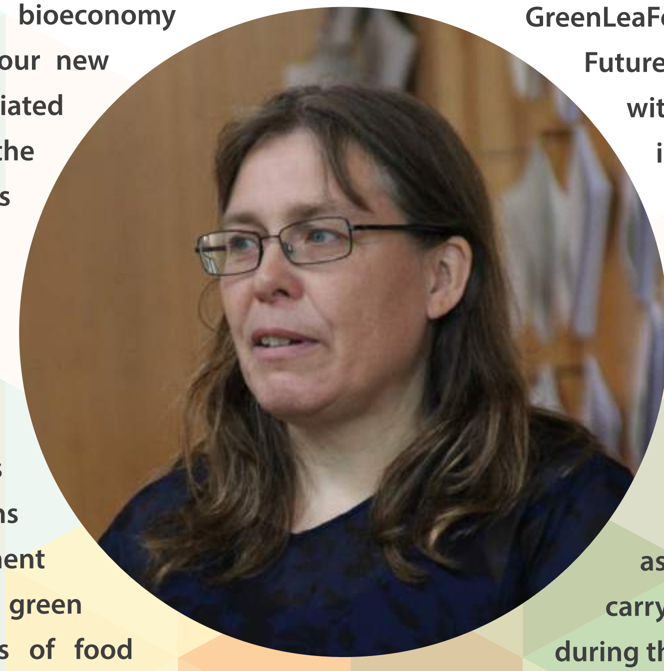
C4F Program director / Eva Johansson

C4F – Crops for the Future

The focus of C4F is to produce high level research in the area of sustainable new plant-based products development, using novel technologies, thereby contributing to a circular bioeconomy in Sweden. During 2021, four new projects have been initiated within C4F, also including the entrance of three new PIs into the program. Thus, we welcome Thomas Prade, Galia Zamaratskaia and Pär Ingvarsson as new PIs within C4F. The topics of the new projects cover research questions related to the development of superabsorbents from green biomass, protein structures of food produced by a mixture of legumes and cereals, and structure-function relationships between model proteins and breeding of high quality forage based on timothy. The new projects are all well connected to the strategy of C4F as well as to other large programs within the research area, such as SLU Grogrund, The Plant Protein Factory, GreenLeaFood, Green2Feed, Mistra Future Food, etc. Together with novel projects started in 2020, covering areas such as plant protein fractionation, Cd and bread-making quality, quality and quantity improvements of oils and proteins in oilseed crops and authophagy, C4F is creating a strong asset of strong research to carry the success of C4F further during the third phase of TC4F. C4F also sees several of the phase 2 projects successfully coming to an end with strong research publications, thesis defenses and sustainable new plant-based products as results from the program.



Text: Eva Johansson

C4F- Crops for the Future

C4F program has been functioning as a research platform since its start, supporting a large number of projects, connected to other large projects. During 2021, about half of the projects were finished and new projects have been initiated, in which the involvement of young researchers (PhD students, postdoc and project leaders) has been further highlighted among the other important criteria. The overall progress of the research within C4F has been in principle as planned in 2021.

A number of peer-reviewed articles of high quality have been published, while a number of manuscripts have been submitted or are in the pipeline for publication. A couple of young researchers, PhD students, or postdocs have been recruited and new young project leaders have been appointed. The Grogrund research school associated with C4F has been established. A number of large grants have been received, partially due to the C4F program, such as Formas food, 8 milj, Energimyndigheten 5 milj, BSRC (bioresource center) – SLU together with Linköping university. Xue Zhao has defended her thesis in October 2021, in which she has developed a simplified method for determination of the branching density in amylopectin.

Research on innovatively designed wheat gluten fibers as potential absorbents of biofluids and impact of climate on Swedish wheat protein quality have resulted in accepted publications. The new results on the use of neutron techniques to monitor Cd stress and water uptake in wheat for food uses have been generated. The results on the relationship between amylopectin internal molecular structure and its thermoanalytical properties have been generated. A 3-D printing technique has been applied in legume research. Studies on purification methods for mung bean protein, spinning protein nanofibrils in flow celles into threads, utilization of SAXs at DESY and texture and microstructure of mixed faba bean protein/starch/fiber gels have generated data for publications.

