elite wheat cultivars by deploying morphological genes
12:00-13:00	*Lunch*
13:00-15:40	**Genome editing in fruits and vegetables** ***Moderator: Angelo Santino, ISPA-CNR, Italy***
13:00-13:30	*Cathie Martin, John Innes Centre, UK* **Engineering vitamin content of tomato by genome editing**
13:30-13:50	*Aurelia Scarano, CNR-ISPA, Italy* CRISPR/Cas9-mediated genome editing on *SlDET1* gene for the nutritional improvement of tomato

13:50-14:10	*Musa Kavas, Ondokuz Mayıs University, Turkey* Generation of male-sterile tomato lines with the CRISPR/Cas9 system
14:10-14:30	*Alessandro Nicolia, CREA-OF, Italy* CRISPR/Cas9-mediated mutagenesis as a strategy to develop resistant tomato plants against Orobanche
14:30-15:00	*Coffee break*
15:00-15:20	*Paola Punzo, CREA-OF, Italy* CRISPR/Cas9 editing of proline metabolism and SOS pathway genes for improving abiotic stress tolerance in tomato
15:20-15:40	*Loredana Moffa, CREA-VE, Italy* Potential of New Plant Breeding Techniques for grapevine breeding
16:00-18:00	**PlantEd Working Groups meetings (WG1-WG5)**
19:00-20:30	*WELCOME COCKTAIL*

Tuesday 21 Sept

08:45-10:25	**Genome editing in plants- the latest technological advancements** ***Moderator: Isabel Mafra, University of Porto, Portugal***
08:45-09:05	*William de Martines, Plant Breeding, Wageningen University, Netherlands* New approaches to gene targeting in plants by exploiting the unique characteristics of CRISPR-Cas12a
09:05-09:25	*Fabio D'Orso, CREA-GB, Italy* Effective CRISPR-mediated knockout mutations in plants require translations reinitiation avoidance
09:25-9:45	*Ellen Slaman, Wageningen University, Netherlands* Applying high-throughput technology to identify CRISPR-Cas9 induced off-target mutations in tomato
09:45-10:05	*Isabel Mafra, REQUIMTE-LAQV, University of Porto, Portugal* Are there available tools to trace genome-edited crops in foods?

10:05-10:25	*Agnes E. Ricroch*, *IDEST, Paris-Saclay University, France* Next biotechnological plants for addressing global challenges: the contribution of transgenesis and New Breeding Techniques
10:25-11:15	*Coffee break – Poster Session*
11:15-12:00	**Joint session with COST Action EPI-CATCH** ***Moderator: Dennis Eriksson, SLU, Sweden***
11:15-11:30	*Federico Martinelli, University of Florence, Italy* Transgenerational effects of chromium stress in *Arabidopsis thaliana*
11:30-11:45	*Michal Lieberman-Lazarovich*, *Agricultural Research Organization, Israel* Epigenetics of heat stress response in tomato
11:45-12:00	*Ueli Grossniklaus, University of Zurich, Switzerland* Standing epigenetic variation is subject to selection and contributes to relevant plant phenotypes
12:00-13:00	*Lunch*
13:00-15:20	**Genome editing in roots and tubers** ***Moderator: Guy Smagghe, Ghent University, Belgium***
13:00-13:20	*Erik Andreasson, Swedish University of Agricultural Sciences, Sweden* Mutations in susceptibility genes through CRISPR/Cas9 genome editing confer increased pathogen resistance in potato
13:20-13:40	*Csaba Eva, Centre for Agricultural Research, Hungary* Edition of potato for reduced PPO activity confers resistance to *Ralstonia solanacearum*
13:40-14:00	*Jeny Jose, Centre for Agricultural Research, Hungary* Molecular and metabolomics analysis of resistant potato varieties as a way forward to generate resistance to *Ralstonia solanacearum*
14:00-14:20	*Mario Tavazza, ENEA, Italy* CRISPR-Cas9 targeting of the *eIF4e-1* gene induces resistance to *Potato Virus Y* in *Solanum tuberosum* L. cv. Desirée
14:20-14:40	*Coffee break*
14:40-15:00	*Priscilla Olayide*, *Swedish University of Agricultural Sciences, Sweden* Identification of suitable targets for gene editing mediated crop improvement: the example of CRISPR/Cas9 directed gene editing in cassava for increased

	β-carotene accumulation
15:00-15:20	*Guy Smagghe*, *Ghent University, Belgium* First report on CRISPR/Cas9-targeted mutagenesis in the Colorado potato beetle, *Leptinotarsa decemlineata*
15:30-17:30	**PlantEd 3rd Management Committee meeting**
19:00-23:00	*SOCIAL DINNER*

Wednesday 22 Sept

08:45-10:55	**Genome editing in oilcrops, algae, trees and other plants** ***Moderator: Tobias Brügmann, Thünen Institute of Forest Genetics, Germany***
08:45-09:15	*Li-Hua Zhu, Swedish University of Agriculture Sciences, Sweden* **CRISPR-Cas9 editing in rapeseed**
09:15-09:35	*Tobias Brügmann*, *Thünen Institute of Forest Genetics, Germany* Establishment of genome editing techniques in trees
09:35-09:55	*Vladislava Galovic, University of Novi Sad, Serbia* Gene editing in poplar using CRISPR/Cas to improve tolerance to *Lonsdalea populi* infection
09:55-10:15	*Hilde-Gunn Opsahl-Sorteberg, Norwegian University of Life Sciences, Norway* Navigating possible seaweed industrial development by crucial genomic tools
10:15-10:35	*Charlotte De Bruyn, ILVO, Belgium* Identification of bitterness related biosynthesis genes in *Cichorium* using CRISPR/Cas9 genome editing
10:35:10:55	*Matthias Fladung, Thünen Institute of Forest Genetics, Germany* Targeted CRISPR/Cas9-based knock-out of the rice orthologs *TILLER ANGLE CONTROL1 (TAC1)* in poplar induced erect leaf habit and shoot growth
10:55-11:20	*Coffee break*

11:20-12:30	**STSM session** ***Moderator: Dennis Eriksson, SLU, Sweden***
11:20-11:30	*Justyna Boniecka, Nicolaus Copernicus University, Poland* Targeted mutagenesis in oilseed rape (*Brassica napus* L.) protoplasts using CRISPR/Cas
11:30-11:40	*Andreja Škiljaica, University of Zagreb, Croatia* Gene editing of *Arabisopsis thaliana* cytosolic/nuclear subclass of Hsp70
11:40-11:50	*Kubilay Yıldırım, Ondokuz Mayıs University, Turkey* *Agrobacterium* mediated CRISPR/Cas9 transformative potential to modify abiotic stresses in poplar
11:50-12:00	*Dejan Stojkovic*, *University of Belgrade, Serbia.* First steps towards bioactivity guided gene editing in chicory for the higher production of targeted sesquiterpene lactones: CHIC project
12:00-12:10	*Melekşen Akın, Igdir University, Turkey* Gene editing in celery: Short Time Scientific Mission at ILVO
12:10-12:20	*André Rosado, Aberystwyth University, UK* Overview of biosafety regulations to support the future regulatory status of precision breeding products in some non-EU countries
12:20-12:30	*Juan Antonio Vives-Vallés*, *University of the Balearic Islands, Spain* Plant Breeders' Rights in the light of the NPBT
12:30-12:35	**Presentation by EU-SAGE**
12:35-12:45	**POSTER PRIZE ceremony**
12:45-13:00	***OFFICIAL CLOSING OF THE CONFERENCE***
13:00-14:00	*Lunch*

Genome editing in cereals

PlantEd

Modulating yield components in barley

Raffaella Battaglia

CREA Research Centre for Genomics and Bioinformatics, Fiorenzuola d'Arda I-29017, Italy

Food security is among the main urgencies that policy and science must ensure to the world population and plant scientists are involved in the identification of sustainable routes to boost crop yield. In this frame, we are working on the identification of key genes controlling fertility and kernel formation in barley. The movement of sugars from the source to the sink tissues impacts many aspects of plant growth. Studying the role of *SWEET* genes in barley we found that male fertility is linked to the activity of the *HvSW4* gene. Kernel development is a further step that determines yield potential. Focusing the attention on the function of selected miRNA genes we have identified two pathways controlling kernel traits. Our data suggest that the miR397a and miR396 genes influence kernel size through the regulation of the *LAC12* and *GRF4* transcripts. Gene editing and over-expression of these genes represent the first step to dissect the molecular pathways controlling seed size in barley. Our data present different molecular pathways controlling fertility and kernel traits, the functional characterization of key genes acting in these pathways put the basis for gene manipulation in the perspective of boosting yield potential in cereals.

Keywords: seed size, laccase, GRF, SWEET, source-sink relation

Precise gene editing of barley using ribonucleoprotein complexes

Goetz Hensel[1,2,3] and Martin Becker[3]

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[2]*Centre of the Region Haná for Biotechnological and Agricultural Research, Czech Advanced Technology and Research Institute, Palacký University Olomouc, 78371 Olomouc, Czech Republic*

[3]*Department of Physiology and Cell Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Seeland, Germany*

Targeted mutagenesis employing sequence-specific endonucleases such as CRISPR/Cas technology has been demonstrated in various plant species. This technique's frontiers still lack predictability of the outcome since CRISPR/Cas-introduced double-strand breaks are resulting in insertions and deletions (InDels), which are itself not predictable. Using the cell's homology-directed repair (HDR) mechanism, a predicted allele exchange can be introduced into the loci by providing a synthetic repair template including the desired gene modification. One way to achieve this precise allele exchange is the use of ribonucleoprotein complexes (RNP). A synthetic sequence-specific gRNA and a Cas protein are assembled in vitro and transferred together with the allele exchange-specific repair template into the cell. To facilitate easy detection of homology-directed genomic modifications, we follow two approaches. Employing Gfp-transgenic barley plants, we use Gfp-specific guides for Cas9/Cas12a-mediated double-strand break induction and application of a custom *Yfp* repair template. After successful HDR, Yfp fluorescence can be used as a readout. In a second attempt, the endogenous *LOX1* gene should be altered by introducing a restriction enzyme recognition site for easier detection of HDR events.
Presented are data comparing SpCas9 and AsCas12a endonuclease in barley epidermal leaf cells considering their different features. To interfere with the ratio between preferred non-homologous end joining (NHEJ) and HDR, RNAi was used to repress key genes involved in NHEJ. RNAi constructs targeting *Ku70, Ku80* and *Ligase IV* genes were generated and used for transient and stable barley integration.

Targeted knockout of barley endosperm-specific storage proteins as a prerequisite for molecular farming purposes

Pouneh Pouramini[1] and Goetz Hensel[2,3]

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[2]*Centre for Plant Genome Engineering, Institute of Plant Biochemistry, Heinrich-Heine-University, 40225 Dusseldorf, Germany*

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The concept for the production of valuable proteins in plants is known as molecular farming. Besides transient expression using viral vector systems in tobacco leaves, the cereal grain is a natural bioreactor ideal for storing proteins at ambient conditions. Therefore, the cereal grain provides a cost-effective, easily scalable expression system for producing high-value proteins in the starchy endosperm. Endosperm-specific expression in barley grains was established using the *Green fluorescent protein* gene driven by the oat *Globulin1* promoter. To increase protein yield and to overcome the competition between endogenous storage protein accumulation and the high-value protein, a targeted knockout of the *Hordein B* family members by using RNA-guided, Cas9-mediated double-strand break induction and error-prone repair was performed. To this end, mutants were generated, and segregating progenies were evaluated to identify transgene-free, azygous *horb1* mutants. Results indicated altered grain morphology, reduced total protein and hordein content, and delayed germination behavior compared to wild type segregants. Deep amplicon sequencing was performed to understand the complex genomic configuration of *horb1* mutant plants. Endosperm-specific overexpression in Golden Promise wildtype and *horb1* mutants were performed, and results will be presented.

CRISPR-Cas9 genome editing for the development of wheat lines with improved nutritional properties

Stefania Masci

Department of Agricultural and Forest Sciences (DAFNE), University of Tuscia, Viterbo, Italy

Although wheat is a staple food for most people, it can cause different adverse reactions among which Non Celiac Wheat Sensitivity. The main cause of this pathology has not been ascertained yet, but there are strong indications that alpha-amylase and trypsin inhibitors (ATI) are the main triggering factors. We have developed durum wheat lines in which specific ATI genes have been silenced and two of these lines are under characterization in regard to their capability to trigger Non celiac wheat sensitivity.
Moreover, although one of the main quality parameters taken into consideration for durum wheat is the yellow index, this is due to lutein that is not a provitamin A precursor. We have used CRISPR-Cas9 to silence 3 key genes of the metabolic pathway of carotenoids (LCYE, HYDB and LOX) that will allow to obtain a "golden durum wheat", highly rich in beta carotene.

