1. Introduction

1.1. Rationale

Increasing world population, shortage of arable land and the resulting growing demand for food, feed and raw materials are major drivers to create plant lines with increased performance, e.g. better resistance to disease and drought (Yan & Kerr, 2002). In addition, plants play a significant role in the developing bioeconomy (Dyer et al., 2008) and emerge as platforms for sustainable production of therapeutics, renewable chemicals and biofuels, purely from sunlight and carbon dioxide (Yan & Kerr, 2002). Such development of plants with specific compositional traits adds impetus to the general interest in enhanced crops (Rajasekaran & Kalaivani, 2013; Saha & Ramachandran, 2013).

Admittedly, the optimization of plant performance using genetic engineering techniques is still lacking systems-level understanding of the effect of genetic modifications (Cusido et al., 2014; Sweetlove et al., 2003). Knowledge-based metabolic engineering approaches are often hampered by the complexity and robustness of plant systems and our still limited understanding of their metabolism (Junker, 2014; Shachar-Hill, 2013). Profound knowledge appears ultimately important to guide metabolic engineers, which becomes immediately clear from the achieved success in breeding superior microorganisms. Meanwhile, industrial microorganisms are optimized on a global scale, through systems metabolic engineering (Ajikumar et al., 2010; Becker et al., 2011; Hwang et al., 2014; Kim et al., 2014a; Kind et al., 2014; Paddon et al., 2013; Poblete-Castro et al., 2013). Particularly, systems metabolic engineering has benefitted from knowledge on metabolic fluxes, i.e. *in vivo* activities of intracellular pathways and reactions, in providing targets for genetic improvement (Kelleher, 2001; Stephanopoulos, 1999). In this regard, the analysis of metabolic fluxes of plant systems promises a huge next step towards

understanding of their metabolic functions and superimposed regulation mechanisms towards superior plant varieties.

In recent years, a powerful collection of flux modeling approaches has been developed to model and simulate stoichiometric metabolic networks of microorganisms. Some methods investigate physiological capabilities by *in silico* analysis of the underlying biochemical conversions, such as elementary flux mode analysis (Schuster et al., 1999; Terzer & Stelling, 2008) and extreme pathway analysis (Papin et al., 2002; Price et al., 2002), whereas others rely on experimental data to deliver necessary constraints, such as ^{13}C-metabolic flux analysis (^{13}C-MFA) (Sauer, 2006; Wittmann, 2007; Young et al., 2011), flux balance analysis (Grafahrend-Belau et al., 2009) and metabolic control analysis (Wang et al., 2004). To engage these methods for plant metabolic flux analysis, the compartmented nature and autotrophic lifestyle of plants, pose specific challenges. However, the emerging state-of-the-art methods and the accumulating information on plant genomes (Michael & Jackson, 2013) now enable systems metabolic engineering concepts to be extended to plant networks for the design of plants with improved performance.

1.2. Objective

The aim of the present work was the advancement of novel *in vivo* and *in silico* flux analysis strategies for future application in plant biotechnology. Hereby, the high degree of complexity and connectivity of plant metabolic networks was a central aspect to be considered. This was achieved by carefully assembling comprehensive genome-based plant networks for the two systems to be studied: *Arabidopsis thaliana* as a model plant and *Oryza sativa ssp. japonica* as agriculturally relevant crop. The *in silico* method of choice for tackling the complex physiology of *A. thaliana* leaves through flux, was elementary flux mode analysis, as it promised the most comprehensive analysis of metabolism. In addition to truly computational modeling of the

created plant network, integrated analysis with experimental data should be evaluated for improved physiological understanding.

Additionally, a comprehensive workflow should be developed for *in vivo* ^{13}C-based metabolic flux analysis of entire plants, including an experimental setup, raw data analysis and flux estimation through parameter fitting. Here, the use of $^{13}CO_2$ as sole carbon substrate, posed specific challenges with regard to tracer application, analytical sensitivity and modeling under isotopically non-stationary conditions. Focus was put on modeling strategies, in particular, ^{13}C-isotopically instationary metabolic flux analysis. Here, the aim was unraveling the metabolic complexity of rice seedlings. The final objective was to apply the developed workflow to gain deeper insights into relevant case-studies, including the prominent herbicide, Imazapyr. Taken together, this work should highlight and expand the potential of plant metabolic modeling in future biotechnological applications.