Based on the study of carbon allocation in rice, the mechanism of methane emission in rice paddies was reviewed, 5 low methane varieties through hybrid breeding with 70% methane reduction from rice paddies were bred, which could generate high biofuel rice straw and build up a win-win cultivation model with low-temperature-adaptive rice in Uppsala.

The latest genome editing technique, CRISPR/Cas9, has been one of the major advanced breeding technologies used in studies on the oil crops and potato within C4F, as an efficient molecular tool for research and crop improvement. The well-established protoplast protocols established within the program have enabled generation of transgene-free mutants of the target crops by CRISPR/Cas9. CRISPS-edited mutation lines of rapeseed and field cress have been obtained for improving the seedcake and oil qualities. RNA-seq analysis in relation to changes in oil and starch on greenhouse grown CRISPR-edited potato with redirected carbon flow has progressed. Moreover, genome sequencing information and RNA-seq profiles have also been used in analyzing evolutionary perspectives of oil accumulation in *Cyperus* tubers.

Successful transient expression of gene combinations to optimize production of novel molecules in pheromone work was achieved and stable transformants of oilseeds were obtained. RNAseq analysis and gene mining of *Lindera* species have been progressed for potentially improving incorporation of medium chain pheromone precursors in camelina.

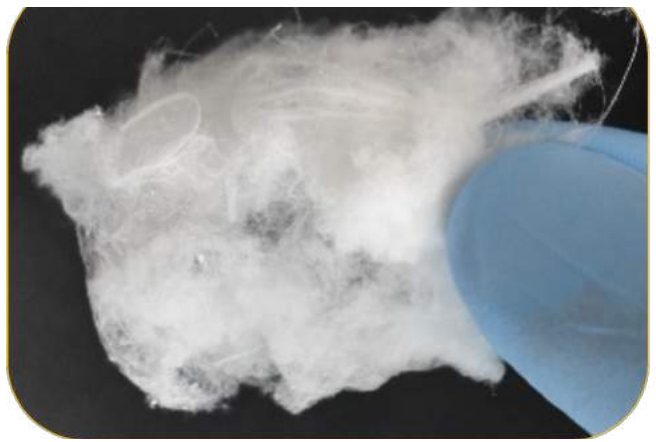
A significant progress in establishing the methods and tools required for elucidating plant autophagy dynamics and the molecular mechanism underpinning the effect of chemical compounds, which we identified previously as selective plant autophagy regulators, has been achieved.

Research outcomes and associated outreach activities deal with new knowledge and information on novel potential uses of plant oils, proteins, starches and other compounds from side streams, which can be used as food, feed and industrial applications. The program contributed to emerging of new research areas such as bio-based composites for food and non-food uses, possibility of crop improvement by regulating autophagy process, and renewable sources of plant produced insect pheromones for pest management. SLU Grogrund has continued to support more novel projects in 2021. Due to Covid-19, physical participation in national and international conferences has not been possible in 2021.

Detailed research findings and progress

Protein fractionation of green biomass, its problems and opportunities in terms of both technologies and economy were elucidated. The major finding is that the fractionation from the pulp has to be further resolved. The structure of protein isolate from lupin and from innovatively processed quinoa was further evaluated by the most novel methods. Functionalization of potato and wheat proteins to obtain superabsorbent materials was further evaluated and described.

A new method was developed to design sustainable microfibers from wheat gluten protein using a non-reducing solvent and electrospinning. The wheat fibers containing 20% of gluten were relatively strong and very promising as blood and biofluid absorbents (Fig. 1). Heat treatment of gluten fibers improved chemical bonding in the proteins and the functional properties of fibers. Varying climate such as, prolonged heat and drought, increased gluten polymers and large monomers, and the ratio of monomers vs polymers, and decreased protein concentration in the wheat material grown during the two years. The cool climate resulted into a longer grain development time, while the hot and dry climate resulted into much shorter grain development period. The yield was impacted negatively, while the grain microstructure was found to be similar due to the heat and drought stresses. The recent neutron results indicated that Cd stress impacted negatively the wheat root development and this differed in the two studied growing media.



