

C4F- Crops for the Future

All the projects within C4F have been conducted in principle as planned with good progress in 2020, while some delays in experimental work happened in some cases due to Covid-19. A number of peer-reviewed articles of high quality have been published, while a number of manuscripts submitted or in preparation are in the pipeline for publication.

The overall progress within C4F

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Newly funded research grants associated with the C4F program have been initiated. Computational modeling tools for plant proteins and especially for the gluten proteins, which have previously not been targeted due to their size, have been developed. Unique research on functionalized plant proteins obtaining absorbent capacities not previously reported, has resulted in patents, several papers in good journals and in a doctoral thesis.

Latest advanced imaging techniques have been used in analyzing plant product qualities for food purposes. The latest genome editing technique, CRISPR/Cas9, has been applied more and more as an efficient molecular tool for basic research and for crop improvement. Efficient protoplast-based methods for producing transgene-free

mutants by CRISPR/Cas9 have been established for important crops included within C4F. By regulating carbon allocation, we have obtained biofuel-rice, rice lines with improved disease resistance, and high fructan or high starch barley varieties, respectively.

Important insight into molecular regulation of autophagy and new tools for modulating and monitoring autophagy in crop models, *Arabidopsis thaliana* and *Nicotiana tabacum*, have been generated (Fig. 1). Research outcomes and associated outreach activities deal with new knowledge and information on novel potential uses of plant oils, proteins, starches and other compounds which can be used as food, feed and different 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, potential medical uses of plant-produced proteins and renewable sources of plant produced insect pheromones for pest management. The plant protein factory is now running in full speed and SLU Grogrund has continued to support more novel projects in 2020. Due to Covid-19, physical participation in national and international conferences has not been possible in 2020.

