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Linking the gene regulatory network with the functional physical structure of whole-genome engineered Arabidopsis mutants : an HR-MAS NMR-based metabolomics approach

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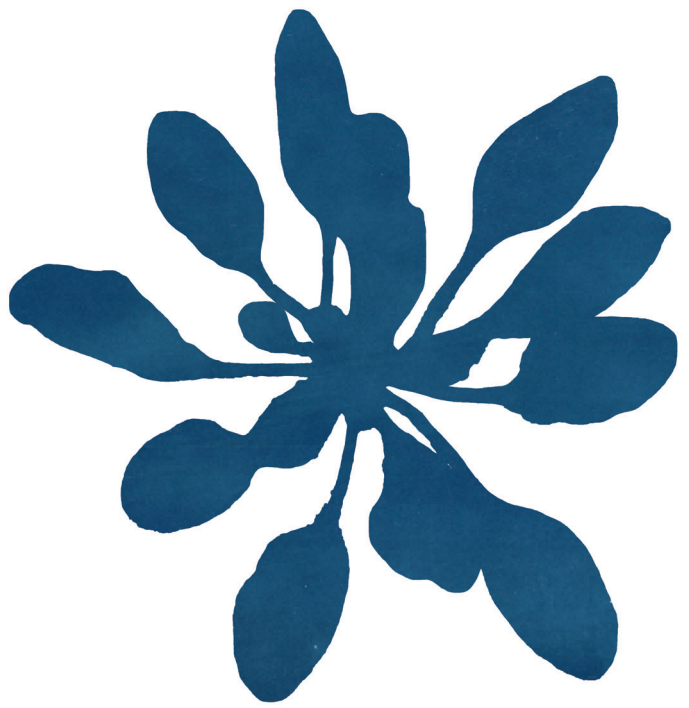
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General discussion and future outlook

To meet the increasing demand for biomass resources for both bioenergy as food, the biomass yield of agriculture products needs to be improved. Agriculture products with increased biomass yield and with a minimal environmental footprint are called smart crops.^[1,2] Smart crops can be developed by genome editing methods.^[3-6] In the PhD thesis, I examined the phenotype of two phenotypically engineered *Arabidopsis thaliana* mutants with increased growth characteristics using a systems biology approach combining omics technologies and bio-imaging methods to understand which pathways are underlying this larger rosette surface phenotype.

5.1 Understanding the phenotype of *Arabidopsis* mutants

A high-resolution magic angle spinning (HR-MAS) NMR-based approach is used to obtain the metabolic profile directly from the leaves of the *Arabidopsis thaliana* plant. The major advantages of HR-MAS NMR over liquid-state NMR is that there is no extraction step necessary which can lead to the loss of signals from non-soluble metabolites. It is also a non-destructive method, which makes it possible to use the sample for other experiments such as transcriptomics analysis.^[7,8] To our knowledge, this is the first time that HR-MAS NMR was applied to resolve metabolic profiles in *Arabidopsis thaliana* leaves, in the native state. Combined with multivariate analysis, HR-MAS NMR-based metabolomics is a powerful tool to investigate the phenotype of plants. It can link the gene regulatory network and the functional physical structure, which is considered as highly complex, using an axiomatic design approach (Figure 5.1A).^[9]

Evolution and development is an interplay between variation at the basis and selection from the higher levels. Metabolic engineering cannot proceed bottom-up only, it needs engineering of a selection criterion to steer the system in the desired direction. Reducing the complexity for highly complicated biological systems gives insight in the system range,

