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Climate-friendly rice - characterisation of rice varieties associated with low-methane emissions and improved bioenergy properties

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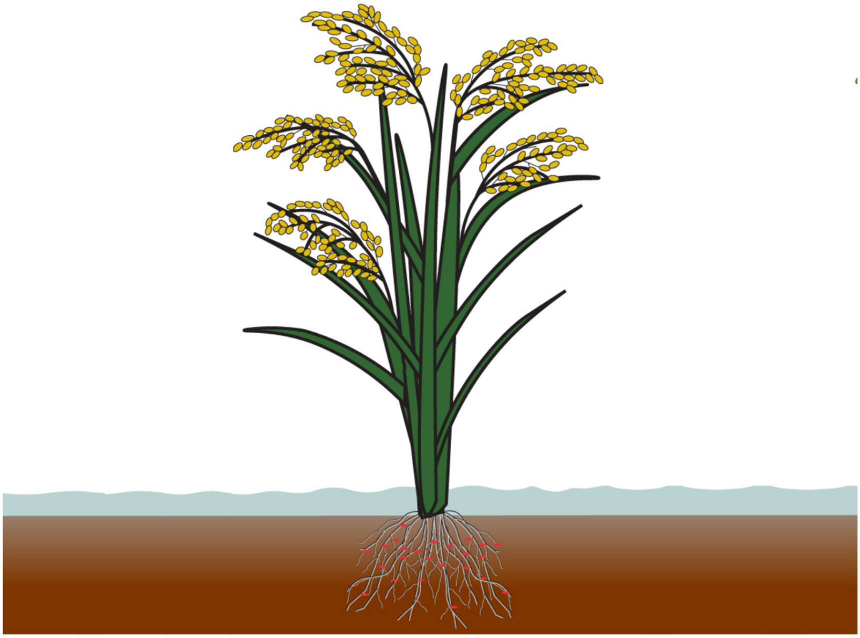

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Cover: Image of rice plant in paddies
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Abstract

Paddy rice cultivation contributes to global warming by emitting methane into the atmosphere. The aims of the work presented in this thesis were to breed climate-friendly rice varieties, to increase rice straw utilisation to reduce the impact of rice cultivation on the environment. The results showed that fumarate and ethanol were the two major exudate compounds from SUSIBA2-rice roots regulating methane emissions from SUSIBA2-rice paddies. A naturally occurring low-methane rice variety was identified and field testing demonstrated that it could be successfully grown in Uppsala, Sweden, opening up possibilities for expanding rice cultivation into high-latitude countries. Key candidate genes important in adaptation of rice to Scandinavian light and climate conditions were identified. By crossing a naturally occurring low-methane rice variety with four high-yielding elite varieties, four independent lines of low-methane rice with high yield potential were developed. Field trials demonstrated that methane emissions from the selected lines were reduced by 70%. Analysis of carbon allocation and molecular analyses of gene activities revealed that sugar transporters such as SUT and SWEET participated in allocation of carbon to aboveground tissues, contributing to the high yield and low-methane properties. The results also indicated that increasing the lipid content in rice straw could increase its value in bioenergy production. Overall, this thesis shows that plant breeding can successfully address several pressing issues affecting sustainability in rice production by reducing greenhouse gas emissions, increasing crop productivity and diversity, and improving bioenergy production from straw.

Keywords: Methane emission, Rice yield, High latitude, Carbon allocation, Root exudates, Rice bioenergy, Sustainable agriculture

Abstrakt

Odling av ris bidrar till utsläpp av växthusgasen metan eftersom risplantor avger kolföreningar från rötterna som tas upp av metanproducerande bakterier. Syftet med denna avhandling var att ta fram klimatvänliga rissorter för att minska risfältens negativa miljöpåverkan, förbättra produktiviteten, samt öka möjligheten att använda sidoströmmar från strå och halm. I ett av avhandlingens delarbeten studerades de kolföreningar som den transgena rissorten SUSIBA2 utsöndrade från rötterna. Undersökningarna visade att förhållandet mellan mängden fumarat och etanol som utsöndrades påverkade avgången av metan. Dessutom undersöktes om utsläppen av metan från risodlingar är sortberoende. Mätningar genomförda på ett stort antal linjer och lokala sorter visade att rissorten Heijing5, som odlas i norra delen av Kina, gav låga metanutsläpp. Detta bekräftades genom fältförsök utförda i Uppsala, och i Heilongjiang provinsen i Kina. Försöken visade att Hejing5 är ett låg-metanris, samt att sorten går utmärkt att odla under svenska klimat och ljusförhållanden. I en storskalig transkriptomanalys påvisades att gener kopplad till tidig blomning och köldstress var differentiellt uttryckta i Heijing5 jämfört med kontrollplantor. Det är möjligt att regleringen av dessa kandidat gener är viktig för anpassningen av Heijing5 till svenska odlingsförhållanden. Från korsningar av Heijing5 och fyra högavkastande rissorter togs därefter fyra oberoende linjer fram som i fältförsök visade sig vara både högavkastande och associerade med reducerade utsläpp av metan. I genomsnitt var metanutsläppen 70% lägre i de framtagna linjerna jämfört med moderlinjerna, samtidigt var fenotypiska karaktärer kopplade till skördenivån, tillexempel frövikt, jämförbara eller högre. I en analys av vilka gener som är aktiva i de framtagna linjerna kunde vi påvisa att gener som påverkar transport av kolhydrater inom en växt var uppreglerade. Analys av fördelningen av olika sockerarter och den totala mängden kol i plantorna visade att de ackumulerade en större andel kol till de gröna delarna av plantan jämfört med föräldralinjerna. Sammantaget visade resultaten att de fyra linjerna har en hög avkastningspotential och är associerade med låga metanutsläpp på grund av att en större andel kol allokerades till stam, blad och frö än till roten. Avhandlingens avslutande delarbete visar på möjligheten att öka energivärdet i risplantornas strå genom att uttrycka en transgen (WRI1) som påverkar sammansättningen av lipider i denna vävnad. Högre energivärden är en efterfrågad egenskap om man vill använda restprodukter från risodlingar tillbioenergi. Sammanfattningsvis visar denna avhandling på möjligheterna att med hjälp av modern växtförädling utveckla en hållbar risproduktion som ger oss möjlighet att minska på avgången av växthusgaser, öka produktionen och anpassa risproduktionen till en framtida bioekonomi.