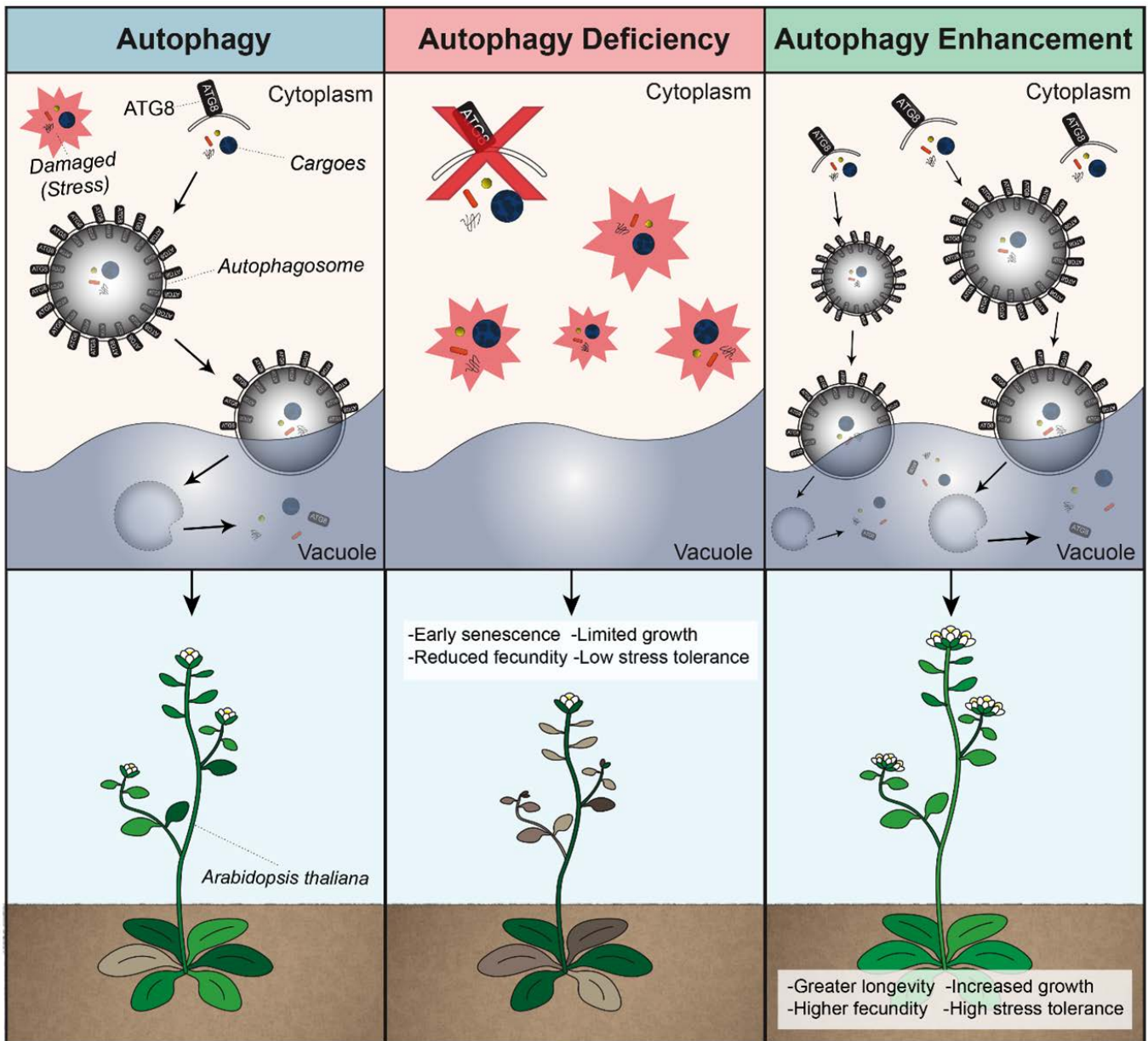


Fig. 1 Autophagy is a major catabolic process in eukaryotes. Upon activation of autophagy, there is a bulk or selective sequestration of cargoes into compartments called autophagosomes, which are double-membraned vesicles that later fuse to an acidic compartment where the cargoes are degraded. The process is coordinated by AuTophagy-related (ATG) proteins, among them is ATG8 that plays a central role in the formation of autophagosomes by becoming directly incorporated into the double membrane. Autophagy deficiency or enhancement has shown to have significant effects on plant growth and stress resistance. Figure prepared by Dr. Adrian Dauphinee.

Detailed research findings and progress

The Problöja project with funding from Vinnova and TC4F was finalized in 2020 with unique research results on which proteins and how to properly functionalize these proteins in order to obtain high superabsorbent properties on the produced materials. We have distinguished the wheat (Fig. 2) and the potato proteins as two target plant protein sources for superabsorbents and also differentiated functionalization routes suitable as sustainable alternatives. The superabsorbents produced contributed high uptake properties for water and salt solutions while the blood absorption was exceptional in speed. The research has resulted in two patents and the formation of a consortia to take the results further to real applications.

The VR and TC4F funded research on computational simulations of plant proteins to understand functional properties has finally started to generate significant results in 2020. The plant proteins under study are extremely difficult to investigate through simulations due to their enormous size, the gluten proteins are building the largest protein polymers in nature.

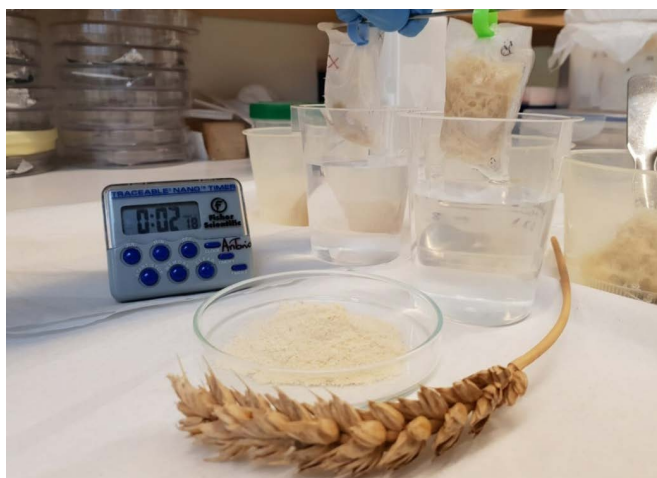


Fig. 2: Superabsorbent production from flour of the wheat grains. Photo by Antonio Capezza



Fig. 3. Plant protein based textiles of various types and their absorption properties; a) paper-like gliadin fiber mat; b) wheat gluten textiles absorbing blood. Photos by Ramune Kuktaite

In collaboration with researchers at KTH and LU, we were able to simulate structural features on these proteins in the plant cell, previously not reported, that explains why some of them are forming internal while others are forming external cross-links while synthesized. Here, the hydrophobicity around cysteines seemed to be of high relevance. The model explaining disulphide bond formation at cell level will contribute significantly to further understanding of disulphide bond formation at processing.

We obtained the knowledge about how the properties and structures of the protein-rich crop foods (gliadin edible foams) are steered by the use of different additives. New intriguing results in the area of plant protein fiber textiles indicated that after specific treatment of the fibers it is possible to tune these fibers for repellent/absorbing applications (Fig. 3) and two manuscripts are in preparation. New results on the climate impact (e.g. heat and prolonged drought) on the protein composition in wheat indicated the genotype being a key player for the strength of the proteins. Differently, a negative effect of heat and prolonged drought was observed for the protein concentration.