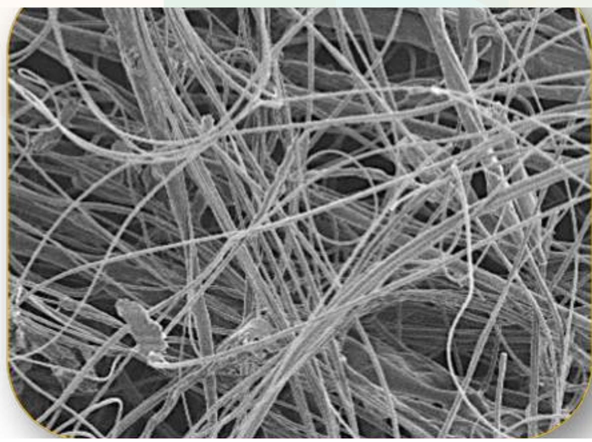


Fig 1: Wheat gluten fibers as potential absorbents of biofluids (Photos by Faraz Muneer)

The results on characterization of secondary structure of 7 different PNFs along with how protein size and purity affect the ability to form PNFs were published. Plant-based PNFs' ability to form stable foams at different pH–values was investigated and the ability of the PNFs to form stable threads was investigated by SAXS.

Faba bean starch, side-stream product, was structurally and chemically characterized and correlated with the thermal behavior. Faba bean starch is amylose rich, has high proportion of long-branch-chain-lengths and a homogenous granular size distribution causing higher gelatinization temperatures and relative viscosities. Isolated faba bean fractions; starch, protein and fiber, were combined to create an edible bio ink. Inks with higher proportion of carbohydrates were more stable. Faba bean protein films were reinforced with cellulose-nanocrystals from pine cones. Pea protein gels at different pH and concentration of unrefined lentil fraction were characterized in terms of texture and microstructure. Results showed that starch was responsible for changes in viscoelasticity and insoluble cell wall fragments acted as fillers in the gels. Similarly, mixed faba bean gels were characterized and the effect of starch and fiber on texture and microstructure of heat induced protein gels was investigated (Fig 2).

The simplified method for determination of the branching density in amylopectin developed by Xue is very significant for connecting starch structure and its physical properties. We strongly believe that the branching density is one of the most important features for the rate of retrogradation. The fact that we now can measure the branching density in a large number of samples makes it possible to provide good statistical evidence for correlations with physical properties. This method has been applied on barley lines with a systematic variation in a transcription factor controlling the starch synthesis.

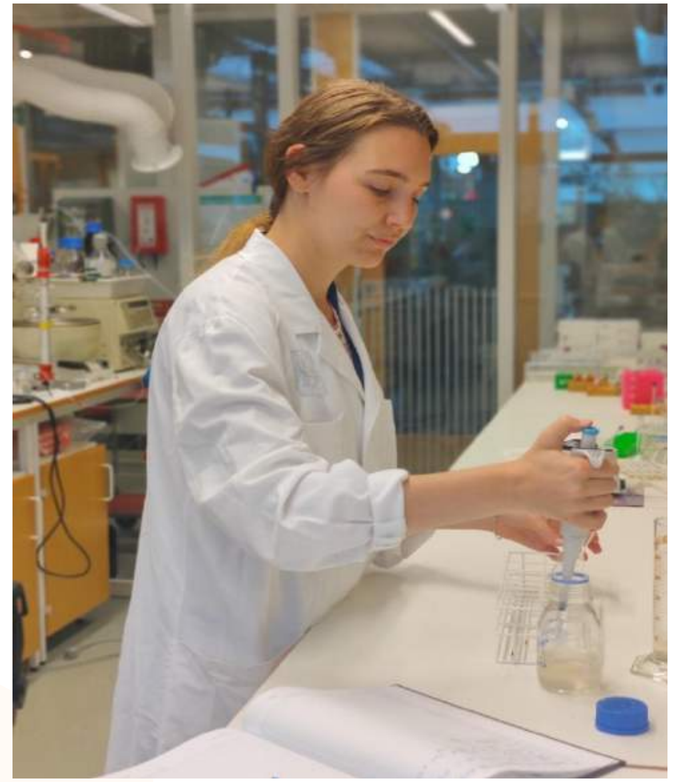
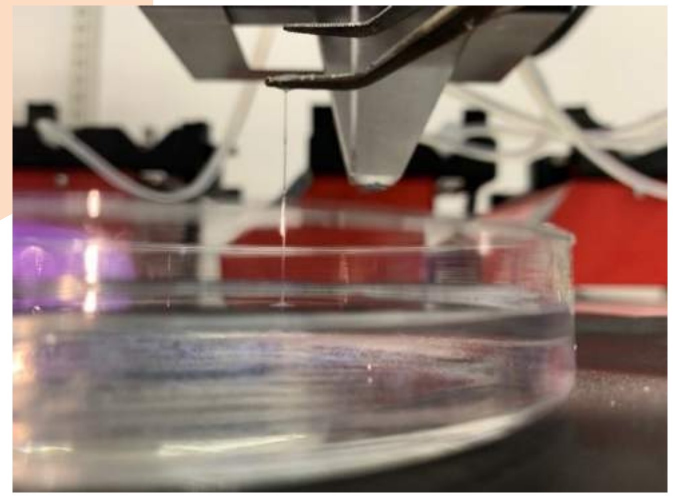