Keywords: Methane emission, Rice yield, High latitude, Carbon allocation, Root exudates, Rice bioenergy, Sustainable agriculture

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Yunkai Jin, Tong Liu, Jia Hu, Kai Sun, Lihong Xue, Mathilde Bettembourg, Pengfu Hou, Peiying Hao, Jintian Tang, Zihong Ye, Chunlin Liu, Peng Li, Aihu Pan, Lushui Weng, Guoying Xiao, Ali A Moazzami, Xiaoping Yu, & Anna Schnürer, & Chuanxin Sun.** (2023). Eco-friendly rice for mitigation of greenhouse gas emissions (submitted)

II. **Jia Hu, Mathilde Bettembourg, Silvana Moreno, Ai Zhang, Anna Schnürer, Chuanxin Sun, Jens Sundström, & Yunkai Jin.** (2023). Characterization of a low methane emissions rice cultivar suitable for cultivation in high-latitude light and temperature conditions (submitted)

III. **Jia Hu, Mathilde Bettembourg, Lihong Xue, Ronggui Hu, Anna Schnürer, Chuanxin Sun, & Yunkai Jin, & Jens Sundström.** (2023). Achieving a high-yield low-methane rice via optimising carbon partitioning (manuscript)

IV. **Yunkai Jin, Jia Hu, Jun Su, Selcuk Aslan, Yan Lin, Lu Jin, Simon Isaksson, Chunlin Liu, Feng Wang, Anna Schnürer, Folke Sitbon, & Per Hofvander, & Chuanxin Sun. (2023).** Improved bioenergy value of residual rice straw by increased lipid levels from up-regulation of fatty acid biosynthesis (submitted)

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1. Introduction

1.1 Rice

Rice (Oryza sativa) is an important staple food for a large proportion of the human population (Khush, 2005; Alexandratos & Bruinsma, 2012). It is the second most consumed cereal after wheat, contributing 80% of the calorie requirements of more than half the global population (FAO, 2008). In 2020, paddy rice production volume worldwide was approximately 756.7 million metric tons, and the primary producers were China, India, Bangladesh, Indonesia and Vietnam, which host 72% of the global production (FAOSTAT, 2020).

The geographical distribution of rice-growing areas worldwide shows that rice can be cultivated from 35°S (Uruguay and New South Wales in Australia) to 50°N (China) (Grist & Lever, 1969). Rice can be cultivated in relatively varying temperature conditions (Xiao *et al.*, 2014; Song *et al.*, 2022), but, the optimal growth temperature for rice is 25-35 °C. Temperatures below or above the optimal temperature have a negative effect on growth, plant physiology, and crop yield. Particular, temperatures above 35 °C restrict the development of roots and shoots, cause poor anther dehiscence and lead to spikelet sterility (Krishnan *et al.*, 2011).

Approximately 40,000 rice varieties have been cultivated worldwide (www.riceassociation.org.uk/type-of-rice). These varieties differ in, *e.g.* panicle length, grain length, grain colour, grain stickiness, plant height, number of tiller, tiller angle and biotic/abiotic resistance. The current scientific consensus is that farmers started planting wild *Oryza rufipogon* populations in the Yangtze River basin in China 13,500 to 8,200 years ago, with that species later evolving into *Oryza. sativa* (Normile, 1997; Vaughan *et al.*, 2008; Huang *et al.*, 2012). Subsequently, migration and trade spread the cultivated *Oryza*. *sativa* worldwide. In parallel, rice was domesticated on the African continent about 3000 years ago, in the Niger river delta, leading to the evolution of *Oryza. glaberrima* (Choi *et al.*, 2019). Nowadays, different rice cultivars are grown in more than 100 countries (Laborte *et al.*, 2017) (Fig. 1). Demand for rice as food is still growing, primarily driven by increases in the global population. Demand is predicted to increase from 7.8 billion tonnes in 2020 to 9.9 billion tonnes by 2050 (Chen *et al.*, 2014). Thus, increasing rice production is essential to maintain food security. At the same time, mitigating the adverse environmental effects of increased rice production, *i.e.* land use, water use, and greenhouse gas emissions, remains a challenge for farmers and the scientific community.