Extending genome editing into elite wheat cultivars by deploying morphological genes

Sadiye Hayta, Mark A. Smedley, Martha Clarke, Cristobal Uauy and Wendy A. Harwood

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Wheat has lagged behind other major cereals in the advancement of gene transformation technology for its improvement. New breeding technologies such as genome editing allow precise DNA manipulation, but its potential is limited by low regeneration efficiencies in tissue culture and lack of transformable genotypes. We developed, in the hexaploid spring wheat cultivar 'Fielder', an efficient and reproducible *Agrobacterium*-mediated transformation system. This high-throughput robust transformation system has been used effectively to introduce genes of interest, for over-expression, RNAi and for CRISPR-Cas based genome editing. Transformation efficiencies of up to 33% in 'Fielder' and 10% in the tetraploid durum wheat 'Kronos' were achieved. A recent development for wheat transformation by the Dubcovsky Lab at UC Davis, USA, is a protein fusion consisting of two developmental regulator genes. The wheat Growth-Regulating Factor 4 (GRF4) and its cofactor GRF-Interacting Factor 1 (GIF1) when overexpressed as a protein fusion improve the regeneration of *in vitro* growing plant cells. When included and tested with our wheat transformation protocol, the transformation efficiencies increased to 77.5% in 'Fielder', 70% in 'Kronos' and we expand that technology into different wheat varieties like Cadenza, Paragon, and elite wheat cultivars.

The GRF4-GIF1 technology results in fertile transgenic plants, giving a "normal" phenotype in low transgene copy plants without the need of specialized promoters or transgene excision, overcoming some of the limitations of transformation technologies with other morphogenic developmental regulating genes. It is an ideal technology to expand the utilization of genome editing technology to wheat varieties previously unable to be transformed or regenerated through tissue culture.

These developmental regulators alleviate some of the cultivar dependence of wheat transformation and, so doing, enable the efficient and rapid transformation and direct genome editing of elite wheat cultivars. Presently, we are using this system in a range wheat varieties looking at gene function and trait improvement using CRISPR-Cas technologies.

Keywords: wheat, genome editing, CRISPR, GRF4-GIF1, morphological genes, elite wheat cultivars.

Genome editing in fruits and vegetables

PlantEd

Fortifying vitamins in tomato using using genome editing

Jie Li[1], Ronan Broad[1], Aurelia Scarano[2], Angelo Santino[2] and Cathie Martin[1]

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[2] *National Research Council, Institute of Science of Food Production (CNR-ISPA), Lecce, Italy*

In 1996 the World Food Summit stated that '**food security** exists when all people, at all times, have physical and economic access to sufficient, safe and **nutritious food** to meet their dietary needs and food preferences for an **active and healthy life**'. Currently, just 17 plant species are consumed as 90% of the global human diet, meaning that many lack adequate vitamins, micro-nutrients and health-promoting phytonutrients. As a consequence of an increasing dependency on processed foods, diets have declined significantly over the past 30 years, with reduced consumption of fresh fruit and vegetables.

Vitamin D is synthesised by humans from dehydrocholesterol, following exposure to UV light, but the major source is dietary. Pro-vitamin D3 (7-dehydrocholesterol; 7-DHC) is synthesised by some plants, on route to cholesterol synthesis. Poor vitamin D status is a major EU public health problem, in all age groups and RDAs of 15 µg per day for (March 2016), cannot be achieved from currently available food sources without supplementation. Appreciation of the prevalence of vitamin D deficiency has become more acute with its recent association with the severity of COVID-19 infection. In Solanaceous plants sterol metabolism has developed a separate path devoted to synthesis of steroidal glycoalkaloid synthesis from cholesterol, distinct from the pathway for brassinosteroid biosynthesis. By editing the gene encoding the steroidal glycoalkaloid-specific isoform of the enzyme synthesising cholesterol from 7-DHC, we have been able to increase the levels of 7-DHC in tomato fruit such that they approach the values for the RDA for vitamin D. We are now working on methods to promote UV-B induced conversion of provitamin D to vitamin D3 in tomato fruit.

Vitamin C (ascorbate) plays a vital role in stress tolerance and amelioration of oxidative stress in both plants and animals. Humans have lost the ability to synthesise ascorbate and obtain it largely from fruit and vegetables. Deficiency in humans impacts dioxygenase-dependent functions (carnitine, neuro-transmitter and collagen synthesis) and immune system function, with severe deficiency (scurvy) resulting in shortness of breath, bone pain, poor wound healing, and eventually death. Ascorbate is also important in iron uptake by plants and mammals. Multiple ascorbate biosynthetic pathways have been proposed in plants but there is now consensus that the Smirnoff-Wheeler pathway (or the L-galactose pathway) is predominant. GDP-L-galactose phosphorylase (GGP) is the first committed and rate-limiting enzyme of the L-galactose pathway and GGP activity is controlled by negative feedback regulation of its translation by a uORF in the 5'UTR of the gene. Genome edits that remove the functionality of the uORF result in increased levels of ascorbate accompanied by defects such as parthenocarpy. We have generated edited lines with 2-5 fold higher levels of ascorbate without the accompanying developmental defects which reduce yield.

Genome editing can make a major contribution to fortifying foods popular with consumers so that all can enjoy a healthier diet.

CRISPR/Cas9-mediated genome editing on *SlDET1* gene for the nutritional improvement of tomato

Aurelia Scarano[1], Fabio D'Orso[2], Giorgio Morelli[2] and Angelo Santino[1]

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[2]*CREA, Research Centre for Genomics and Bioinformatics (CREA-GB), Rome, Italy*

The tomato *SlDET1* gene is the orthologue of the *Arabidopsis* nuclear protein DE-ETIOLATED 1 (DET1) gene and it has been proposed encoding a negative regulator of the phytochrome signal transduction. Mutations in *SlDET1* gene, such as a C-to-T mutation in exon 11, or an alternative splicing causing a 9 bp-deletion in exon 11, have been found in tomato *high pigment (hp2)* mutants. The phenotypes of *hp2* mutants are characterized by light hypersensitivity, displaying elevated levels of anthocyanins in the seedlings, shorter hypocotyls, and more deeply pigmented fruits compared with wild-type plants. Mutations in the 5'-terminal part of the gene generate instead very severe phenotypes in terms of plant growth and survival. In *hp2* mutants, throughout fruit ripening, genes related to chloroplast biogenesis and structural genes involved in phytonutrients (e.g., carotenoids and flavonoids) biosynthesis are up-regulated. Such up-regulation put the plastid biogenesis as an important determinant of phytonutrient overproduction in the *hp2* mutant fruits.

In this study, we developed a strategy to introduce mutations by CRISPR/Cas9 on *SlDET1* that may allow the accumulation of bioactive compounds of nutritional interest in fruits, but resulting in less severe phenotypes than those known so far. For this purpose, sgRNA guides were designed on two different sites of exon 11, and were assembled coupled in our construct. To estimate the rate of targeting efficiency of our sgRNA guides, we used the hairy roots transient assay in tomato, which is a fast and reliable tool to study the possible editing mediated by CRISPR/Cas9. A range of mutations on *SlDET1* has been observed with the both guides, confirming that they can be applied to the stable transformation. Further work is now in progress on phenotypical and molecular characterisation of stable transformants.

These results can contribute to the generation of tomato mutants that could be different from the previously described *hp2* ones, with the high-pigmented fruits during development and ripening. The CRISPR/Cas9-mediated genome editing represents a novel biotechnological strategy to generate new different tomato lines with high levels of important phytonutrients for human health, such as carotenoids and flavonoids, thus improving the nutritional value of this worldwide important crop.

Generation of male-sterile tomato lines with the CRISPR/Cas9 system

Musa Kavas[1], Zafer Seçgin[1] and Kubilay Yıldırım[2]

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Tomato (*Solanum lycopersicum*), a member of the Solanaceae family, is the second most important horticultural crop worldwide. In addition to the health, economic and nutritional importance, tomato is used as a model plant in fleshy fruits improvement studies and genome manipulation studies. CRISPR/Cas9 system has been successfully used in various genome editing studies in tomato varieties. *Aconitaz hydratase* (ACO) gene, present in the Tricarboxylic Acid (TCA) cycle, breaks the citrate reversibly into isocitrate and is associated with the production of Acyl-Coa, hence considered associated with pollen formation. This study aims to knock out the *SlACO4* gene using the CRISPR/Cas9 gene-editing system and to evaluate the effect of this editing on pollen formation. Two guide RNA (gRNA) were designed to target *SlACO4* genes using the CRISPR/Cas9 system, and genome-edited plants were produced using *Agrobacterium*-mediated gene transfer. Results of CRISPR/Cas9 system by testing two gRNA targeting *SlACO4*. Insertions and deletions were observed in the genome-edited plants obtained according to the sequence result. Targeting the *SlACO4* gene resulted in an 85% decrease in the number of seeds per fruit in mutant plants. Consequently, the CRISPR / Cas9 first time in the tomato genome using regulating means has determined that the *SlACO4* gene is related to pollen formation in this study.

Keywords: Tomatoes, Aconitase hydratase, CRISPR/Cas9, *Agrobacterium*-mediated

CRISPR/Cas9-mediated mutagenesis as a strategy to develop resistant tomato plants against Orobanche

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The Orobanche (broomrape) are parasitc weeds belonging to the genus *Orobanche* spp and *Phelipanche* spp. They represent a serious threat for several crops cultivated in the Mediterranean basin, and some regions of Asia and Europe. In Italy, the tomato (*S. lycopersicum*) cultivation can be significantly affected by the diffusion of the species *Phelipanche ramosa*, that cause relevant yield and qualitative losses.
The release of strigolactones (SLs) in the root exudates is the main germination stimulant that trigger the germination of *P. ramosa* seeds in infested soils.
By the use of CRISPR/Cas9 mutagenesis the knock-out mutants for the four main genes *(SlD27, SlCCD7, SlCCD8, SlMAX1*) that leads to the biosynthesis of SLs, have been produced in tomato in the same genetic background. Phenotypic analysis of the mutant progenies Slccd7 and Slccd8 have evidenced the expected phenotype of the SL- plants and a significant reduction in *P. ramosa* infection in soilless experiments.
In order to preserve the SL biosynthetic pathway within tomato plants, a parallel CRISPR/Cas9 mutagenesis of the genes involved, or potentially involved, in the release of SLs in root exudates (*SlPDR1*, *SlPDR2* and *SlPDR3*) has been carried out and the first T0 plants of Slpdr1 and Slpdr2 have been obtained.
Globally, our CRISPR/Cas9 mutagenesis pipeline will provide novel genetic material that: a) will generate CRISPR-alleles that can potentially be introgressed in commercial tomato variety in order to confer resistance to *P. ramosa*; b) will contribute to elucidate some biological aspects of the SLs biosynthesis and transport in *Solanaceae* that still need further investigation.

Keywords: CRISPR/Cas9, tomato, strigolagtone, orobanche

CRISPR/Cas9 editing of proline metabolism and SOS pathway genes for improving abiotic stress tolerance in tomato

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Osmotic stress caused by high salinity and water deficit is a major limiting factor to crop yield. Here, we used CRISPR/CAS9 technology to obtain stress-tolerant tomato plants through editing of genes involved in proline metabolism and the Salt Overly Sensitive (SOS) pathway. Proline is a multifunctional amino acid whose production is enhanced under osmotic stress to maintain cell turgor pressure. To generate plants with increased proline levels, we selected the gene encoding *PYRROLINE-5-CARBOXYLATE DEHYDROGENASE1 (P5CDH1*), involved in proline catabolism, as target to be edited for producing loss-of-function mutants. The SOS pathway controls ion homeostasis by reducing the concentration of toxic Na+ ions in plant cells during salt stress. The Na+/H+ antiporter SOS1, a key component of this pathway, is characterized by an activation domain and an auto-inhibitory domain. To obtain mutants producing constitutively active SOS1, we targeted the protein autoinhibitory domain that keeps the protein inactive in control conditions. Hairy root system was used to test the constructs carrying two single guide RNAs (sgRNAs) for each gene. Root genotyping through high resolution DNA fragment analysis showed high mutation efficiency in the target sites. To obtain stable transformed plants, *Agrobacterium tumefaciens*- mediated transformation of *Solanum lycopersicum* (cv Red Setter) cotyledons was performed. We selected mutations resulting in premature stop codons in p5cdh1 and large deletions in the inhibitory domain of *sos1* by DNA sequencing. These results show that the CRISPR/Cas9 system and the tested sgRNAs can be successfully used to edit the two selected tomato genes. Progenies of independent P5CDH1 transformants were used to measure proline content. Interestingly, *p5cdh1* mutants showed higher proline levels in leaves compared to wild-type. Phenotyping of *p5cdh1* and *sos1* in stress conditions is in progress to validate differential tolerance to suboptimal environmental conditions in these lines.