Fig 2. above: PhD-student Anja Herneke working with protein nanofibrils in the lab (Photo by Klara Nilsson).
below: Thread made from spinning mung bean protein nanofibrils. (Photo by Anja Herneke)



The results are very promising and will be published together with data describing gene activities and beta-glucan levels.

Biomethane production in *WRI1*-rice straw has been detected with a 20% increase compared with wild type without any grain yield defect. A win-win cultivation modus of Nordic climate adapting rice has been shown. We have also shown that endogenous fumarate and ethanol dictate methane emission in rice paddies, and obtained low-methane high-yield rice lines based on our discovery through hybrid breeding for sustainable agriculture with less environmental impact. Three manuscripts have been submitted in these research areas.

Manuscripts on the results of proteomic analysis in relation to oil in *Cyperus* and *WRINKLED1* overexpression in rice, respectively, have been re-submitted, while a manuscript on decipherment of transcription factor domains important for induction of fatty acid synthesis has been submitted. A couple of manuscripts are in preparation regarding interactions between transcription factors affecting seed structure, seed filling and seed maturation, oil induction in wheat endosperm with seed X-ray imaging and nutritional evolution, evolution of oil accumulation in *Cyperus* and genome editing as a methodology to redirect carbon flow by modifying promoters of transcription factors.