2. Theoretical Background

2.1. *Arabidopsis thaliana* as important model plant

Although *Arabidopsis thaliana* (thale cress) is often considered a simple weed, this small flowering plant is, without doubt, the most thoroughly studied plant species available. Especially its short generation time, highly reduced genome, regeneration through self-pollination and small stature make *Arabidopsis* an excellent candidate for research in plant biology (Koornneef & Meinke, 2010). Consequently, in the pre-genomics era *A. thaliana* emerged as the pivotal plant species in many biological research fields including physiology, biochemistry, molecular biology and evolution. Furthermore, the early sequencing and annotation of its complete genome (Arabidopsis Genome Initiative, 2000) allowed *A. thaliana* to establish itself in disciplines such as functional genomics, systems biology and biotechnology (Van Norman & Benfey, 2009). Despite its agricultural irrelevance, it has become impossible to imagine plant research without *A. thaliana* and likely, this simple weed will remain a major contributor to future plant research (Fig. 2-1).

Figure 2-1: *Arabidopsis thaliana* in plant research

Left: Plant sampling of *Arabidopsis thaliana* rosette for biotechnological research. Right: Genetically modified *Arabidopsis thaliana* in the flowering stage. Both photos belong to BASF SE, Ludwigshafen, Germany.

2.2. Rice biotechnology - towards genetically superior transgenic rice

2.2.1. *Oryza sativa* as model crop

Oryza sativa (rice) is a model crop with both agricultural and economical relevance (Fig. 2-2). Since the genome of rice was fully sequenced in 2005 (International Rice Genome Sequencing Project, 2005), its appearance in functional genomics, proteomics, systems biology, green biotechnology and crop improvement has rapidly emerged (Coudert et al., 2010; Itoh et al., 2005; Jiang et al., 2012; Kim et al., 2014c; Xu et al., 2005; Yin & Struik, 2008). Pertinent characteristics responsible for this growing interest in *O. sativa* as a model crop, include its large significance to human nutrition (FAO, 2013), the relatively small genome size (Goff, 1999) and straightforward transformation as compared to other grass species (Yan & Jiang, 2007). In addition, there is a considerable genomic homology between rice and other cereal species, which increases the likelihood that rice-specific behavior could lead to elucidation of orthologous functions in other cereals (Goff, 1999).

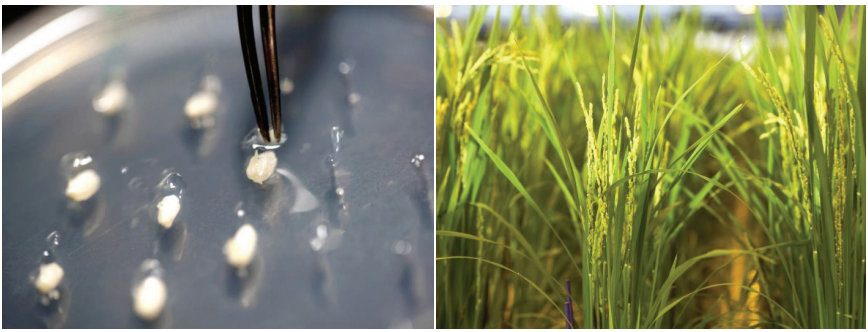

Figure 2-2: *Oryza sativa* in plant research

Left: *Agrobacterium*-mediated genetic transformation of rice seeds in a petri dish. Right: *Oryza sativa* in the greenhouse. Both photos belong to BASF SE, Ludwigshafen, Germany.

2.2.2. Importance of rice in biotechnology

Due to urbanization, soil degradation and climate change, arable land is diminishing fast, whilst the demand for crops rises with the growing world population, diet shift and bio-fuel consumption. Consequently, there is an urgent need to improve crop yields, as we otherwise will not be able to feed the world in the near future. This is especially true for rice, as it is the most important food crop in the world (FAO, 2013). The classical Latin word for rice, 'oryza', and 'sativa', meaning cultivated, provided rice with its species name. *Oryza sativa* has been cultivated for more than 10 000 years and is a monocot angiosperm, i.e. a flowering plant with one seed-leaf (Molina et al., 2011; Sang & Ge, 2007). It further belongs to the family of *Poaceae*, or true grasses, and contains two major subspecies: *japonica* and *indica*. *Japonica* varieties are sticky, short-grained and usually cultivated in dry fields in upland Asia, whereas the *indica* varieties are typically non-sticky, long-grained and mainly lowland rice cultivars, grown mostly submerged throughout tropical Asia (Garris et al., 2005). Together, these two subspecies provide more than one third of the global population with their daily calories.

Technological advances during the green revolution enabled the distribution of pesticides, synthetic fertilizers, irrigation technologies and high-yield varieties acquired through conventional breeding techniques. This already led to a vast increase in productivity between 1960 and 2000. However, since then, yield has not improved significantly (Ray et al., 2013; Zhu et al., 2010). We have come to a time, where traditional breeding techniques have to be supplemented with knowledge from genome analysis, systems biology and plant biotechnology to realize a second green revolution through genetic engineering of crops (Sakamoto & Matsuoka, 2004). Genetic transformation of plants has come a long way since the first transgenic rice plant was generated about 25 years ago (Toriyama et al., 1988; Zhang et al., 1988; Zhang & Wu, 1988). Especially the development of reproducible genetic transformation protocols, either through direct DNA transfer or by *Agrobacterium*-mediated transformation technologies (Zhu et al., 2010), enabled the construction of genetically engineered rice varieties with improved characteristics.