Potential of New Plant Breeding Techniques for grapevine breeding

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New Plant Breeding Techniques (NPBTs) aim to overcome traditional breeding limits for plant improvement to biotic and abiotic stresses, satisfying the European Policies requirements that promote chemical input reduction and a more sustainable agriculture. Applying genome editing via CRISPR/Cas9 in grapevine, we focused our attention on susceptibility genes: to control powdery mildew we chosen to knock-out two genes belonging to *MLO* (*Mildew Locus O*) family *VvMLO7* and *VvMLO6*. In parallel we also focused our attention on abiotic stresses, in particular drought stress, performing a knock-out of two different gene classes: I) two different isoforms of *GST* (*Glutathione S-Transferase*) that are involved in increasing of ABA (abscisic acid), lateral root branching and osmolytes accumulation; II) two different isoforms of *PME* (*Pectin Methyl Esterase*) involved in regulation of woody hydraulic proprieties modifying pit membrane thickness and porosity.
In parallel to genome editing, we used cisgenesis to move the resistance locus *RPV3-1* (*Resistance to Plasmopara viticola*) into economically important grape cultivars. This locus is formed by two different genes, TNL2A and TNL2B, that were inserted together (with native promoters and terminators).
To avoid one of the drawbacks linked to classical *Agrobacterium tumefaciens* mediated transformation, hence the insertion of unrelated transgene, we exploited an inducible excision system based on a Cre-Lox recombinase technology. The system is controlled by a heat-shock inducible promoter that will be activated once the transformation event(s) will be confirmed allowing the removal of CRISPR/Cas and selection markers both in genome editing and cisgenic approach.

Genome editing in plants- the latest technological advancement

PlantEd

New approaches to gene targeting in plants by exploiting the unique characteristics of CRISPR-Cas12a

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One of the promises of genome editing is to efficiently make directed changes in a plant's DNA through gene targeting. Unfortunately, this has remained largely unachievable. Previous attempts at gene targeting commonly use a DNA repair process known as Homology Directed Repair (HDR) to incorporate changes. HDR is a high-fidelity recombination-based DNA repair mechanism, but it remains inefficient in plants. Therefore, it is of interest to investigate other repair mechanisms, that have previously been thought to be error-prone, as a tool for gene targeting. Specifically, alternative nonhomologous end- joining (aNHEJ) is a good candidate to exploit as a high-fidelity repair mechanism. aNHEJ relies on microhomologies to direct DNA repair; control of these microhomologies allows us to direct repair. With the use of different CRISPR enzymes it is possible to steer repair to high-fidelity repairs. One of these enzymes, Cas12a, cuts DNA and leaves overhangs that seem to stimulate a high-fidelity repair that resembles either aNHEJ or a simple ligation in plants. In our research we show for the first time in plants this new type of repair using Cas12a. By designing target sites to leave complementary microhomologies, it was possible to transiently direct precise DNA repair and make large DNA deletions in agroinfiltrated *Nicotiana benthamiana* without any trace of undesirable sequences. Furthermore, we show strong evidence for the integration or replacement of large stretches of DNA using this new type of repair. Understanding and optimization of the repair pathways shown in these studies will finally bring us close to the promise of efficient gene targeting in plants

Effective CRISPR-mediated knockout mutations in plants require translation reinitiation avoidance

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CRISPR/Cas9 system is a powerful technology which enables scientists to generate a huge genomic diversity for plant breeding. Despite its simplicity, a successful editing on target genes requires an optimal experimental design and a careful editing evaluation which should take into consideration all possible factors.
One of the pitfalls which may interfere with the generation of a complete KO mutant is the translation of the edited alleles from alternative ATGs inside the coding sequence producing truncated proteins which may have partial, altered or even unknown activity. Unfortunately, this aspect is largely underestimated in plants and it can cause a misleading phenotype evaluation.
In this work, we show an example of translation reinitiation in a tomato gene (*LeHB1*) after CRISPR-mediated editing. Using a protoplast-based transient expression system which allows the production of GFP fusion proteins, we found that internal ATGs downstream of the mutations induced by CRISPR can be used as a translation initiation site with the production of an N-terminally truncated protein.
As the phenotypic evaluation of mutants is time consuming, it should be advisable to screen the edited alleles and select only those with effective knockout effect. To this end, we propose a simple and fast method to assess the actual lack of production of truncated proteins encoded by the modified alleles.

Applying high-throughput technology to identify CRISPR-Cas9 induced off-target mutations in tomato

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CRISPR-Cas technology has brought the ability to introduce deletions and insertions at virtually every position in the genome and has the potential to significantly accelerate plant breeding. However, the specificity of this technology in plants has not been thoroughly investigated. One of the main reasons for this is the lack of high throughput research systems in plants. To overcome this limitation, we developed a method for tomato protoplast transfection in a 96 well-format. By coupling this method to next-generation sequencing of amplicons of predicted off-target sites, we investigated the efficiency, specificity and mutagenic spectrum of 89 sgRNAs. This high throughput data revealed off-target mutations for 13 of those gRNAs. These off-target events mostly occurred at genomic locations that had one mismatch compared to their respective targets. No off-target mutations were found at positions that had three or more mismatches compared to the target. Additionally, we were able to identify other events that occur at low frequencies, such as the integration of fragments of vector or genomic DNA in CRISPR-induced double-stranded breaks. The protoplast system also allowed us to initiate the development of the unbiased off-target detection method GUIDE-seq, which is not dependent on *a priori* off-target prediction. This method was originally developed for mammalian cells and is based on the integration of a double-stranded DNA oligonucleotide (dsODN) in double-stranded breaks. We optimized the integration of this dsODN in Cas9-induced breaks in tomato protoplasts and have demonstrated the potential to detect off-target activity using this method. This research provides more insight into the specificity of plant genome editing and shows the potential of high-throughput methods for developing novel CRISPR techniques in plants.

Are there available tools to trace genome-edited crops in foods?

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In the past decades, biotechnologists have revolutionised the agro-food business with the introduction of genetically modified organisms (GMO) with desirable agronomic traits, improving the yield and quality of crops. More recently, biotechnologists have exploited genome editing methods, such as CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR associated protein), to modify the characteristics of organisms important for food and feed production [1]. GMO are highly regulated in several worldwide countries and, particularly, in European Union, while the regulatory scenario regarding genome-edited crops is currently not well defined globally. Besides the genome editing precision and range of off-target and other unintended effects, especially in comparison with random mutagenesis, have been one focus of the discussion regarding regulatory status of genome-edited organisms, another critical issue has been the feasibility of developing methods for detecting genome-edited organisms [2]. Currently, several issues with regard to the detection, identification and quantification of genome-edited products have been mostly at theoretical level [3]. Nonetheless, it is worth to refer the recent advances on this issue [2,4,5], which continues placing real-time quantitative PCR (qPCR) as a key tool, as it has been for GMO analysis. The first detection method of canola with a single base pair edit conferring herbicide tolerance, the first commercialized genome-edited crop, relied on qPCR, providing sensitive (0.05%) and specific results compatible with the standards of practice, equipment and expertise typical in GMO laboratories [2]. An editing site-specific qPCR method was developed based on the unique edited sequence in CAO1-edited rice plants, which specifically identified CAO1-edited from other CAO1-edited rice and wild types of rice with high specificity and sensitivity [4]. The conjunction of both qPCR approaches with the use of locked nucleic acids (LNA) in primer design was crucial to increase assay specificity [2,4]. The combination of qPCR with high-resolution melting analysis (HRM) enabled successfully identifying gene-edited rice plants with small target DNA in/dels or even single base pair insertion/deletions with a sensitivity down to 1% [5]. Although not quantitative, HRM analysis allows high throughput screening and genotyping of targeted gene-editing mutants [5]. Droplet digital PCR (ddPCR) is a breakthrough technology that has been applied to detect gene-edited rice [4,6]. It relies on partitioning individual amplifications into separate compartments, as well as the detection of their endpoint amplification products, providing ultrasensitive and absolute nucleic acid quantification without a standard curve [6]. ddPCR enabled identifying wild-type, homozygous and heterozygous mutations induced by CRISPR/Cas9 in the rice *TGW6* gene [6]. Additionally, ddPCR allowed quantitative analyses of genome-edited rice meeting the performance requirements for GMO detection methods [4].

Therefore, the answer is yes, qPCR and ddPCR have provided promising tools to detect and identify genome-edited crops in foods.

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Next biotechnological plants for addressing global challenges: the contribution of transgenesis and New Breeding Techniques

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The aim of this survey is to identify and characterize new products in plant biotechnology since 2015, especially in relation to the advent of New Breeding Techniques (NBTs) such as gene editing based on the CRISPR-Cas system. Transgenic (gene transfer or gene silencing) and gene edited traits which are approved or marketed in at least one country, or which have a non-regulated status in the USA were collected, as well as related patents worldwide. In addition, to shed light on potential innovation for Africa, field trials were examined on this continent. The compiled data are classified in application categories, including agronomic improvements, industrial use and medical use, namely production of recombinant therapeutic molecules or vaccines (including against Covid-19). Our data indicate that gene editing appears to be an effective complement to 'classical' transgenesis, whose use is not declining, rather than a replacement, a trend also observed in the patenting landscape. However, increased use of gene editing is apparent. Compared to transgenesis, gene editing increased the proportion of some crop species and decreased others amongst approved, non-regulated or marketed products. A similar differential trend is observed for breeding traits. Gene editing also favored the emergence of new private companies. China, and prevalently its public sector, overwhelmingly dominates the patenting landscape, but not the approved/marketed one which is dominated by the USA. Our data go in the sense that regulatory environments will favor or discourage innovation.

Joint session with COST Action EPI-CATCH

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Transgenerational effects of chromium stress in *Arabidopsis thaliana*

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It is already well-known that plants remember previous events of environmental stress and may use these memories to activate a better response when these events occur again. However, plant transgenerational stress memory is a complex phenomenon that is still far to be fully elucidated. One of the most important plant stresses due to heavy metals is excess of Chromium (Cr) that is largely used in many anthropic activities. The aim of this work was to gain insight into the molecular mechanisms of transgenerational stress memory in plants in response to heavy metals using A. thaliana and chromium stress as a model system. Phenotypic data showed that chromium stress have significant transgenerational effects in terms of root length, stress tolerance and seed germination. The comparison of leaf transcriptomic responses between F0 and F1 confirmed that a Cr stress transgenerational memory occurs due to epigenetic modifications. Functional data mining identified key candidate genes involved in transgenerational stress memory such as those involved in response to iron starvation and homeostasis: bHLH TF family (ORG2, ORG3, bHLH100, MATE transporter, BRUTUS. Findings will be very useful to identify genes usable in biotechnological approaches for enhancing phytoremediation of Cr contaminated soils.

Keywords: *Arabidopsis thaliana*, chromium, stress memory, transgenerational, plants

Epigenetics of heat stress response in tomato

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Heat stress is a major environmental factor limiting crop productivity, thus presenting a food security challenge. Various approaches are taken in an effort to develop crop species with enhanced tolerance to heat stress conditions. Since epigenetic mechanisms were shown to play a regulatory role in mediating plants' responses to their environment, we investigate the role of DNA methylation in response to heat stress in tomato (*Solanum lycopersicum*). We recently found that the *ddm1b*, a DNA methylation-deficient tomato mutant, is significantly less sensitive to heat stress compared with the background tomato line, M82. Under conditions of heat stress, this mutant line presents higher fruit set and seed set rates, as well as a higher survival rate at the seedling stage. On the transcriptional level, we observed differences in the expression of heat stress-related genes, suggesting an altered response of the *ddm1b* mutant to this stress. Following these preliminary results, further research would shed light on the specific genes that contribute to the observed thermotolerance of *ddm1b*.