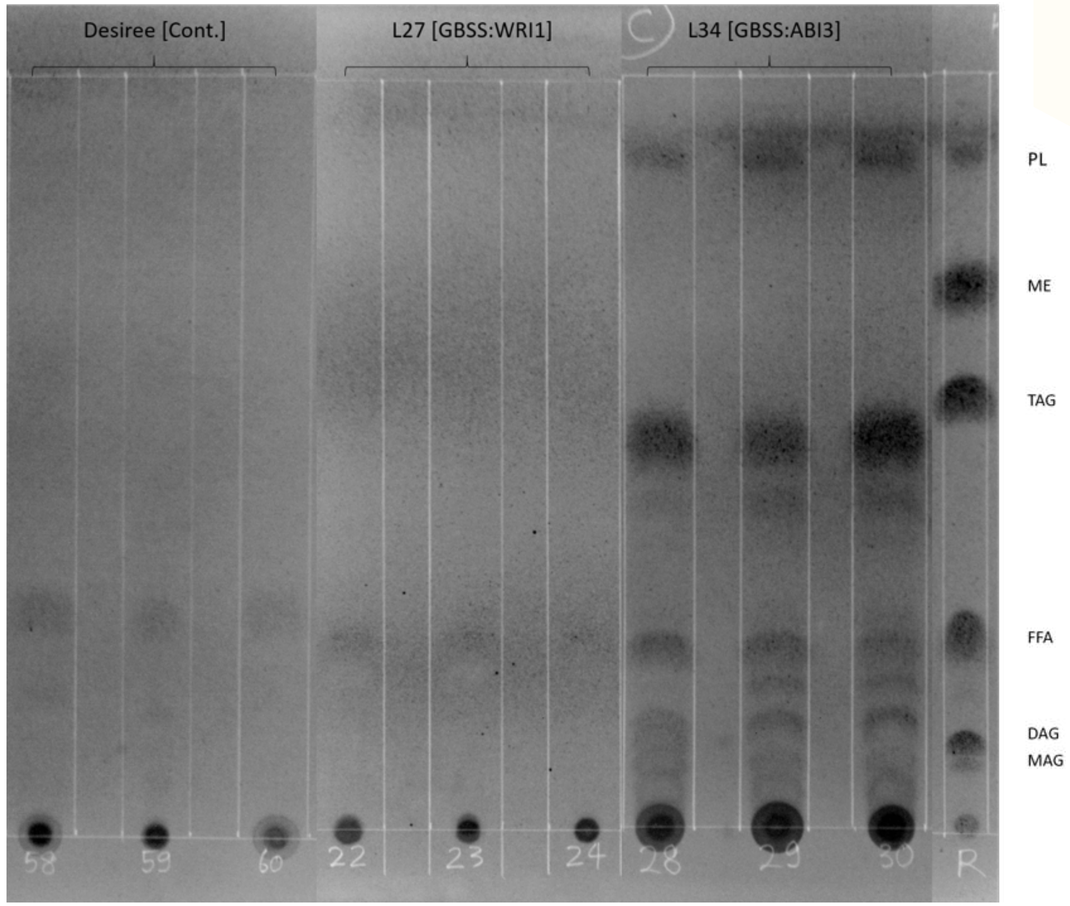


Fig. 3 Lipid accumulation induced by CRISPR-Cas9 modulation of promoter of StABI3 from seed to tuber expression (Photo by Shrikant Sharma)

Fig. 4 CRISPR-edited rapeseed (left) and filed cress (right) plants grown in biotron (Photos by Li-Hua Zhu)

Efficient protoplast regeneration protocols with successful transfection and generation of CRISPR-edited lines of field cress and rapeseed, respectively, have been published. Using these protocols, a number of mutation lines targeting on glucosinolate genes have been now obtained for both species. Further screening for homozygous lines in biotron (Fig 4) is ongoing, which would provide materials for phenotypic, chemical and molecular analyses of these lines on the target trait. Cloning of new target genes on new target traits are ongoing on both species.

One manuscript in pheromone research has been published, one is under revision and one is under submission regarding detecting release of pheromones from engineered plants and validating the whole production process of pheromone precursors from field trial as oil constituents including their processing and evidenced application in pest control. Characterization of novel genes and compounds related to insect pheromone production are progressing in collaboration with LU partners (Fig 5).

We have established a new method for non-invasive imaging of autophagic reporters in the model organism Arabidopsis thaliana (Fig 6). The method and our initial observations on the cell-type specific autophagy are summarized in the manuscript currently under review in Scientific Reports.

Using the established state of the art method we have obtained results on the differences in autophagic activity in the shoot and roots upon different conditions impacting the pathway activity, i.e. inhibition of the TORC kinase complex, nitrogen or carbon starvation.