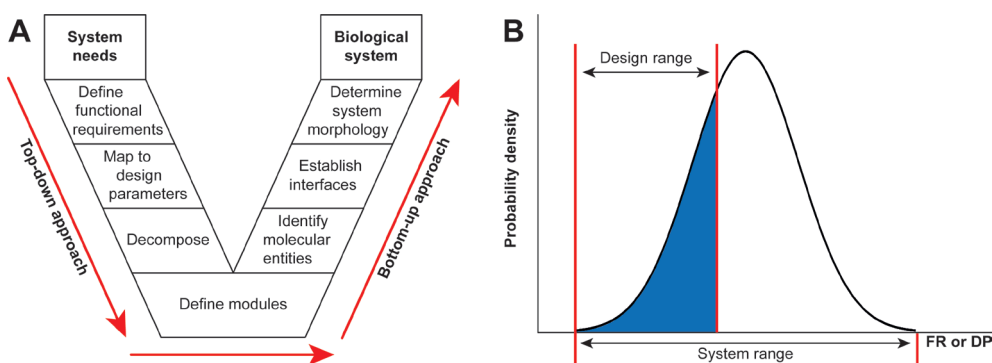


Figure 5.1: V-model can be used to understand the molecular interactions leading to the phenotype of plants. The first step is to define the systems need and to define the functional requirements. The functional requirements are mapped to the design parameters such as molecules, cells etc to decompose phenotype. When the physical modules are clear, they can be put together to have a biologically functioning system by identifying the molecular entities, establish the interfaces and finally to determine the system morphology.^[4] B) The system range represents the different phenotype, which is a probability density function. The design range is the set of phenotypes which can be obtained.^[4]

the range of phenotypes that can be realized by variation at the molecular level, and how functional requirements, such as a proper balance between sources and sinks, determine the specific range of phenotypes that fulfil the requirements of the biological design, the design range imposed by selection from the higher levels in the biological hierarchy (Figure 5.1B).

5.2 *Arabidopsis* mutants with enhanced growth characteristics

In this thesis, I apply the HR-MAS NMR toolbox combined with systems biology to investigate the *Arabidopsis thaliana* mutants with enhanced growth characteristics to understand which pathways are leading to enhanced growth. The results show that the VP16-02-003 and VP16-05-014 mutants have a reduced ability to defend against stresses. The growth-defence trade-off indicates that the energy which is not used for defence is available for enhanced growth.

The disadvantage of this reduced ability to defend against environmental stress is that the plants need to be cultivated in a protected environment. A protected environment can be achieved with controlled-environmental agriculture (CEA) technologies, like greenhouses.^[10] Greenhouses take up a lot of the available space for agriculture. A way to solve this is to make use of vertical farming (Figure 5.2). In vertical farming, plants are cultivated in stacked layers and in an environment where light, temperature and humidity can be regulated.^[10,11]



Figure 5.2: An example of vertical farming with different levels to grow plants (© Adobe Stock).

5.3 *Arabidopsis* Low Chlorophyll Fluorescence 1 mutant

In addition to the studies presented in this thesis, I applied the HR-MAS NMR-based metabolomics workflow to the *Arabidopsis* Low Chlorophyll Fluorescence 1 (LCF1) mutant.^[12] Like the VP16-02-003 and VP16-05-014 mutants, the LCF1 mutant is obtained with artificial zinc-finger genome interrogation. LCF1 has a high operating efficiency of photosystem II (ϕ PSII) and low chlorophyll fluorescence. From an early study, we know that the PSII/PSI ratio is decreased in this mutant relative to Col-0. Also, the rosette surface area and biomass yield are reduced, but the starch content per gram fresh weight is increased compared to the wild-type.^[12] The LCF1 mutant expresses a truncated MORC2 protein which encodes an ATPase of the Microrchidia (MORC) family. This gene is involved in large-scale epigenetic transcriptional regulation. There is converging evidence that the truncated protein forms the basis of the LCF1 phenotype. To better understand the alterations in the pathways leading to the phenotype of this plant, the HR-MAS NMR-based metabolic workflow was applied on the leaves of the LCF1 mutant at seven different time-points throughout the light/dark cycle. The results of this study will be published separately.

Figure 5.3 shows the score plots of the multivariate analysis of the *Arabidopsis* wild-type Col-0 and the LCF1 mutant at $t = 4$ hours into the light period. The three component PCA model explains 84.4% of the separation ($Q^2 = 0.6$). For the OPLS-DA model, the R^2X , R^2Y and Q^2 (cum) were 0.822, 0.952 and 0.733, respectively. Both models have a good quality and an accurate prediction. The sample classes are clustered together in both the PCA and the OPLS-DA score plot.