Genome editing in roots and tubers

PlantEd

Mutations in susceptibility genes through CRISPR/Cas9 genome editing confer increased pathogen resistance in potato

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The use of pathogen-resistant cultivars is expected to decrease yield variations and fungicide use in agriculture. However, in potato breeding, increased resistance obtained via resistance genes (R-genes) is hampered because R-gene(s) are often specific for a pathogen race and can be quickly overcome by the evolution of the pathogen. Susceptibility genes (S-genes) on the other hand are important for pathogenesis, and loss of S-gene function in plants confers increased resistance. Here we present the mutation and screening of seven putative S-genes in potatoes, including two DMR6 potato homologues. Using a CRISPR/Cas9 system, which conferred co-expression of two guide RNAs, tetra-allelic deletion mutants were generated and resistance against late blight (caused by *Phytophthora infestans*) was assayed in the plants. Functional knockouts of *StDND1*, *StCHL1*, and *DMG400000582* (*StDMR6-1*) generated potatoes with increased resistance against late blight. Plants mutated in *StDND1* showed pleiotropic effects, whereas *StDMR6-1* and *StCHL1* mutated plants did not exhibit any growth phenotype, making them good candidates for further agricultural studies. Additionally, we showed that *DMG401026923* (here denoted *StDMR6-2*) knockout mutants did not demonstrate any increased late blight resistance, but exhibited a growth related phenotype, indicating that *StDMR6-1* and *StDMR6-2* have different functions. Interestingly, an increase resistance to Early blight (caused by *Alternaria solani*), has also been recorded. Finally, field trial data from Sweden will be presented.

Edition of potato for reduced PPO activity confers resistance to *Ralstonia solanacearum*

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Polyphenol oxidases (PPOs) represent an important defence mechanism to protect plants against herbivores and certain pathogens. In potato, contrasting evidence indicates that sometimes not the increase but the decrease of PPO activity may result in pathogen resistance, perhaps due to metabolic perturbation. To complicate matters, some pathogens, including the *Ralstonia solanacearum* bacterium that causes wilt disease in plants, possess their own PPO enzymes.

Our aim was to study the relationship between PPO activity in potato and its *Ralstonia resistance*. To this end, the potato cultivars 'Désirée' and 'Balatoni Rózsa' were edited via CRISPR/Cas with a conserved target for numerous PPO genes (*Pot32, Pot33, Pot72, Stuppo3, Stuppo7*). DNA-sequencing indicated that the *Pot32*, *Pot33* and *Pot72* genes were mutated in a plant line from 'Balatoni Rózsa' which reduced its PPO activity and the browning of the tubers and roots. This plant line was more susceptible to *Ralstonia solanacearum* compared to the original cultivar. In addition, we produced an edited 'Désirée' line which also had considerably reduced PPO activity in the tubers, and yet it was highly resistant to *Ralstonia*. This line was not mutated in the *Pot32*, *Pot33* or *Pot72* genes, so possibly another PPO gene was affected. Genomic, transcriptomic and metabolic level characterisation of these two lines are under way. Different activity and tissue-specificity of the potato PPO genes might explain their contrasting effect on resistance to *Ralstonia*.

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Molecular and metabolomic analysis of resistant potato varieties as a way forward to generate resistance to *Ralstonia solanacearum*

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The bacterium *Ralstonia solanacearum* (Rs), belonging to quarantine pathogens in the EU, is known to cause wilt disease in potato and in across 200 other plant species. For the lack of an effective chemical protection against *Rs*, it is essential to understand existing resistance mechanisms and to increase the genetic resistance in potato. Here, we studied known resistant potato varieties in comparison to the susceptible model cultivar 'Désirée' by first confirming their resistance/susceptibility, followed by metabolomic analysis and transcriptome sequencing with qPCRs to evaluate the gene expression at 2 and 6 days post-inoculation, respectively.
Of nine known *Rs*-resistant/tolerant varieties that were available and tested, 'Calalo Gaspar' (CG) and 'Cruza' (CR) were confirmed to be the most resistant in an in vitro infection assay. The metabolomic data showed an increase in chlorogenic acid and its isomers and also indicated salicylic acid as a key player of defence signalling against *Rs* with reduced jasmonic acid and abscisic acid in both varieties after infection. From transcriptome analysis, several marker genes for salicylic acid signalling and differentially regulated candidate genes that could be contributing to *Rs* resistance/susceptibility were identified, among others *DMR6*, *WRKY72* and *WAT1*. KEGG analysis highlighted that the phenylpropanoid pathway and the glutathione metabolism were downregulated in CG contrary to in CR where both these pathways were upregulated post-infection. GO analysis highlighted that several peroxidases and antioxidants decreased in CG upon infection and several chitinases and cell-wall metabolic processes were enriched in CR. Thus, CG and CR seem to have common as well as differing responses to *Rs* infection. qPCRs validated higher amounts of phenylalanine ammonia-lyase in both resistant varieties compared to 'Désirée' which exerted decreased post-infection expression. Regulation of antioxidants and cell-wall building processes appear to contribute to *Rs*-resistance. Our understanding of the resistance pathways in CG and CR has not just identified reasons but also ignited potential targets for generating genome-edited, *Rs*-resistant potato.

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CRISPR-Cas9 targeting of the *eIF4E-1* gene induces resistance to *Potato virus Y* in *Solanum tuberosum* L. cv. Desirée

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Translation initiation factors and, in particular, the *eIF4E* gene family is the primary source of recessive resistance to potyviruses in many plant species. However, no such resistances to this virus genus have been identified in potato *(Solanum tuberosum* L.) germplasm. As in tomato, the potato eIF4E family consists of four genes, *eIF4E-1* and *-2, eIF(iso)4E,* and *nCBP*. In tomato, *eIF4E-1* or *eIF4E-2* knockout (KO) confers resistance to a subset of potyviruses, while the *eIF4E-1/2* double KO, although conferring a broader spectrum of resistance, leads to plant development defects. Here, the commercial tetraploid potato cv. Desirée, owning the *Ny* gene conferring strain-specific HR to *Potato virus Y* strain O, was used to evaluate the impact of CRISPR-Cas9-mediated *eIF4E-1* KO in broadening the PVY resistance spectrum. After a double protoplasts transfection-plant regeneration process, transgene-free *eIF4E-1* KO potatoes were obtained. The *eIF4E-1* KO plants challenged with a PVY-N isolate showed a reduced viral accumulation and amelioration of virus-induced symptoms. Our data show that it is possible to obtain transgene-free potato plants with a broader spectrum of resistance to PVY by KO of a gene of the *eIF4E* family.

Identification of suitable targets for gene editing mediated crop improvement: the example of CRISPR/Cas9 directed gene editing in cassava for increased β-carotene accumulation

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As improved and simplified applications of CRISPR/Cas technology are being continuously reported, and this break-through discovery is maintaining the promise to revolutionise plant genome engineering, correct identification of the right target genes to obtain durable desired traits becomes even more important.
We describe the application of our approach, based on comparative gene expression analysis of genotypes with contrasting trait phenotypes, for discovery of target genes suitable for genome editing-mediated improvement in different plant crops. We finally report on the utilization of target genes, identified by this approach, for biofortification of cassava via CRISPR/Cas9 technology.
Cassava is an important staple crop in the tropics in that it is a good source of carbohydrate; however, it has low micronutrient levels. Genetic improvement of cassava by conventional breeding has been a challenging task due to complexities such as its heterozygosity, limited flowering capacity of several varieties and the lengthy cropping cycle. The CRISPR/Cas9 system is an efficient tool in genetic modification of crops to generate important traits such as high nutrient density. In this study, the CRISPR/Cas9 gene editing technology was used to knockout three genes, selected through genetic and metabolic comparative studies, in the cassava cultivar TMS60444. Using the editing vector pDIRECT_22C, a deletion mutation was generated in beta-carotenoid hydroxylase (CHYβ), lycopene epsilon-cyclase (LCYε) and 9-cis-epoxycarotenoid dioxygenase (NCED1) genes of the carotenoid synthesis pathway. Friable embryogenic calli of cassava cultivar TMS60444 were transformed with LBA4404-LCYε, LBA4404-CHYβ, and LBA4404-NCED gene constructs by *Agrobacterium* mediated transformation to generate transformants with higher carotenoid content.

First report on CRISPR/Cas9-targeted mutagenesis in the Colorado potato beetle, *Leptinotarsa decemlineata*

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Leptinotarsa decemlineata (Say), commonly known as the Colorado potato beetle (CPB), is an agricultural important pest for potatoes and other solanaceous plants. The CRISPR/Cas system is an efficient genome editing technology, which could be exploited to study the biology of CPB and possibly also lead to the development of better environmentally friendly pest management strategies. However, the use of CRISPR/Cas9 has been limited to only a few model insects. Here, for the first time, a CRISPR/Cas9 protocol for mutagenesis studies in CPB was developed. A gene with a clear phenotype such as the *vestigial* gene (*vest*), known to be involved in wing development in other insect species, was selected as a good indicator for the knockout study. First, *vest* was functionally characterized in CPB by using RNAi technology for knockdown studies. Once the expected deformed wing phenotypes were observed, a CRISPR/Cas9 work flow was established for mutagenesis in CPB. By co-injecting the Cas9 protein and a *vest*-guide RNA into 539 CPB eggs of <1 h old, sixty-two successfully developed to adults, among which mutation in the *vest* loci was confirmed in 5 of the 18 wingless CPBs (29% phenotypic mutation efficiency). The mutation in *vest* resulted in a clear phenotype in the CPBs, which developed to adulthood with no hindwing and elytron formed. Altogether, this study provides for the first time a useful methodology involving the use of the CRISPR/Cas9 system for mutagenesis studies in one of the most important pest insects.

Genome editing in oil crops, algae, trees and other plants

PlantEd

Genome editing of oilseed crops

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Protoplast approach is important for both basic research and plant breeding. It is particularly useful in developing directly transgene-free mutants by CRISPR/Cas gene editing. However, difficulty in protoplast regeneration has restricted its application in genome editing for most important crops. Although CRISPR/Cas9 has been used in some crops for trait improvement, the published results so far relied mainly on stable transformation mediated by *Agrobacterium* or particle bombardment. Most published studies using protoplasts for gene editing by CRISPR/Cas9 in crops ended in transfection with no shoot regeneration from the protoplasts. Proof-of-concept protoplast regeneration protocols for CRISPR/Cas9 genome editing are still lacking for most crop species. We have been working with developing protoplast regeneration protocols for oilseed crops with focus on field cress (*Lepidium campestre*) and rapeseed (*Brassica napus*) in recent years. We have now successfully developed efficient protocols for isolation, regeneration and transfection of protoplasts for both species and are in good progress for carinata (*Brassica carinata*). Using the optimized protocols, we have been able to efficiently edit target genes through CRISPR/Cas9 and got stable mutations lines for field cress and rapeseed.

Establishment of genome editing techniques in trees

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While crop plants have been subject to breeding for thousands of years and many molecular biology tools are now available, the molecular toolbox for forest trees is still being set up. Nowadays, research is also supported by simpler and faster genome sequencing.
At the Thünen Institute of Forest Genetics, Grosshansdorf, Germany, genome editing techniques are being applied to tree species, most notably fast-growing poplar hybrids, which serve as model trees. In poplars, CRISPR/Cas-based genome editing works very efficiently when aiming gene knock-outs. New techniques are gaining relevance, such as DNA-free editing techniques, nickase-mediated precise base editing, or methods to integrate donor sequences by homology-directed repair (HDR).
Most forest tree species have not been edited so far. The new TreeEdit project financed by the Federal Ministry of Food and Agriculture via the Fachagentur Nachwachsende Rohstoffe focuses, for the first time, on an important German forest tree species, the European beech *Fagus sylvatica*. For this species, the achievement of the three basic steps of *in vitro* cultivation, genetic transformation and CRISPR/Cas editing is planned.
Developing new genome editing techniques for forest trees and establishing these techniques in European beech have great potential to move forest tree breeding forward. Urgent challenges such as climate change have been clearly noticeable in recent years through extreme droughts, such as in 2018, and are obvious as bare patches on the landscape. Forest adaptation is needed so that rapid and cost-saving genome editing can contribute to the functional elucidation of the genetic background of, e.g., drought stress tolerance.