Fig. 4. Cultivation of rice in Uppsala

We have characterized starch from faba beans in comparison with wheat starch and initiated tests of mixed gels of protein, starch and fibers from faba beans. We have also performed tests on addition of arabinoxylan to bread and the result is under evaluation. Two manuscripts on pea protein and nanofibrils are prepared and one has been submitted. Due to Covid-19, the X-ray scattering analysis on nanofibrils, connected to the VINNOVA project, was delayed, while the pre-experiments in the flow cell has been done at KTH.

The branching density is one of the most important features for the rate of retrogradation, while retrogradation is critical for product quality aspects such as product appearance and shelf life. We have developed and published a useful method for determination of the branching density in amylopectin in order to connect starch structure and its physical properties. With this method, we can measure the branching density in a large number of samples for providing a good statistical evidence for correlations with physical properties. The method has been applied on barley lines with a systematic variation in a transcription factor controlling the starch synthesis. The results are under evaluation. The starch composition and amylose structure have been studied on the CRISPR/Cas9 edited lines

with reduced or eliminated the synthesis on amylose in potato and one manuscript on this is under review.

Basic research regarding health effects of dietary fibres as well as health effects of phenolic compounds in food products was the fundament for the side stream project, associated with to an EIP Agri. project. While it is established that dietary fibres have a range of beneficial health effects, as do phenolic compounds, the difference in physiological response depending on the type of fibre and the role of the phenolics is yet to be understood. By investigating the fibres and phenolics in the broccoli leaves, this would highlight the levels of these components in this resource for new food products, and contribute to future interest in utilization of the unharvested broccoli leaves. The results from previous investigations of side streams of broccoli were summarized and published and the licentiate thesis Content of dietary fibre and phenolic compounds in broccoli side streams was defended in 2020. We have evaluated broccoli leaves left in field for their possible use as new healthy ingredients in food products. The results showed that broccoli leaves contain valuable components which can be interesting to be used as food ingredients, and that the connection between fibres and phenolics is important to further elucidate.

We have found that the overexpression of the *AtWRI1* gene in rice can increase the oil content in straw by ca 2 folds compared with wild type, indicating a high potential for biofuel purpose. A manuscript on this is in preparation. We have also found that overexpression of one important gene in Nipponbare rice could significantly increase the resistance to rice blast and planthopper, providing a new potential for reducing usage of fungicides and pesticides (Fig. 4). At least 10 barley varieties with high fructan or high starch have been developed and SNP markers on the traits have also been developed for identifying superior individuals from cross populations for further breeding.

We have proven that genome editing is a useful method to redirect carbon flow by modifying promoters of transcription factors (TFs) and to clarify interactions between TFs in different seed development stages. The work with oil induction in wheat endosperm has been extended with seed X-ray imaging and nutritional evolution. Underground oil regulation has been complemented with transcriptomes and metabolic evaluation of *Cyperus* metabolism. We have enabled the synthesis of 12:1 – 16:1 pheromone precursors, where 14:1 and 16:1 are on levels for commercial application.

Research is now intensified on *Lindera* plant species (oil containing 10:1, 12:1 and 14:1 fatty acids) on finding novel genes and enzymatic to utilize for improved *Camelina* lines (basic research with applied outcome). Further focus is on wax esters which have been developed with the added benefit of carrying fatty alcohols being true pheromone blend constituents. Mating disruption tests for two different major pests have been conducted using pheromones derived from oil of our first *Camelina* line cultivated on

a larger scale. Improved *Camelina* lines with the same target compound have been produced and additionally *Camelina* lines with novel target compounds destined for upscaling and pheromone production. Final experiments with collaborator and later incoming postdoc have been delayed due to COVID-19. One manuscript has been published and three manuscripts are under preparation regarding the results presented above.

For wax ester (WE) production, samples from crosses between 35S:FAR.kana x 35S:PES2.kana have been sent for WE analyses. To simplify selection of double expressors, 35S:FAR.hyg x PES.kana crosses with different selectable markers have been done. Tobacco transformations and crosses were finalised. Some FARxPES crosses displayed an extreme dwarf phenotype, not observed in parents. WE analysis in generated crosses/transformants will be done during 2021.

We have finalized the crambe project and produced valuable transgenic lines with improved oil qualities, which can be further explored for any possibility to perform larger field trials in areas where interest in cultivating GM crops exist through international collaborators. For other oilseed crops, we have put significant efforts in developing protocols for protoplast regeneration of field cress and rapeseed in order to build a strong Swedish base for genome editing of these species in a near future. We have early reported the establishment of a highly efficient protoplast regeneration protocol. We have now established an efficient protoplast-based genome editing method by CRISPR/Cas9 for field cress. Using this method, we have generated mutant lines of field cress (Fig. 5) that have mutations in GTR1 and GTR2 genes for reducing the glucosinolate content in seeds by blocking transport of glucosinolates from

vegetative tissues to seeds. Further molecular, chemical and phenotypical studies on these lines will be done once homozygous lines are available. Significant progress on development of protoplast regeneration protocol for rapeseed has also been achieved. Two manuscripts about these results are in preparation.