Both PCA and OPLS-DA models were constructed for every time-point in the light/dark cycle. The OPLS-DA model is used to identify sixteen biomarker candidates and quantified the metabolite levels of these metabolites. This includes the organic acids fumaric acid, malic acid and lactic acid, the sugar fructose, the cofactor NADPH, the organic osmolyte

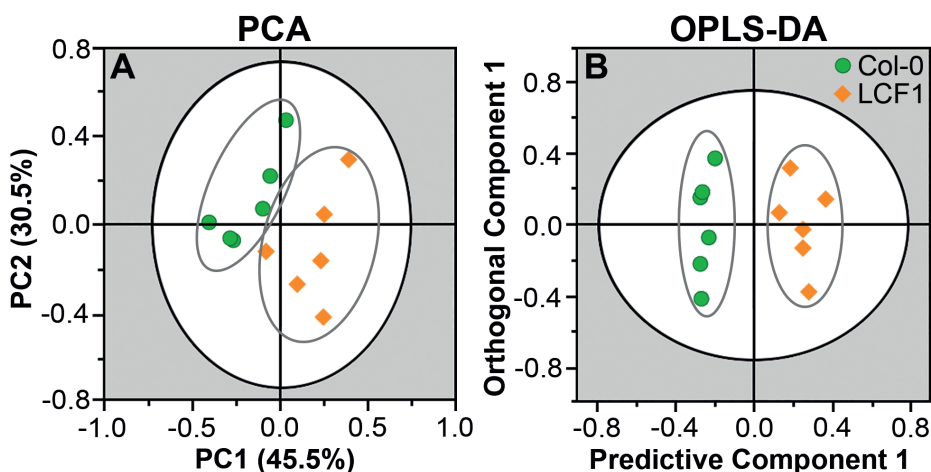


Figure 5.3: The score plots for the PCA (A) and OPLS-DA (B) analysis from the metabolic profiles obtained from the whole *Arabidopsis thaliana* leaves of Col-0 (●) and LCF1 (◆) at $t = 4$ hours into the light period.

betaine, the amino acids L-aspartic acid, L-glutamic acid, L-leucine, L-lysine, L-phenylalanine, L-threonine, L-tyrosine, L-valine and the non-essential amino acid GABA and the non-proteinogenic amino acid ornithine. The next step is to perform literature research and pathway modelling to investigate the function of these biomarkers and relate them to the phenotype of the LCF1 mutant.

5.4 Future outlook

In the future, the HR-MAS NMR-based metabolomics workflow can be used to investigate other *Arabidopsis thaliana* mutants. In addition, it can be applied to smart crops to probe the primary metabolites and selected secondary metabolites. It is also possible to make some improvements in the HR-MAS NMR technology for example by improving the resolution, localized HR-MAS NMR and HR-MAS NMR on living plants. In addition, the results of metabolomics can be supplemented with information about altered metabolic steady-states in the system using fluxomics. Also, the results from the metabolomics can be integrated with other omics technologies in a multi-omics approach.

Crops for bioenergy

In my PhD project, I investigated *Arabidopsis thaliana* mutants obtained using zinc-finger artificial transcription factor (ZF-ATF)-mediated whole genome interrogation and used HR-MAS NMR-based metabolomics to understand the underlying pathways resulting in the phenotypes of the plants. For instance, several plants are interesting to use as biological resources for bioenergy. Traditional cereal crops like maize (*Zea mays*) and sorghum (*Sorghum bicolor*) are interesting for starch-based ethanol production. Also, sugar-producing plants like sugarcane (*Saccharum officinarum*) and sugar beet (*Beta vulgaris*) can be used for direct fermentation of ethanol. But there are also crops dedicated to bioenergy production, such as Switchgrass (*Panicum virgatum*) and woody plants, including hybrid poplar, willow and pines. For biodiesel, soybean (*Glycine max*), canola (*Brassica napus*) and sunflower (*Helianthus annuus*) are interesting crops. Alternative bioenergy plants like grasses, trees and green algae are interesting to investigate using the systems biology approach.^[13]