Gene editing in poplar using CRISPR/Cas to improve tolerance to *Lonsdalea populi* infection

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Bark canker disease is among the devastating and difficult to manage problems for a wide variety of valuable forest tree species. Canker makes woody plants vulnerable to microorganism attack and their penetration into wood tissue. Practical implementation of biocontrol organisms under field conditions remains a bottleneck while the application of antibiotics to control this disease on a large scale should be carefully considered. The best way to control the canker disease on the woody plants is to develop resistant cultivars. Therefore, genes responsible for the bark canker disease tolerance in trees should be identified and introduced into tree breeding programs to fasten the tolerant plant development and control the disease spread. As complete genome of *Populus trichocarpa* was sequenced access to many genes that might be used in bark canker disease tolerance are available. In current study, *Populus x euramericana* trees having sticky, brown-colored fluid oozes in vertically cracked bark of the trunks, were investigated to identify the responsible pathogen of poplar bark canker disease. The results of 16S genome analysis have indicated that *Lonsdalea populi* (formerly *Lonsdalea quercina* subsp. *populi*) is the main bacteria causing the canker disease in the lately investigated trees. This anaerobe, gram-negative bacteria was realized to invade vascular tissue, mainly floem, of the trees and become domestic species for many valuable forest species. Therefore, RNA sequence-based transcriptome analysis was conducted between the *L. populi* infected and non-infected healthy black poplar stalks and leaves to identify gene regulation network and response behind *Lonsdalea* infection. Among many differentially expressed genes, WRKY transcription factors were the most upregulated ones in the leaves of infected plants. The PtrWRKY73 was isolated from poplar with PCR, cloned into *Agrobacterium* and transferred into *Arabidopsis* for its overexpression in transgenic plants. The results of this transgenic study revealed increased resistance to a virulent strain of the gram-negative bacterial pathogen. PtrWRKY73 was planned to be knockout with plant genome editing tools (CRISPR/Cas9) in the genomes of canker susceptible and resistant poplar clones. By this way, we aimed to discover the gene regulation role of WRKY on the plant-pathogen interaction. The gene sequence of PtrWRKY73 was obtained from databases and many guide RNAs (gRNA) targeting the exon parts of the gene were identified via CrisprP software. Among these gRNAs, two of them were selected due to their high GC content, low off target capacity and appropriate folding ability. Both gRNAs were inserted into a Cas9 containing pHSE401 vector and transformed into *Agrobacterium* EHA105 and GV3101 strains. gRNA/Cas9 complex was stably inserted into poplar tissue. After regeneration of the PtrWRKY73 knockout transgenic poplars, the effects of this transcription factor on gene regulation network in response to *L. populi* infection will be measured with RNA

seq analysis. This knowledge could facilitate understanding of the bark canker disease tolerance strategies in poplar trees and identify important transcription factors that can be used in breeding programs.

Keywords: Poplar, CRISPR, WRKY TFs, Biotic stress

Navigating possible seaweed industrial development by crucial genomic tools

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Macroalgae meet sustainability demands, since not competing for land, fresh water or fertilizer. Europe's coastline is further impressing 185 000 km long, making up over half of our globe's total coastline. Macroalgae also called seaweeds, are expected to be important for future solutions, given their broad growth regions along long coastlines, important ecological roles and impressive biomass production. Kelps (*S. japonica* and *S. latissimi*) are important industrial species in Asia, and sugar kelp (*Saccharina latissima*) has a large unexploited potential along the European and North American shores. To secure a sustainable exploitation, cultivation might be the best solution to protect wild populations from over- harvesting. Also, to meet expected increased sea temperature and reduced salt content, breeding might be essential to secure sugar kelp's survival helping its adaptation. European regulatory frameworks are still being developed and to secure a knowledge-based foundation, research efforts need to be stepped up.

Macroalgae have been studied for centuries, but only recently supported by 1) detailed microscopy studies to explain cell divisions and growth of early stage embryos, 2) the first genome sequences and 3) the first transcriptomic analyses. However, lack of many full genome sequences, well annotated genomes and established gene transformation protocols restrict further advancements. These species are also of fundamental importance since they phylogenetically and biologically have similarities to both land plants and animals. Novel insight is therefore expected from untangling genes involved in cell division and growth, and their regulation, adding to both land plant and animal biological understanding.

The genome sequences will further be of major importance to identify promoters and understand genetic regulation as they lack land plants' central stem cell promoting genes such as *WUSCHEL* and *SHOOTMERISTEMLESS,* and missing regulatory genes such as the *CLE* genes, while having multiple copies of Calpains similar to animals. There was recently a considerable break-through in wheat transformation from precision insertion by CRISPR/Cas9 of a combined microRNA and an affected central stem cell gene. This combination increased transformation frequencies by a 20-fold and made transformation feasible in genotypes earlier not transformable. This reminds us of how important improved gene transfer protocols are, and how basic science unexpectedly might contribute to practical applications like plant breeding. Additionally, this shows how regulatory restrictions might hamper the uses, as this use of CRISPR is avoided in breeding since stopped by patent restrictions/regulatory frameworks.

We still have limited knowledge of the genetic origin and variation in kelp genomes in European waters, also affecting our possible breeding options. Genome editing is seen as a promising tool by the Phycomorph COST action's 200 pages PEGASUS document's suggested regulatory baseline to the European commission. However, the limiting factors for genome editing is still gene transfer to be able to use the technique and full genome sequences to evaluate possible off target effects. We are working on building this foundation combining our cell division model with genomics, combining transcriptomics with an aimed pan-genome sequence. We would like to present how this is all related and draw attention to its impact by sound science-based knowledge to secure targeted regulatory framework and how illustrate how impact depend on knowledge to possibly develop a sustainable macroalgae industry.

Identification of bitterness related biosynthesis genes in *Cichorium* using CRISPR/Cas9 genome editing

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Cichorium intybus var. *sativum* (chicory) and var. *foliosum* (witloof) are economically important crops with a high nutritional value due to many specialized metabolites, including sesquiterpene lactones (SLs). However, sesquiterpene lactones are responsible for a bitter taste, limiting the use of *Cichorium* for industrial purposes. Editing specific genes from the SL pathway in *Cichorium* would lead to changes in the SL metabolite pathway and thus result in altered bitterness.

The genes *germacrene A synthase* (*GAS*), *germacrene A oxidase* (*GAO*), *costunolide synthase* (*COS*) and *kauniolide synthase* (*KLS*) are already known to control production of SLs. To identify new genes in the SL biosynthetic pathway in *C. intybus,* a comprehensive genome-wide screen was executed. By this, paralogs of the known members (*GAS*, *GAO*, *COS* and *KLS*) were identified and gene families were annotated. Previous genome-wide transcriptome profiling studies demonstrated that a Methyl Jasmonate (MeJA) treatment may trigger the upregulation of genes encoding enzymes involved secondary metabolic pathways, including the SL biosynthetic pathway. Therefore, we performed a comprehensive transcriptome analysis of MeJA treated samples in three species (*C. intybus* var. *sativum*, *C. intybus* var. *foliosum* and *Lactuca sativa*), to identify MeJA-inducible members of gene families putatively involved in the SL biosynthetic pathway. Combining the genome-wide gene family annotation with their MeJA-inducibility allowed to identify ten *CiGAS*, six *CiGAO*, four *CiCOS* and ten *CiKLS* candidate genes putatively related to the SL biosynthetic pathway. These candidate genes were used in co-expression assays in the heterologous host tobacco (*Nicotiana benthamiana*), to confirm their involvement in the SL biosynthetic pathway. Catalytic activity of two *CiGAO* paralogs and four *CiCOS* paralogs is already shown. The catalytic activity of other *CiGAO* and *CiKLS* candidate genes are analyzed at the moment.

Inducing CRISPR/Cas9 knock-outs of these selected SL candidate genes in *Cichorium* and analyzing the SL metabolite production of obtained mutated plants could validate their gene function in planta and alter bitterness production. Therefore, a CRISPR/Cas9 protoplast transfection method was developed to create mutated *Cichorium* genotypes (De Bruyn et al., 2020). Targeting one paralog of *CiGAS*, *CiGAO* and *CiCOS* revealed a range of mutated genotypes. Using ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS) it was shown that mutant containing a mutation in both *CiGAO* alleles have a lower amount of some SL metabolites, compared to plants with no mutation or plants with only one mutated allele. Now protoplasts transfected with multiple CRISPR/Cas9 sgRNAs targeting multiple paralogous SL candidate genes are regenerating into plants. Regenerants will be screened by HiPlex amplicon sequencing to verify CRISPR/Cas9 mutation efficiency. Analyses on the mutants by UHPLC-MS/MS will provide insights in the function of the SL candidate genes, the formation of the SL compounds and hence the bitterness in *Cichorium*.

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Targeted CRISPR/Cas9-based knock-out of the rice orthologs *TILLER ANGLE CONTROL 1 (TAC1)* in poplar induced erect leaf habit and shoot growth

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Pyramidal-, erect- or upright-growing plant forms are characterized by narrow branch angles of shoots and leaves. Putative advantages of upright-leaf and shoot habit could be a more efficient penetration of light into lower canopy layers, thus increasing the photosynthetic potential of the entire plant. Alternatively, plants with upright-growing shoots need less individual space on a default field size.
Pyramidal genotypes have already been reported for various tree genotypes including Italian Cypress, Lombardy poplar, and pillar peach. For peach, the paralogous rice ortholog *TILLER ANGLE CONTROL 1 (TAC1)* has been proposed to be the responsible gene for upright growth. However, it not really has been demonstrated for any of the pyramidal tree genotypes that a knock-out mutation of the TAC1 gene is the causal origin for pyramidal plant growth.
In our work, we have identified a putative rice *TAC1* ortholog and its most homologous paralog in the genomes of *P. trichocarpa* (Potri.014G102600 [“TAC-14”] and Potri.002G175300 [“TAC-2”]), *P. tremula*, and *P.* × *canescens* clone INRA 717-1B4, respectively. By applying the CRISPR/Cas9-system, we successfully knocked-out the two putative Pc*TAC1* orthologs in INRA 717-1B4. The mutants obtained were phenotyped over a period of three years in the glasshouse. Our results indicate that the homozygous knock-out of just TAC-14 is sufficient to induce pyramidal plant growth in *P.* × *canescens*.
By applying CRISPR-based TAC-14-knock-out in poplar elite clones, planting of up to twice as many of pyramidal poplar individuals on short rotation coppices (SRCs) could lead to twice as high wood yield, without any breeding, simply by increasing the number of trees.

STSM session

PlantEd

Targeted mutagenesis in oilseed rape (*Brassica napus* L.) protoplasts using CRISPR/Cas

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Oilseed rape (*Brassica napus* L.) seeds are used for the production of one of the most health promoting oils in the food industry. To improve agronomic traits of this polyploid species with a relatively high number of paralogous genes, CRISPR/Cas technique, allowing for targeting multiple homologous genes at once, is very beneficial. Therefore, the general aim of this work was to establish an efficient protocol for CRISPR/Cas-mediated genome editing in oilseed rape. Protoplast isolation, transfection with plasmids encoding CRISPR/Cas reagents and subsequent regeneration of transfected protoplasts were carried out. Genome editing events were detected by multiplex PCR amplicon sequencing. The CRISPR/Cas target loci encode proteins involved in the metabolism of guanosine tetraphosphate and guanosine pentaphosphate (unusual nucleotides jointly referred to as [p]ppGpp or alarmones of the stringent response – RelA/SpoT Homeologs [RSH]), and are likely to be involved in proper seed development and maturation. Protoplasts were successfully isolated from 4-5 weeks old in vitro grown plants of three cultivars. After protoplast transfection (polyethylene glycol [PEG; 100% or 80%]-mediated transfection) with a GFP expressing vector (pKAR6), we could determine the transfection efficiency between 8,27% and 32,56% (analyzed by fluorescence microscopy), depending on cultivar and concentration of PEG used for the transfection. The efficiency of some transfection experiments was additionally checked by flow cytometry. The value of correlation for the results obtained using these two different methods equaled 0,77 or more, implying that the flow cytometry method, after further adjustments, can be successfully used as a protoplast transfection efficiency screening technique. Using multiplex amplicon sequencing, we could determine the rate of particular CRISPR/Cas-mediated edits at one of the loci was in the range of 1-3%. Further amplicon sequencing on regenerated calli samples is ongoing.