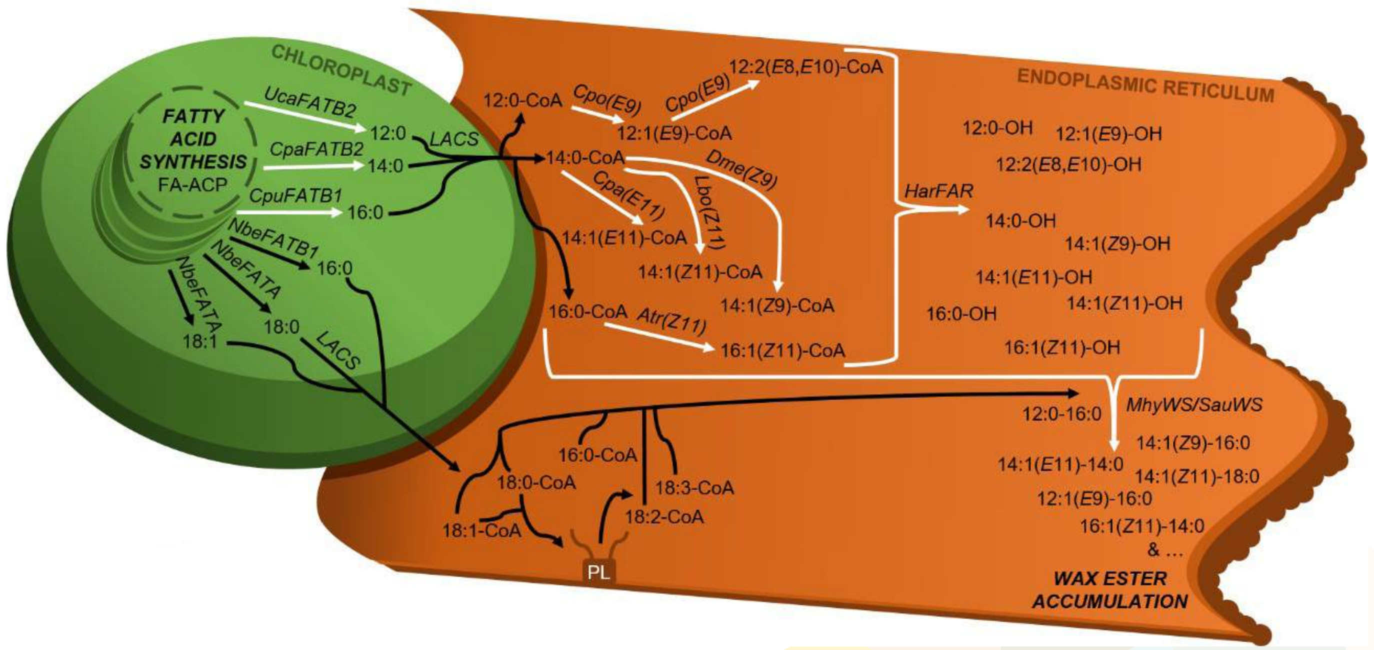


Fig 5. Strategy for producing novel medium chain wax esters with applications for pheromone production. White arrows indicate novel activities added to the plant. (Photo by Kamil Demski)

We have developed SPIRO, a state of the art automated plant phenotyping platform, which enables rapid assessment of the autophagy modulation on plant seedlings under standard growth conditions. The platform and its application is described in the manuscript currently under review in the Plant Cell.

We have established the first prototype of non-invasive bioluminescence reporters for plant autophagic activity in collaboration with Planta LLC in order to introduce a fungal bioluminescence pathway that recruits plant metabolite caffeic acid to produce substrate for bioluminescence reporter. The reporter has been modified to decrease its activity upon plant autophagy upregulation. We have successfully obtained bioluminescence activity in plant cells and are currently working on modifying intracellular localization of the pathway components to be optimal for autophagic activity detection.

We made a significant progress in elucidating the mechanism of action of the small organic compounds that we have previously identified as specific regulators of plant autophagy. We have short listed a number of proteins potentially interacting with these compounds in plant cells and are currently confirming the occurrence of the interaction and its impact on plant autophagy. These information will not only enable efficient tuning of plant autophagic activity using the compounds, but will also guide implementation of genetic tools for regulating the same targets and might contribute to establishing new molecular markers for breeding.

Currently most of the experiments are carried out using the model plant Arabidopsis. The C4F program will allow us to then expand our conclusion also on crop species closely related to Arabidopsis, i.e. *Brassica napus* and *Lepidium campestre*. For this we established a collaboration with Dr. Selvaraju Kanagarajan (Prof. Li-Hua Zhu's group, SLU, Alnarp), which will be carried out in a form of a jointly supervised PhD project.

In what way the research has contributed to social benefit

Within C4F program, some projects are closely connected to or have been transferred to UDIs or EIPs, one way to transfer TC4F knowledge into product-based projects, thus benefiting to the society. Whereas some other projects have potential significant social benefits, thus benefiting the society in long-run. For instance,
The plant protein factory was transmitted to a pilot facility at the faculty, to be available for uses of interested users. A patent of the superabsorbent material was filed.