Particularly usefull for current and future genetic manipulation towards desired plant traits is the recent progress on the front of genome editing, which permits site-specific changes to rice DNA through the use of designer nucleases (Li et al., 2015; Petolino, 2015; Yu et al., 2015).

Despite the substantial increase in crop protection during the green revolution, crop loss, caused by various biotic and abiotic factors, is still eminent. The introduction of agronomically useful genes in rice, could significantly reduce these losses. Examples include resistance to drought (Jeong et al., 2010; Joo et al., 2014; Qian et al., 2015), salinity (Campo et al., 2014; Ghosh et al., 2014; Sahoo et al., 2014), extreme temperatures (Li et al., 2013; Qin et al., 2015; Yang et al., 2013), oxidative stress (Kim et al., 2014b; Lee et al., 2013; Park et al., 2013), mineral deficiency (Takahashi, 2003), insect predation (Qi et al., 2009; Quilis et al., 2014), fungal infestation (Chujo et al., 2014; Qian et al., 2014), viral invasion (Ma et al., 2011; Sasaya et al., 2014; Shimizu et al., 2013) and bacterial infection (Goto et al., 2014; Lu et al., 2014). One prominent example is the introduction of endotoxin-producing genes from *Bacillus thuringiensis* into rice, as it offers protection against lepidopteran pests, which are responsible for 2–10 % of the total rice yield loss in Asia (High et al., 2004). Without a doubt, this list is not comprehensive and merely provides a glimpse on the variety of stress-resistance genes recently engineered into rice.

Furthermore, nutritional improvement of rice can help reduce malnutrition, as it is currently the staple food in most developing countries (Bajaj & Mohanty, 2005; Bhullar & Gruissem, 2013). The most prominent example hereof is the bio-fortification of rice with provitamin A, giving the grains a golden color, hence golden rice (Bhullar & Gruissem, 2013). Vitamin A deficiency is an important cause of eye-defects, leading to permanent blindness when untreated. In developing countries around 250 million pre-school children and a substantial proportion of pregnant women are estimated to suffer from severe vitamin A deficiency (WHO, 2015). Even prior to blindness, vitamin A-deficiency increases child mortality as a result of enhanced susceptibility to measles, diarrhea, and malaria (UNICEF, 2009). Other examples of nutritionally enriched rice varieties

include bio-fortification with folate (Storozhenko et al., 2007), iron (Masuda et al., 2012; Wirth et al., 2009), zinc (Johnson et al., 2011), essential amino acids (Lee et al., 2001; Lee et al., 2003; Long et al., 2013; Wakasa et al., 2006) and improved oil quality (Anai et al., 2003). Additionally, transgenic rice can be a production platform for heterologous proteins that can be applied as edible vaccines (Suzuki et al., 2011; Yang et al., 2012; Zhang et al., 2009) and medicine (Xie et al., 2008).

Despite the many successes in developing transgenic rice with improved nutritional content or resistance to both biotic and abiotic stress, a ceiling in yield improvement has been reached. Therefore, additional strategies towards higher yields, such as maximizing the conversion efficiency of CO_2 and light into biomass, are desperately needed (de Bossoreille de Ribou et al., 2013). Recent efforts towards developing such transgenic lines have mainly focused on photosynthetic efficiency by improving transient pools of sink starch (Gibson et al., 2011; Smidansky et al., 2003), introducing the C_4 photosynthetic machinery and the cyanobacterial CO_2 concentrating mechanism into rice (Price et al., 2013; von Caemmerer et al., 2012), as well as manipulating single photosynthetic functions such as RuBisCO (Lin et al., 2014; Parry et al., 2013) and sedoheptulose 1,7-bisphosphatase (Zhu et al., 2010). However, there is limited knowledge about the carbon conversion efficiency in rice, or any other crop (Alonso et al., 2007; Goffman et al., 2005), so that redirection of metabolic carbon flow to achieve higher yields has not been successful yet (de Bossoreille de Ribou et al., 2013). Overall, it can be concluded that recent strategies to improve yield have been hampered by lacking knowledge on the systems-wide physiology of rice (Long, 2014; Yamamoto et al., 2009). Therefore, improving the systems-level understanding of plants will form a stepping stone towards the development of high-yield crops (de Bossoreille de Ribou et al., 2013).