Keywords: stringent response, oilseed rape, *Brassica napus* L., CRISPR/Cas, protoplasts transfection and regeneration, flow cytometry, multiplex PCR amplicon sequencing

Acknowledgments: The work is to high extend the result of the COST_STSM_46999 project. The research experiments conducted by JB, which results were an inspiration to conduct the work, were financed from the funds of the National Science Centre in Poland (2017/01/X/NZ1/01981 [JB]) and the Ministry of Science and Higher Education in Poland (1189-B research grant [JB]).

Gene editing of *Arabidopsis thaliana* cytosolic/nuclear subclass of HSP70

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Climate change is marked by severe temperature fluctuations, weather extremes such as droughts or flooding and an increase in abiotic and biotic stress, all of which affects the growth, development and yield of crop plants. In eukaryotes, the 70-kDa heat shock protein (HSP70) family performs protein folding, chaperoning and protection from adverse effects of stress. Members of the cytosolic/nuclear subclass of HSP70 family, such as HSP70-4, were also shown to be involved in the Mediator complex, a multiprotein complex involved in different processes such as transcription regulation at the level of RNA pol II assembly, chromatin architecture, RNA processing and epigenetic regulation. Association of heat-shock proteins in the Mediator complex opens up new pathways to understanding how plants cope with gradual and abrupt environmental changes. In the last decade, CRISPR/Cas technology has provided a highly specific tool for generating plants with site-specific mutations, free of unwanted mutations scattered across the genome. The purpose of this study was an introduction to CRISPR/Cas gene editing in *Arabidopsis thaliana*, followed by preparation of virtual reagents and protocols for generation of knockout mutants of *HSP70* gene family. Keeping in mind the redundancy of HSP70 proteins, an objective was to prepare detailed protocols for generation of both single (*HSP70-4*) and multiple gene knock-out mutants.

Acknowledgement: This STSM was carried out online in collaboration with Dr. Martina Juranić of Wageningen University & Research, The Netherlands, under COST Action CA18111 *Genome editing in plants - a technology with transformative potential*.

Agrobacterium mediated CRISPR/Cas9 transformative potential to modify abiotic stresses in poplar

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Global warming, climate change, and environmental pollution are threatening forest tree species to extinction. Every species has their unique response to combinations of different abiotic stresses. Drought and high salinity are among the most important stressors that alter plant water status and severely limit the growth and development of forest trees. Although much is known about how plants acclimate to each of these individual stresses, little is known about how they respond to a combination of these two stress factors occurring together, namely a multifactorial stress combination. Stress-responsive transcription factors (DREB, bZIP, WRKY, NAC, GRASS etc.,) are known to coordinate the gene regulation in plants and play essential roles in multifactorial stress responses and tolerance. Therefore, these transcription factors (TFs) are important targets for developing tolerant crops and trees against multifactorial stress combinations. In the current study, 6 different types of TFs (Grass 16-17, WRKY 73, DREB71, NAC44, and bZIP) that was previously associated with the salinity and drought tolerance in poplar species was aimed to knockout with CRISPR/Cas9 for understanding their functional and regulatory role on multifactorial stress tolerance.

The sequences of poplar' TFs were retrieved from the popgenie.org database and processed with Benchling software to find out the guide RNAs (gRNA) that can target exon parts of TF genes. Among many PAM sequences (NGG) containing gRNAs the best ones were selected according to their GC content (<%40), off-target capacity in poplar genome, and 3-dimensional folding structure with crRNA-tracr RNA complex. gRNAs were also designed to be 20 bp in length and to have one restriction enzyme (RE) cutting site in the Cas9 target site. In this way, a total of 12 gRNA (two gRNAs for each TF) were designed and synthesized. gRNAs were inserted into pHSE401 Cas9 containing plasmid and transformed into *Agrobacterium* EHA105 strains. gRNA/Cas9 complexes were then stably inserted into poplar callus via agroinoculation and subsequent antibiotic selection.

After regeneration of the transgenic poplar trees, CRISPR-mediated mutations in the genome will be confirmed with PCR-RE digestion and loss of RE cutting site assays. Then TF-mutated transgenic poplars will be subjected to drought and salt stresses combinations to see their responses to these abiotic factors. The effects of each TF on gene regulation network will be measured with RNA seq. analysis. By this way, the effects TFs on multifunctional stress tolerance will be explained and their functional role on gene regulation will be exhibited for further research.

Keywords: Poplar, CRISPR, Transcription Factor, Drought, Salinity

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First steps towards bioactivity guided gene editing in chicory for the higher production of targeted sesquiterpene lactones: CHIC project

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Cichorium intybus L. (*Asteraceae*) or chicory is a leafy biennial or perennial plant, with a strong fleshy taproot, that can grow up to 75 cm in length. Chicory has a long history being used as medicinal plant. The major sesquiterpene lactones (STLs) of chicory belong to the class of guaianolide sesquiterpene lactones and are commonly derived from a single sesquiterpene, germacrene A.

Chicory as a multipurpose crop for dietary fibre and medicinal terpenes is a European research and innovation project by companies, institutes and universities from 11 European countries and New Zealand. The ambition of CHIC is to tailor chicory genes by NPBTs in such a way that they alter their product specificity, which will lead to a chicory variety producing pharmaceutically important terpenes. For these purposes, STLs previously identified in chicory were assessed for their pharmaceutical potential.

Antimicrobial activity of STLs (costunolide, lactucopicrin, lactucin, 11β,13-dihydrolactucin, 8-deoxylactucin) was tested via analysing the type of growth inhibition (biostatic or biocidic) using modified microdilution method. Impact of compounds on biofilm formation was determined on yeast *Candida albicans* 475/15 and resistant strain of bacterium *Pseudomonas aeruginosa*.

Costunolide, lactucopicrin, 11β,13-dihydrolactucin, 8-deoxylactucin compounds found in chicory roots, showed the most prominent effect against resistant strain of *P. aeruginosa*. All tested standard compounds demonstrated very good antifungal activity against *Candida* species (MIC 0.03-1.00 mg/mL). Parthenolide showed the most promising activity in inhibiting *C. albicans* biofilm formation.

By using NPBT, novel chicory varieties with high-value terpenes that showed antibacterial, antifungal and antibiofilm activities will be made.

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Gene editing in celery: Short Time Scientific Mission at ILVO

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The basic principles of constructing a vector were learned. The vectors which were constructed with 2 different guide RNAs were grown in *E. coli*. Then, plasmid DNA extraction from *E. coli* was performed. Protoplasts were isolated, and protoplast transfection was performed with vector-based protocol to knock out and modify the nucleotide sequence of Apig1 protein. Protoplast transfection success was confirmed by the presence of GFP under microscope. The transfection/mutation efficiency was determined by the ratio of transfected versus untransfected protoplasts. The vectors were efficiently grown in the bacteria. Plasmid DNA extraction was successful. Protoplasts were able to be isolated and transfected successfully with the vectors. The transfection efficiency was estimated about 10%. A network for future collaborations was established and ideas were exchanged for possible future projects. This study was funded by the COST Action CA18111 “Genome editing in plants – a technology with a transformative potential” (PlantEd), through the Short-Term Scientific Mission (STSM) Number 45539.

Keywords: celery, genome editing, STSM, PlantEd

Overview of biosafety regulations to support the future regulatory status of precision breeding products in some non-EU countries

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There is no international consensus whether precision breeding products will be subject to regulation as Living Modified Organisms (LMOs) / Genetically Modified Organisms (GMOs), or not. The purpose of this study is to provide an overview of biosafety regulations of LMOs / GMOs in 27 countries near the European Union, that will serve as a baseline contributing to further discussions about the potential future regulatory status of precision breeding in these targeted countries. This is done by revising national biosafety legal frameworks, and results from a recent online survey to key stakeholders, including biosafety regulatory officers and biotechnology researchers, in the targeted countries. Our results classify countries in four main groups based on their approach to define LMOs / GMOs under domestic biosafety legislation. As such, the key criterion for the clustering of countries is whether the national legislation has adopted the legal definition of GMO under European Union law, Directive 2001/18/EC on the deliberate release into the environment of GMOs, or the LMO definition under the Cartagena Protocol on Biosafety to the Convention on Biological Diversity. Our study also shows that few countries have authorized the use of LMOs / GMOs for primarily R&D activities, whereas many other countries have established a ban for LMOs / GMOs import and cultivation. In addition, our results indicate that only one country (Israel) has adopted a legal instrument to determine the regulatory status of precision breeding products, and initials discussions are currently being made in 11 of the targeted countries. Finally, our study identifies 25 research institutes currently working with precision breeding technologies in plants under containment and/or confinement in 10 of the targeted countries. This study is part of the COST Action CA18111 “Genome editing in plants – a technology with a transformative potential” (PlantEd), through the Short-Term Scientific Mission (STSM) Number 47467.

Plant Breeders' Rights in the light of the NPTBT

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According to art. 1(iv) of the UPOV Act of 1991, *""breeder" means - the person who bred, or discovered and developed, a variety, […]"*. Similarly, art. 11.1 Council Regulation (EC) No 2100/94 of 27 July 1994 on Community plant variety rights states that *"[t]he person who bred, or discovered and developed the variety, […] referred to hereinafter as 'the breeder', shall be entitled to the Community plant variety right."* Behind these statements lies the idea of preventing that a *"mere act of discovery"* (Würtenberger et al., 2015, p. 29) is considered to be a sufficient basis for the entitlement to plant variety protection, so discouraging acts of biopiracy over plant genetic resources (Würtenberger et al., 2015, p. 29). Nowadays, that standard is presumed to be subjected to new tensions because of the emergence of NPBTs. Even the equilibrium between IP titles could be altered (Vives-Vallés, 2018). The UPOV Convention is revisited in order to find out how much breeding or *development* is needed, and NPBTs approached to assess how much threat do they really pose to the system.

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POSTER SESSION

PlantEd

1. **Wheat cell suspensions as a possible tool for CRISPR/Cas9 constructs evaluation**

   Michalski K., Linkiewicz A.M.

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1

Wheat cell suspensions as a possible tool for CRISPR/Cas9 constructs evaluation

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Utilization of site-specific nucleases (Cas9, TALEN, ZNF, etc.) for precise plant genome modifications has become a hot topic within last few years. Despite huge optimization effort the method still shows one major drawback, namely the necessity to test activity of every nuclease construct before using it *in planta*.

Several strategies of construct efficiency validation were proposed, based mainly on in vitro approaches. Among them, the transfection of seedling-derived protoplast became the most commonly used. It is a straightforward method consisting of PEG-mediated of construct plasmid into plant protoplasts and subsequent genomic DNA isolation and mutation efficiency quantification.

During our work with CRISPR/Cas9 nucleases we noticed that PEG-mediated protoplast transfection gives results that do not corelate with *in planta* findings when it comes to transformation of polyploid cereal species, like triticale or wheat. Therefore, here we present a proof of concept for a novel approach based on *Agrobacterium*-mediated transformation of wheat cell suspension cultures. We present a protocol for suspension establishment and transformation, as well as results of native gene modification based on Sanger sequencing and bioinformatic analysis. We discuss advantages and possible future development of this method as it is more consistent with *in planta* techniques.

2

microRNA abundance can be modulated by CRISPR/Cas9 system in polyploids

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microRNAs (miRNAs) are a class of small noncoding RNAs, which modulate the abundance and spatiotemporal accumulation of target mRNAs at post-transcriptional level and through that play important roles in several biological processes in plants. Here we show that in polyploid species, CRISPR/Cas9 system can be used for fine-tuning miRNA expression, which is even more useful if compared to knock-out of function. We established the complete pipeline for CRISPR-Cas9-mediated modulation of microRNA expression in potato and validated it on three miRNAs from potato. The pipeline consists of design and assembly of dual sgRNA CRISPR-Cas9 constructs, transient transfection of protoplasts following fast and efficient screening by high resolution melting analysis to select functional sgRNAs and stable transformation of potato with functional sgRNAs to select transgenic lines with desired mutations and microRNA abundance based on sequencing and qPCR. We show that miRNA-editing using dual sgRNA approach results in different types of mutations between transgenic lines but also in different alleles of the same plant, which are target site-dependent. The most abundant were short deletion, although we also detected 1-nt insertions and longer deletions. miRNA abundance correlates with the frequency and type of introduced mutations, as more extensive mutations in more alleles result in lower miRNA abundance. In all transgenic lines with Cas9 expression, we detected mutations, suggesting high efficiency of Cas9-editing. By amplifying several regions of T-DNA, we also showed that miRNA-editing efficiency with our dual sgRNA approach is genotype-dependent, although it was relatively high in both tested genotypes.