Faba beans, oat, peas, rapeseed, potato are Swedish crops with a good nutritional profile, which can be a good plant based protein alternative to soy-products and gluten. Legumes can help with nitrogen fixation when incorporated into an intercropping system, which will thus enrich the soil and reduce over fertilization.
The starch research aims to improve product quality in food as well as non-food applications. Starch with increased amylose content have nutritional benefits since it has more slow carbohydrates. Slow carbohydrates can potentially decrease our insulin response and thereby reduce the risk to develop type II diabetes.

Our research on retrogradation can in the long run reduce food waste by prolonging product shelf life. Crystallizations and other inter-molecular interactions are also important for material applications, that will be studied further in this project.

Social benefits include two open-access scientific publications that communicating a new knowledge on the latest findings in the area of bio-based absorbents and wheat quality breeding for climate stability. The paper focusing on innovative green and sustainable materials from plant protein by-products is a high value information for the Swedish society and the world aiming to be independent from petroleum resources in nearest future. The paper focusing on wheat gluten protein quality aspects in a varying climate is a valuable information to obtain when breeding wheat crops for future climate stability and aiming to make Sweden self-securing of quality wheat.

The new research has discovered the mechanism of methane emission in rice paddies, and based on the research, we have bred 5 independent low methane rice lines, with 70% methane reduction, which could dramatically mitigate the effect of rice production on global warming. The research has achieved two international patents and at least two high profile publications.

Novel CRISPR-edited mutation lines of oilseed crops with improved oil qualities contribute to increased plant oil production for food, feed and industrial purposes, and consequently reducing the fossil use and benefiting the environment.

Insect pheromones for pest management is non-toxic and produced from a renewable resource contrasting to pesticides. Camelina derived pheromone blends have now been proven to work in the field.