Improvement of the HR-MAS NMR technology

Among improvements to be made in the HR-MAS NMR technology, an obvious one is to use an NMR spectrometer with a higher magnetic field strength to improve the sensitivity and resolution of the NMR spectra. This can result in the observation of lower abundant metabolites at higher field strength. Soon we will have capabilities to perform HR-MAS NMR at 1.2 GHz, which will improve the range and resolution of the method relative to the current practice in this thesis, where experiments were performed at 400 MHz.^[14]

It also can be interesting to study specific structures of the leaves, such as the veins, lamina or the petiole or other parts of the plant. Recently, Sarou-Kanian *et al.* developed a new method using ¹H HR-MAS slice localized spectroscopy (SLS) and HR-MAS chemical shift imaging (CSI) to determine the distribution of metabolites along the anteroposterior axis of *Drosophila melanogaster*.^[15] Here a MAS probe coupled with a three axes gradient system is used, together with pulse sequences for SLS and CSI. The same technology can be applied for the investigation of plant material.

Fluxomics and multi-omics integration

Metabolomics provides a snapshot of the metabolic status of a sample at a specific time. For most enzymes involved in metabolism, knowledge about the *in vivo* kinetics is necessary to predict metabolic fluxes. Metabolic fluxes are the result of the interplay of gene expression, protein concentration, protein kinetics and regulation, and depend on metabolite concentrations (red arrows in Figure 5.4). Metabolic flux analysis, also called fluxomics, can be used to determine the metabolic reaction rates. Fluxomics can thus help to understand complex metabolic pathways and their regulation for characterisation of the phenotype of the plant.^[16,17] Fluxomics can be done by introducing a ^{13}C -labelled precursor into the metabolic network or by supplying $^{13}\text{CO}_2$ and follow the redistribution of the label into other metabolites by either NMR or mass spectrometry.^[18,19] The redistribution can be followed throughout time during dynamic labelling or after reaching steady-state in a steady-state labelling approach.^[19] In the current fluxomics protocols, an extraction step has to be performed which has the disadvantage of losing components during preparation. It can thus be interesting to develop an HR-MAS NMR-based fluxomics approach which is not available at the moment.

In a multi-omics approach, the results from the various omics technologies, such as genomics, transcriptomics, proteomics, metabolomics and fluxomics, are integrated to unravel the complexity of a biological system (Figure 5.4).^[20-22] A major practical challenge of multi-omics is to handle different data formats and the high data dimensionality property of each data set. To integrate the different information layers, bioinformatics tools are necessary to track the different components for every layer, such as genes, proteins and metabolites at the same time.^[20]

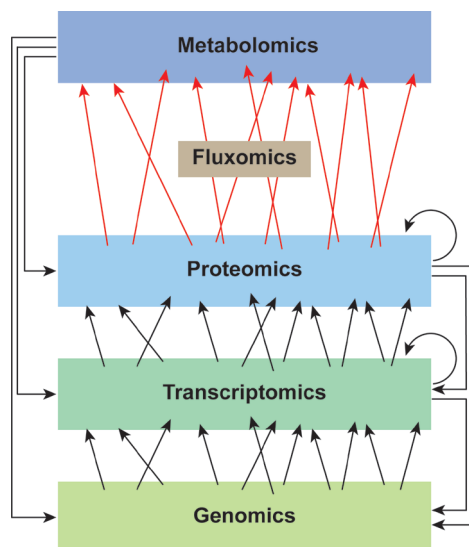


Figure 5.4: The results of genomics, transcriptomics, proteomics and metabolomics are integrated in a multi-omics approach to unravel the complexity of a biological system. In fluxomics (red arrows), the interplay between gene expression, protein concentration, protein kinetics and regulation and the metabolite concentrations are studied.

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