3

TILLING-by-sequencing and genome editing for the functional validation of candidate domestication genes in common bean (*Phaseolus vulgaris* L.)

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Common bean (*Phaseolus vulgaris* L.) is the most important grain legume for human consumption providing up to 15% of total daily calories and 36% of total daily protein in parts of Africa and the Americas. As a legume, it also has a role in sustainable agriculture owing to its ability to fix atmospheric nitrogen.
Wild common bean is organized in two geographically isolated and genetically differentiated wild gene pools (Mesoamerican and Andean) that diverged from a common ancestral wild population more than 100,000 years ago. From these wild gene pools, common bean was independently domesticated in Mexico and in South America nearly 8,000 years ago, and these domestication events were followed by local adaptations resulting in landraces with distinct characteristics. Domestication led to morphological changes in seed and leaf sizes, in the growth habit and photoperiod responses, variation in seed coat color and pattern that distinguish culturally adapted classes of beans. This unique example of parallel domestication is the subject of the PARDOM project that, starting from the *Phaseolus* replicated experiment, aims at understanding common bean genome evolution and adaptation.
In the framework of the PARDOM project, we are developing TILLING-by-sequencing and genome editing technological platforms for the functional validation of candidate domestication genes in common bean.
For the development of the TILLING-by-seq platform, DNA from seeds of a *P. vulgaris* TILLING population developed in the Mesoamerican genotype BAT93 (Porch et al. 2009; Cominelli et al. 2018) was extracted. A three-dimensional pooling system of 54 pools, each of 96 samples, at resolution of a population of 1728 individuals was used for NGS targeted sequencing based on custom capture probes. For the genotyping, a total of 719 genes of interest were chosen, based on the presence of one or more signals of domestication, differential expression between the

Andean genotype and Mesoamerican genotype, known involvement in the phenomenon of shattering, seed development and in the cytokinin hormonal pathway. Among these genes, 27 had a complete CDS sequence coverage, whereas for the others the first 1-3 exons were covered, for a total of approximately 491Mb.
The validation of candidate genes for domestication is currently in progress also via forward genetics, following the identification of target regions in coding sequences for genome editing based on CRISPR/Cas9 technology. Fifteen target candidate domestication genes have been selected, based on the presence of one or more signals of domestication. In order to verify sgRNAs editing efficiency a prescreening in hairy roots is planned. Given the challenges posed by common bean transformation (biolistic transgenesis), the genome editing approach is being simultaneously carried on also on soybean (*Glycine max*) orthologous genes.

4

Obtaining potato *Solanum tuberosum* plants that simultaneously express genes *desA* and thaumatinII

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Potatoes *Solanum tuberosum* are one of the most common agricultural crops in the world. However, its yield depends on environmental factors. Potatoes are sensitive to the negative effects of low temperatures and frosts during germination. Drought also has a negative effect on the yield of potato tubers. One of the possible mechanisms of plant resistance to abiotic factors is to increase the content of unsaturated fatty acids in the composition of membrane phospholipids. Desaturases are enzymes that promote the formation of double bonds in fatty acids and thus convert them from saturated to unsaturated.

However, the study of infection of plants with pathogens of fungal etiology is relevant. Certain mechanisms of control at the cellular level of pathogens are present in some plants. For example, increased expression of the gene thaumatin in *Thaumatococcus daniellii* plants.

The gene *desA* encoding Δ12-acyl-lipid desaturase of cyanobacteria *Synechocystis* sp. PCC 6803 and the gene thaumatinII of the plant *Thaumatococcus daniellii* was used in the work. Carried out the genetic transformation of *Agrobacterium tumefaciens* - mediated method.

The expression of two transgenes in a plant organism at the same time was investigated. The expression of the thaumatin II gene was proved by RT-PCR. It is known, that during the expression of the gene thaumatin there is an increase in the expression of protein kinases, so checked the expression of the desaturase gene indirectly by the activity of the protein of the reporter gene (reporter gene LicBM3 thermostable lichenase bacterial *Clostridium thermocellum*). A positive lichenase reaction was observed, which indicates the possibility of using two transgenes in potato plants to increase their stress resistance. Further research is underway.

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5

New technologies in achieving heat and drought resilient oilseed production, the case of camelina

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Camelina [*Camelina sativa* (L.) Crantz] also known as “false flax” or “gold of pleasure”, is a self-pollinated, annual oilseed that belongs to the *Brassicaceae* family. Camelina is native species of Eurasia, which is gaining interest world-wide due to its better cold, heat and drought tolerance, and less susceptibility to disease and pests than oilseed rape. The most of research work on camelina has been carried out in northern America and continental Europe. Consequently, there are not many data on evaluation of suitability of camelina genotypes for cultivation in southern Europe. Two breeding groups (IFVCNS and BOKU) and one group focusing on the agronomy development of the crop (DISTAL) just recently started research activities focusing on development of new genotypes more adapted for southern regions of Europe and evaluation of their productivity in these, more arid regions.

The hexaploid oilseed crop *Camelina sativa*, which has three closely related expressed subgenomes, is an ideal species for investigation of gene dosage as an important cause of phenotype variation. Targeted mutagenesis of the three delta-12-desaturase (FAD2) genes was recently achieved in camelina by CRISPR-Cas9 gene editing, leading to combinatorial association of different alleles for the three FAD2 loci. As a result, a large diversity of camelina lines was obtained with various lipid profiles, ranging from 10% to 62% oleic acid accumulation in the oil. Using the same approach, the different allelic combinations of genes associated with heat or drought stress tolerance may provide a unique source of genetic variability for creation of climate resilient camelina. ‘Omics’ studies which are in progress will identify the genes of interests, proteins, and metabolites in developing seeds that are impacted by heat or drought stress. Such studies, along with effective agronomic management system would pave the way in developing crop genotypes/varieties with improved productivity under drought and/or heat stresses. This would lead to prevention of high risk scenarios in the future production of oilseed crops, due to inability of the staple oilseed crops to adapt to high temperatures and drought.

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6

Functional conservation of nascent polypeptide associated complex in plants

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The development of plant flowers represents a complex process controlled by numerous mechanisms. The double homozygous knock-down mutant of both β subunits of nascent polypeptide associated complex (NAC) in *Arabidopsis thaliana* (further referred to as *nacβ1 nacβ2*) had defective phenotype including abnormal number of flower organs, shorter siliques with a reduced seed set, and inferior pollen germination rate together with a lower ovule targeting efficiency. Moreover, a delayed development of plants and lower chlorophyll content was observed. Previously, we characterised the NACβ subunits in *Arabidopsis thaliana*, deciphering their subcellular localisation, expression profiles, interactomes and effects of their silencing on gene expression including transcriptomic and proteomic analysis. NAC subunits are present in all eucaryotes, showing high sequence homology between evolutionary distant species suggesting their high functional conservation. In plants, numbers of genes encoding NAC subunits vary between individual species, with basal land plant *Marchantia polymorpha* being the sole known example with only one NACα and NACβ. Using CRISPR-Cas9 system, we aim to analyse the functional conservation of NAC subunits by characterising their function in a basal plant organism and compare the results between *Marchantia polymorpha* and *Arabidopsis thaliana.*

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7

Genome editing of *Ocimum basilicum* L. through CRISPR/Cas9 to induce resistance to pathogen *Peronospora belbahrii*

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Downy mildew disease, a very common and devastating disease of sweet basil (*Ocimum basilicum*), is caused by the obligate biotrophic oomycete fungus *Peronospora belbahrii.* Symptoms affect the entire leaf system, with chlorosis and lesions of the leaf near the central vein and the appearance, on the underside, of an abundant grey sporulation. The leaves gradually become necrotic and fall off (necrotrophic phase). The cultivations of basil in greenhouses and in open fields, destined respectively for fresh and processed consumption, have been heavily damaged by infections of *Peronospora belbahrii* for a decade (Minuto et al., 2004), causing crop losses close to 100%.

The increasing use of chemical agents to counter these adversities does not adapt to the prospect of agriculture increasingly directed towards a sustainable model. For this reason, in basil conferring resistance to pathogens is a priority in genetic improvement programs. With the recent development of genome editing technologies, in particular the CRISPR/Cas9 system, it is possible to attend to the genetic level quickly and efficiently to modify specific DNA sequences codifying characters of agricultural interest, such as turning off genes that make basil vulnerable to pathogens. Of particular interest is the susceptibility gene *DMR6* (*Downy Mildew Resistance 6*) whose mutation has been shown to confer resistance to oomycetes, in *Arabidopsis thaliana* (Zeilmaker et al., 2015), in *Solanum lycopersicum* (Thomazella et al., 2016) and in sweet basil cv. Genoveser (Hasley et al., 2021). This work describes the isolation of the entire orthologous sequence of *DMR6* (*ObDMR6*, 1260bp), in *Ocimum Basilicum* cv. FT Italiko, used as culinary herb to make the famous Italian “pesto sauce”, and the application of CRISPR/Cas9 technology to modify the susceptibility gene.

For this purpose, the binary vector pDirect_22c (Cermark et al., 2017) was used, optimized to create single or multiple genetic knockouts. Two target sites (gRNA) on ObDMR6 exon 2 were identified, using the CRISPRdirect tool (Naito et al., 2015) and used for the creation of a cloning cassette for the simultaneous expression of the 2 gRNAs, starting from a single transcript, to obtain targeted mutations in two points of the gene. The obtained construct was used in genetic transformation experiments of *O. basilicum* mediated by *Agrobacterium rhizogenes* and *tumefaciens*, which led to obtaining 96% of hairy roots positive for Cas9 integration and 82.3% of edited regenerated plants respectively. The resistance of the edited clones to the pathogen *P.*

belbahrii will have to be evaluated through *in vitro* infection assays and the identification of off-target mutations.

Keywords: CRISPR/Cas9, Genome editing, *Ocimum basilicum*, sustainability, downy mildew, cv. FT italiko

8

Genome editing of wheat - challenges and prospects for tackling changing environment

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Developing wheat able to sustainably produce high yields when grown under biotic/abiotic stresses is an important goal, in order to obtain food security in the face of ever-increasing human population and unpredictable global climatic conditions. However, random mutagenesis or genetic recombination as conventional ways for wheat improvement, are time-consuming and cannot keep pace with increasing food demands. Targeted genome editing (GE) technologies, like zinc-finger nucleases, transcription activator-like effector nuclease, and clustered regularly interspaced short palindromic repeats (CRISPR)/(CRISPR)-associated protein 9 (Cas9)) have been successfully used in editing wheat genome to get heritable variations for creating diversity and precision breeding. The tetraploid durum wheat (*Triticum turgidum* ssp. durum L.) and the hexaploid bread wheat (*Triticum aestivum* L.) are the most widely cultivated types, both with large genomes, developed as a consequence of ancient hybridization events between ancestral progenitors. The highly conserved gene sequence and structure of homoeologs among subgenomes in wheat often permits their simultaneous targeting using CRISPR-Cas9 with single or paired single guide RNA (sgRNA). Since its first successful deployment in wheat, CRISPR-Cas9 technology has been applied to a wide array of gene targets of agronomical and scientific importance, such as α-gliadin genes to lower gluten grain content, *TaGW2* to increase grain weight, *TaZIP4-B2* to understand meiotic homologous crossover, *TaQsd1* to reduce preharvest sprouting, *TaMTL* and *CENH3* for haploid plant induction etc. In the future, genes important for abiotic stress tolerance of wheat should be also targeted by GE technologies. During the last decade, identification of sources for abiotic stress tolerance in the IFVCNS wheat collection was performed under different projects, complemented with molecular analyses for identification of candidate genes of importance for wide adaptation of wheat to changeable environments. The final aim is the exploitation of IFVCNS wheat collections and the newest breeding technologies, such as genome editing, epigenetic tools, genome selection etc. for creation of highly productive resilient wheat varieties, as well as ideotypes specific for certain agro-ecological conditions.