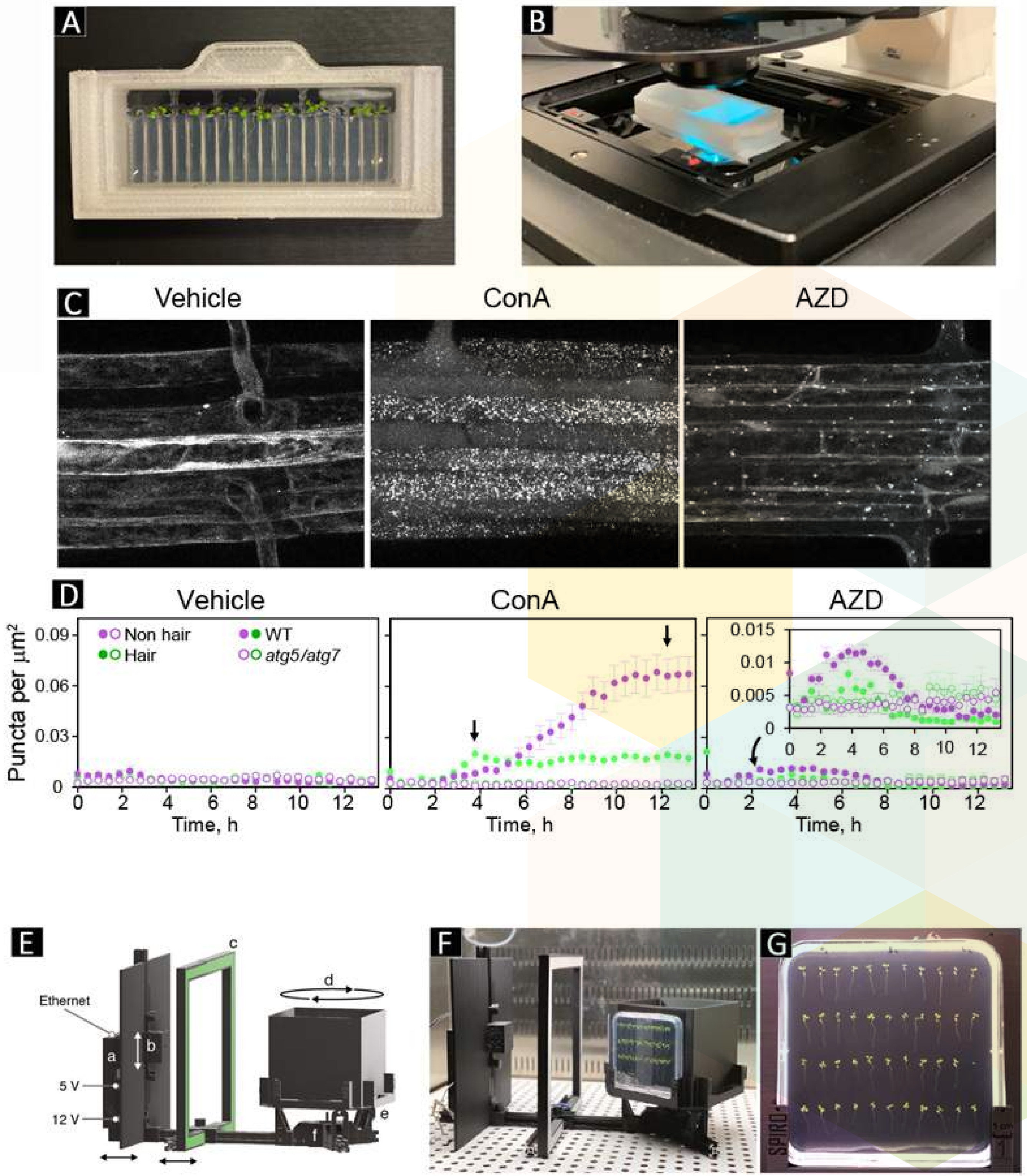


Fig 6. State-of-the-art tools developed for the project. (A-D) RoPod toolkit for non-invasive monitoring of autophagic activity in Arabidopsis thaliana roots. **A.** RoPod, a 3D printed chamber custom designed for growth and imaging of Arabidopsis seedlings. **B.** RoPod mounted on a stage of a confocal laser scanning microscope, not that seedlings are grown and imaged inside the same chamber, which alleviates mechanical stress usually imposed by mounting seedlings on the glass slides for microscopy assays. **C.** Detection of the fluorescent reporter for plant autophagy in the epidermal root cells using RoPod toolkit. Under non-inducing conditions (vehicle) the reporter is diffusely distributed in the cytoplasm. Treatment with AZD8055 (AZD) triggers formation of puncta-like autophagosomes in the cytoplasm. Implementation of Concanamycin A (ConA) leads to accumulation of autophagic bodies in the plant vacuoles. **D.** Time resolved dynamics of cell-specific autophagy obtained using RoPod toolkit and the fluorescent reporter illustrated in C. (E-G) SPIRO, Smart Plate Imaging Robot, an automated platform for phenotyping plant seedlings grown on Petri plates. **E.** a schematic representation of the imaging platform that comprises Raspberry Pi-driven components: camera, stepper motor and LED light source. Petri plates are mounted on the cube-shaped staged and imaged at user-defined time intervals. **F.** Photo of a SPIRO robot inside a standard growth incubator. The small footprint of the robot allows automated phenotyping under normally used conditions. **G.** An example of high-resolution image obtained by the SPIRO, the downstream pipeline includes automated image processing for seed germination and root growth detection.

At least one example on how C4F takes basic research to application to be used

The most of the projects in C4F have a character of more towards applied research, meaning that we have tried to transfer the known knowledge from basic research in oil, protein and starch as well as material science into potential applications in one way or another. Some examples are:

1. Protein factory or superabsorbent material
2. Bio-based sustainable absorbents
3. Wheat gluten quality for breeding climate stable wheat
4. Cd stress in wheat- management strategies
5. By understanding the structure and properties of the different fractions ; protein , starch and fibre new foods can be developed and optimised. Mixtures will also be valuable to show how pure fractions is needed. Previous knowledge on the structure and behaviour of the faba bean fractions was used in the bio-ink development in the 3D-printing project.
6. Starch is a useful part in a composite material where the starch can provide oxygen barrier functionality and something else gives strength and water resistance.
7. A single gene characterised in rice could be used to generate a high-bioenergy-rice for fuel production by increasing the oil content in rice straw.
8. Pheromones from a plant production source for pest management is a good example of going from basic science on specific pheromone compounds their genetic background in e.g. moths to applications in production and pest management.
9. Identified target genes in model species or the same species from basic research have been used in improving target traits in oil crops by CRISPR/Cas9.

Text, photos and illustrations: Li-Hua Zhu and C4F researchers

Vice program leader Li-Hua Zhu