Using transcriptomics, bioinformatics and gel-shift analysis, we have narrowed down the number of potential autophagy-regulating transcription factors (TFs) from 2,300 to two main candidates: ANAC046 and ANAC055. The impact of ANAC046 on the expression of AuTophagy-related (ATG) genes has been validated by RT-qPCR. ATG protein level analyses are ongoing. Newly generated ANAC055-overexpressing plants will be employed to explore the role of ANAC055 and its ATG targets in Arabidopsis stress response.

A photoaffinity labelling (PAL) assay for use in planta was developed to identify the protein targets of autophagy enhancers identified through our chemical screen. Through collaboration with the Chemical Biology Consortium of Sweden (CBCS), diazirine photo-activatable probes were added to the molecules. Arabidopsis seedlings were used to develop the PAL assay and samples have been sent for proteomic analysis through support from an EPIC-XS grant (VIB, Belgium).

The current methods for measuring autophagy require laborious sampling of plant material, which do not allow to track dynamics of the pathway. We aim to establish non-invasive methods for quantifying autophagic activity in planta. For this, we engineered two types of reporters: (i) dual-luciferase reporter that shows decrease in luminescence upon upregulation of autophagy; (ii) split-luciferase-based reporter, which luminescence increases proportionally to autophagic activity.



Fig 5. Gene-edited field cress plants with pods and flowers. Photo by Li-Hua Zhu.

We have finalized the hemoglobin project, associated with a SSF project. The expression of the human Mband A1M proteins in the leaves of *Nicotiana benthamiana* have been successful and purified proteins have shown functions similar to the native ones. Two articles about these results have been published, which have attracted attention from food and medical industries. We are now finalizing the work with XTEN attached to fetal hemoglobin to stabilize the protein and a manuscript is in preparation. There is a high potential to use our methodology to express important proteins from various sources for producing plant-based proteins for diverse applications.

In what way the research has contributed to social benefit

The ultimate goal of C4F is to contribute to social benefits in some ways. 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.

The UDI project "Plant Protein Factory" that is an outcome of TC4F ended in 2020. It generated a lot of results that have to be evaluated and several of the partners are interested in a continuation for further development into reality. The Faculty together with the department has the idea for a continuation for the Plant Protein Factory pilot to be stored and run as an academic facility.

The Problöja project on superabsorbents also has results that calls for a transfer to applications which will be searched for. Moreover, the new knowledge obtained on composite materials can be further explored in development of nutritious food (protein rich and dietary fiber rich) with specific structures and making Sweden more self-sufficient and bio-based textile materials. Varying climate impact results on the protein quality can

be further used in wheat breeding programs striving towards breeding of climate resilient crop. Faba beans, oat, peas, rapeseed, potato are Swedish crops with a good nutritional profile that are concerned within the C4F program. They 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 new type of starch with improved product quality is for food and 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 intermolecular interactions are also important for material applications, that will be studied further in this project.

Potential use of side streams of broccoli leaves would contribute to an increased resource efficacy in the broccoli production and better production economy, which would in turn contribute to increasing Sweden's degree of self-sufficiency as more food can be harvested in the same production area.

The new concept of C-flux has generated new cereal lines that can add values for biofuel production for living purpose and reduce dependence on fossil oil, reduce usage of fungicides and pesticides and increase production of yield and quality. Insect pheromones for pest management is non-toxic and produced from a renewable resource contrasting to pesticides will be beneficial to sustainable agriculture.

The general public is already aware of the pivotal role of autophagy in medicine, including its anti-aging and anti-neurodegenerative effects. We strive to convey information about the importance of studying plant autophagy for improving crop fitness and productivity.

Novel transgenic or 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.

Successful expression and characterization of functional heme-binding proteins in plants contribute to social benefits through providing more plant-based proteins for potential nutritional and medicinal applications in future. Our publications in this project have attracted some industrial contacts for exploring potential investment.



Vice program leader Li-Hua Zhu

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 good examples are:

1. Protein fiber textiles
2. Establishment of Plant Protein Factory
3. Protein-rich food
4. SUSAP – Sustainable superabsorbents
5. Plant protein fiber textiles and absorbents
6. Monitoring Cd absorption in wheat
7. Climate stable wheat
8. Initiation of EIP project in broccoli.
9. A single gene can be used to generate biofuel rice for food purpose
10. 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.
11. Understanding the relationship between microstructure of starch, proteins and fibers and their different attributes in food and different industrial applications.
12. Expression and characterization of important human heme-binding proteins in plants for potential nutritional and medicinal purposes.