2.3. Plant metabolism

2.3.1. Spatial separation and temporal shift in carbon and energy acquisition

The primary site for assimilation of atmospheric CO_2 and energy-gain from light through photosynthesis, is the plant leaf. From here, photosynthetic assimilates, mainly sucrose and some amino acids, are transported to the roots and actively growing plant parts, where they function both as carbon and energy-source (Fischer et al., 1998; Lalonde et al., 2004). To fulfill their task with maximum efficiency, leaves are organized into specialized tissues and cell types, each playing a distinct physiological role (Fig. 2-3). These various cell-types exhibit a highly conserved intracellular organization. Like for all eukaryotes, their DNA is stored in the nucleus, which is surrounded by the cytosol. Next to the vacuole, the cytosol houses specialized organelles, such as microbodies, chloroplasts and mitochondria (Taiz & Zeiger, 2006). This high level of intracellular organization is also reflected in the allocation of specific metabolic functions to distinct organelles, e.g. chloroplasts and mitochondria are the energy-producing sites of the cell, responsible for photosynthesis and respiration, respectively (Araújo et al., 2014; Weber & Linka, 2011), whereas, peroxisomes, a type of microbody, neutralize highly toxic hydrogen peroxide and participate in photorespiration (Hu et al., 2012).

During the day, the assimilation of carbon fuels leaf metabolism and allows the accumulation of starch as well as the export of sucrose to non-photosynthetic tissues. Since plants have to resort to an alternative energy source at night, the stored transitory starch is degraded to maintain leaf metabolism in the dark. The dramatic metabolic shift from photoautotrophy during the day, to a heterotrophic life style at night in photosynthetic organs, is not apparent in heterotrophic plant tissues, as they are continuously provided with sucrose, delivering both carbon and energy to the cells. Both these temporal features and the previously discussed spatial organization should be considered in systems-wide plant investigations, as whole plant physiology is a tight cooperation between its organs, tissues, cells and organelles throughout the day-night cycle.

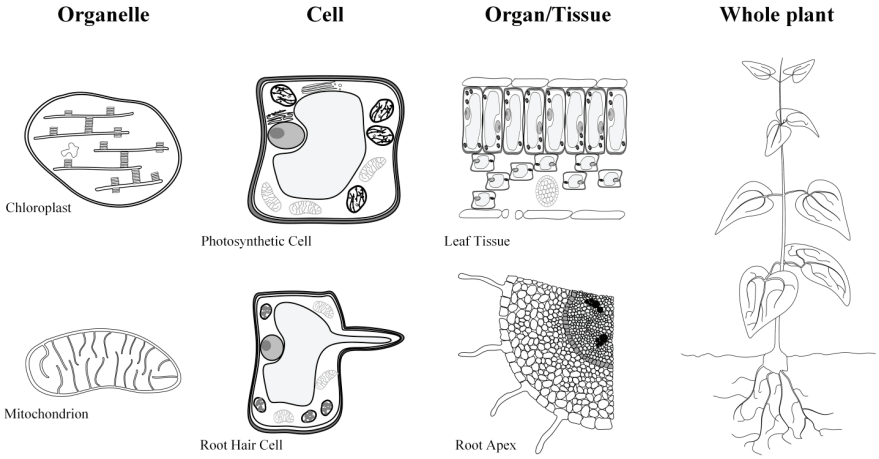

Figure 2-3: Organizational layers of plant anatomy

Whole plants are composed of different tissues, e.g. leaf tissue and root apex, which in turn consist of distinct cell types. Photosynthetic cells are characterized by large vacuoles and the presence of many chloroplasts and mitochondria, whereas root hair cells have 'hair-like' outgrowths and can, next to a vacuole and nucleus, contain amyloplasts and mitochondria. Specific metabolic functions are allocated to distinct organelles, such that whole plant physiology is a tight cooperation between its organs, tissues, cells and organelles throughout the day-night cycle. [1]

2.3.2. Cellular metabolism of photosynthetic C_3-leaf

At the heart of the cellular metabolism of photosynthetic leaf tissue is the Calvin–Benson–Bassham (CBB) cycle, also known as reductive pentose phosphate cycle or C_3-cycle (Fig. 2-4). The CBB cycle starts with the carboxylation of ribulose 1,5-bisphosphate by RuBisCO, yielding two molecules 3-phosphoglycerate. These are subsequently reduced in a two-step process to build glyceraldehyde 3-phosphate. The third phase of the cycle is responsible for the regeneration of ribulose 5-phosphate from glyceraldehyde 3-phosphate through several enzymatic steps, which are strongly connected to the non-oxidative pentose phosphate pathway. Typically, one molecule 3-phosphoglycerate is gained for every three successions. As the CBB

[1] This figure and all components hereof are hand-drawn by the author of this work.