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9

Improving perennial ryegrass adaptability and resilience (EditGrass4Food)

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The aim of the project is to improve adaptability and resilience of perennial ryegrass for safe and sustainable food systems through CRISPR-Cas9 technology. Genes involved in the mechanisms of freezing tolerance and biomass growth under water deficit will be investigated and targeted via gene editing.
"EditGrass4Food" is a EEA-Norway Grant (Baltic Research Program) project that has started in May 2021. It is coordinated by the University of Latvia and the partners are: Norwegian University of Life Sciences, Lithuanian Research Centre for Agriculture and Forestry and Tallinn University of Technology.
Perennial ryegrass (*Lolium perenne*) is the dominant forage grass species in Europe. However, perennial ryegrass exhibits poor performance under unfavourable environmental conditions, thus the changing climate poses a substantial challenge to ryegrass cultivation in the Baltic/Nordic region. In this project, we intend to utilize unique pre-breeding material, developed by the members of our consortium and CRISPR-based editing to validate candidate genes involved in northern adaptation of perennial ryegrass. We will investigate changes during abiotic stress periods at the transcriptome level to reveal gene regulatory pathways. Improving perennial ryegrass for winter hardiness, persistence and biomass formation under water limited conditions will help breeders in the Nordic/Baltic region to prepare for meeting new demands due to climate change.

10

Engineering haploid inducer lines in chicory

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Cichorium intybus var. *sativum*, also known as chicory, is an economically important crop that is mainly cultivated for the high inulin content in its roots, which has many applications in the food industry. Hybrid breeding, in which two homozygous lines are crossed to obtain high yield heterozygous offspring, can increase the root yield of chicory. To develop these homozygous chicory lines, we set out to create haploid inducer lines. Here, we describe a stepwise approach to find naturally occurring mutations that may lead to haploid induction or to create them through CRISPR.

In a first step, we selected four genes involved in haploid induction in model organisms (*CENH3*, *KNL2*, *MTL* and *DMP*) and identified their orthologues in chicory. About 90 primer pairs were developed to cover as much of the coding sequence of these genes as possible, for screening via amplicon sequencing.

Second, we aimed to identify naturally occurring alleles of these four genes, and to identify highly conserved regions where natural variation does not occur, and thus induced targeted mutagenesis is needed. To this end, we screened about 1600 plants, comprising 35 chicory varieties and 25 closely related witloof (*Cichorium intybus* var. *foliosum*) varieties. Preliminary data analysis revealed that the two *CENH3* orthologs are highly conserved, while *KNL2*, *MTL* and *DMP* chicory orthologs have both variable regions, as well as highly conserved regions in the coding sequence, usually coinciding with important protein domains.

Third, to induce mutations at highly conserved regions in these genes, gRNAs were developed and DNA vectors containing the CRISPR components were transfected into chicory protoplasts. Transfection efficiencies typically ranged from 10-50%, and seem to be dependent on the vector size. These protoplasts are being regenerated and grown into full plants, and will be screened for CRISPR induced mutations via amplicon sequencing.

Finally, plants showing interesting mutations, either found through screening natural variation, or induced via CRISPR, will be checked for a haploid induction phenotype. This will be done via hybridization crosses followed by ploidy assessment via flowcytometry of the offspring.

11

CRISPR-mediated multiple editing of *Becurtovirus* genome enabled curly top disease resistance in sugar beet and inhibited viral mutant escape and formation

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The curly top disease is a serious, yield-limiting factor for agricultural production in especially arid and semiarid regions of the world. In recent years, BCTIV (*Beet curly top Iran virus, Becurtovirus*, *Geminiviridae*) mediated curly top disease limited the production of numerous crops such as sugar beet, cowpea, common bean and tomato throughout the Middle East. CRISPR-Cas9 technology has been recently used to reduce geminivirus replication in infected plants and to develop curly top tolerant plants. Therefore, in the current study, 20 guide RNAs (gRNA) targeting the genic and intergenic regions of BCTIV genome were designed and transferred into a Cas9 containing Agrobacterium plasmid. The leaves of sugar beets were agro-inoculated for transient expression of gRNA/Cas9 complexes. Each leaf was then agroinfected with BCTIV to see its replication and spread efficiency on gRNA/Cas9 applied plants. PCR and qPCR results of the transient expression experiment indicated %30 to 85% reduction in viral spread and replication in gRNA/Cas9 applied plants. Restriction site mutation assay and sequencing analysis have confirmed the mutations created on the virus genome. However, the rolling cycle amplification (RCA) test indicated that some mutant viruses created by CRISPR can escape and create disease symptoms in sugar beet plants. To overcome this limitation, multiplexed gRNA-based CRISPR-Cas9 approach was designed to completely inhibit viral replication, spread and mutant evolution. In this design, the most effective four gRNAs on viral replication was selected to target all the genes (REP, CP, MP, ssDNA) on the BCTIV genome simultaneously. These gRNAs were transferred into a Cas9 containing Agrobacterium plasmid with golden assembly. Transient expression of this multiplex gRNA on BCTIV infected sugar beet leaves completely eliminated the viral replication and spread on the plants. PCR, qPCR and RCA assay on the viral genome did not detect any viral spread and escape mutants in multiplex gRNA/Cas9 applied plants. The multiplexed viral genome-editing technique could be an effective way to trigger a high level of disease tolerance on plants and eliminate mutant viral formation.

Keywords: BCTIV, Geminivirus, Curly top,Sugar beet CRISPR/Cas9

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Organic varieties, a breath of fresh air for plant breeding and plant variety protection in the EU, or their trojan horse?

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The concepts of 'organic heterogeneous material' and 'organic variety suitable for organic production' under Regulation (EU) 2018/848 are examined in the light of the available means of interpretation. Their fit into the current systems of seed certification, listing as well as plant variety protection, are also assessed. Finally, the relation between those concepts and Genetically Modified Organisms and techniques, including New Plant Breeding Techniques, is also approached. No absolute positions seem reasonable on this debate. On the one hand, those concepts push for a greater flexibility into EU seed and variety systems and standards, a breath of fresh air requested by some players (e. g., Gutzen, 2019, p. 46) and scholars [e.g., Litrico and Violle (2015) or Louwaars (2018)]. On the other hand, the possibility of a harmonious coexistence between these new wedges and the extant systems is, to the least, questionable [already warned by, e. g., Gutzen (2019, p. 57)]; besides, the potential benefits stemming from those new concepts will be restricted to the discipline of organic agriculture. Furthermore, thanks to the arrival of these new concepts, the gap between organic agriculture and modern plant biotechnology, instead of disappearing or, at least, diminish [on the advantages of combining those cropping systems, see, e. g., Renobales Scheifler (2009) or Purnhagen et al. (2021)], will likely increase further.

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Investigation of role of ORA59 transcription factor during *Pyrenophora teres* f. *teres* infection in barley

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Barley (*Hordeum vulgare* L.) is in the four most important cereals in the world. It has a significant role in the world's food supply and livestock feed and it is a model plant for research. Deployment of resistant cultivars is the most economic and eco-friendly method to control plant diseases. *Pyrenophora teres* f. *teres* (PTT), the causal agent of net form of net blotch disease of barley, is one of the most important fungal pathogens of barley.

The plant hormones has a key role in defence mechanism against plant pathogenes. The defence reaction induced by salicylic acid (SA) and jasmonic acid (JA) are the two most important pathways, which enable the plant to respond to the pathogenic attack properly. The activation of the JA-signalling pathway is required for resistance against necrotrophic pathogens. SA can antagonize JA signalling and vice versa. Their interaction provides an opportunity to the fine-tuning of the response. The APETALA2/Ethylene-Responsive Factor (AP2/ERF) superfamily of transcription factors (TFs) are implicated in the responses to both biotic and abiotic stress. ORA59 is one of the member of this family, which has been shown to increase its expression as a result of infection in model plants (Arabidopsis). The production of ORA59 is stimulated by JA and repressed by SA.

Therefore, we investigate the role of *ORA59* gene in defence mechanism against *Pyrenophora teres* f. *teres* infection in barley where it has not been studied yet.

Two barley genotypes cv. Golden promise and cv. Mv Initium was involved in the experiment. H-947 *Pyrenophora teres* f. *teres* isolate was used for artificial infection. The barley *ORA59* gene (HORVU4Hr1G000700.2 in Plant Ensembl) was identified based on Blast searches, it showed 69% AA identity to the *Arabidopsis* protein.

In our experiment, significant increase in the barley *ORA59* gene expression was observed after seven and fifteen days of *Pyrenophora teres* f. *teres* infection.

The connection between *ORA59* expression and PTT resistance of barley genotypes was studied by gene knock-out using CRISPR/Cas9 system. *ORA59* edited barley plants were produced by *Agrobacterium*-mediated transformation of immature embryos of Golden Promise. One of the transformed plant proved to be *ORA59* edited.

The resistance of gene edited barley plants against PTT was characterized by detached leaves assay. Two leaf pieces were separated from three shoots of the *ORA59* edited plant and placed on agar and then infected with H-947 PTT isolate. As a result of infection, necrosis appeared in the detached leaf test on the leaves, which were recorded according to the Tekauz scale on days 1, 2, 4, 6, and 9 after infection. Based on the lesion type, the AUDPC curve of ORA59 shoots was calculated. The three samples, unable to produce the transcription factor ORA59, showed significantly higher infection than the wild type.

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Deciphering rubber biosynthesis using genome editing and artificial miRNA in *Hevea brasiliensis*

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The rubber tree is the only source of natural rubber exploited on an industrial scale. Natural rubber has been placed on the list of strategic materials for Europe since 2017. The *cis*-1,4 polyisoprene is biosynthesized from sucrose produced by photosynthesis in the leaves and translocated to specialized cells called laticifers. After loading, sucrose is metabolized into isopentenyl pyrophosphate (IPP), a monomer used for elongation of the polymer biosynthesized in the rubber particles of latex cells. All genes involved in the NR biosynthesis pathway have been identified in the genomic sequences of the Chinese rubber clone Reyan 7-33-97 [1] and in clone PB 260 [2], and particularly the genes encoding the Rubber Elongation Factor (REF1–8) and Small Rubber Particle Protein (SRPP1–10) families. Difficulties persist in establishing a functional model for the final step of polyisoprene chain polymerization. Routine somatic embryogenesis procedure [3-5] has allowed developing an efficient *Agrobacterium tumefaciens*-mediated genetic modification [6-8]. Several functional studies by endogenous or exogenous gene overexpression have been successfully carried out allowing the discovery of new functions in Hevea, notably in laticifier cell differentiation [9-11]. The identification of a highly expressed *HbMIR408* gene allowed the development of a gene silencing strategy using artificial miRNA leading to a partial inactivation of the *uidA* transcript, present in a transgenic line overexpressing this gene [12]. The same approach is underway with the use of sgRNAs targeted against the *uidA* gene in order to obtain total inactivation. The partial or total extinction of the expression of the genes coding for the proteins of the biosynthetic complex could make it possible to disentangle the role of each protein that constitutes it by taking into account the functional redundancy.

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Knockout of *CPL-3* gene in tomato using genome editing

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Tomato (*Solanum lycopersicum* L.) is one of the important vegetables in the world due to its commercial and dietary value and its widespread production Turkey is the fourth-largest producer of tomatoes. It is a rich source of several important health-promoting nutrients such as Vitamin C and E, minerals and carotenes including ß-carotene and lycopene. But on the other side crop faces several biotic stresses and cause reduction of yield. There is a decrease in crop production worldwide 20-40 % due to biotic stresses. However, tomato faces huge yield losses due to several infectious diseases caused by *Pseudomonas syringae*. Pv. tomato (Pst). Several genes of tomato plants have been identified related to a positive and negative regulator of the immunity gene. Among those negative and positive regulators of defense genes of tomato, there is a negative regulator of an immune gene, which suppresses the disease resistance genes, C- Terminal domain phosphatase Like 3 known as *CPL-3* involved in the suppression of immune gene which causes resistance against several pathogenic diseases. In this study, we find out *CPL-3* gene in tomato. Further, we are trying to knock out the *CPL-3* (RNA polymerase II C-terminal domain phosphatase) gene using Crispr-Cas9 that is involved in downregulating immune responsive genes of crop plants under biotic stress conditions. By knocking out *CPL-3*, tomato plants will continue to express immune-related genes in response to biotic stresses, hence immunity against a pathogen will be achieved.

Keywords: Tomato, Diseases, Quality, Immunity, Crispr-Cas9, *CPL-3*