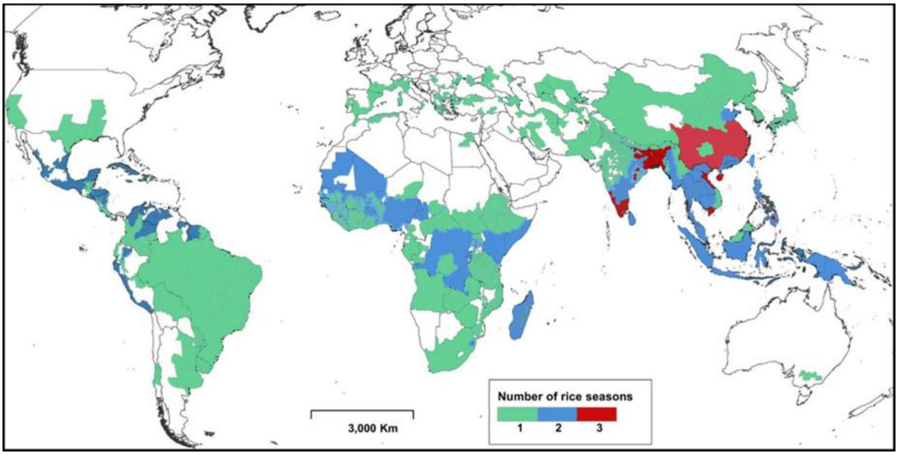

Figure 1. Rice cultivation areas worldwide and number of growing seasons. Adapted from Laborte *et al.*, 2017

1.2 Methane emissions from rice paddies

Since the pre-Industrial period, human activities are estimated to have increased Earth’s global average temperature by about 1°C (NASA, 2022). With current emission rates, mean surface temperatures are expected to reach 2°C by the middle of this century and increase to 2.1-3.5 °C by 2100 (IPCC, 2021). Global warming and climate change pose a severe threat to life on earth through increased average temperatures and frequency of extreme weather events, such as heat waves, droughts and floods. At the global scale, the key greenhouse gases emitted by human activities are carbon dioxide, methane, nitrous oxide, and fluorinated gases, which account for 76%, 16%, 6% and 2% of the total, respectively (IPCC, 2014). Among the different human activities, agriculture, forestry and other land use emit 24% of the total greenhouse gases. Agriculture, an essential human activity, is estimated to account for roughly 70% of total anthropogenic nitrous oxide and 50% of total anthropogenic methane emissions (Ozlu *et al.*, 2022). Thus, agricultural activities have been identified as a significant driver of climate change (Montzka *et al.*, 2011; Vermeulen *et al.*, 2012).

Methane is the second most important greenhouse gas after carbon dioxide, and has a climate impact that is 25 times greater than that of carbon dioxide (EPA, 2020). Globally, 45-80% of total methane emissions come from human activities (Liu & Whitman, 2008) (Table 1), mainly rice cultivation, livestock farming, coal burning and biomass combustion. Methane is also emitted from several natural sources, of which wetlands are the largest source accounting for 15-40% of total methane emissions (Table 1).

Table 1. Source of atmospheric methane. Adapted from Liu & Whitman, 2008

Sources	**Methane emissions (Tg methane per year)**	**Percentage (%)**
Natural sources		
Wetlands	92-237	15-40
Ocean	10-15	2-3
Methane hydrates	5-10	1-2
Anthropogenic sources		
Ruminants	80-115	13-19
Energy generation	75-110	13-18
Rice agriculture	25-100	7-17
Landfills	35-73	6-12
Biomass burning	23-55	4-9
Water treatment	14-25	2-4

Methane emissions have risen 10-fold in the past decade due to increased agricultural activity (Saunois *et al.*, 2020), and methane emissions from rice paddies account for 7-17% of the methane emitted from anthropogenic sources (IPCC, 2013). The warm, waterlogged soils and anaerobic conditions in rice paddies provide an ideal habitat for methane-producing archaea. It has been estimated that production of 1 kg of rice results in emission of 100 g of methane into the atmosphere (Davamani *et al.*, 2020). The process of methane production from rice paddies consists of two steps: 1) complex organic compounds (polysaccharides, proteins, organic acids and neutral fats) are degraded to carbon dioxide, hydrogen and acetate by hydrolytic and acidogenic bacteria; and 2) carbon dioxide, hydrogen and acetate are reduced to methane by methanogenic archaea (Neue *et al.*, 1996; Thauer *et al.*, 2008). Up to 90% of the methane produced is transported and diffuses from the soil to the atmosphere through the rice aerenchyma, while the remainder is released through ebullition or oxidised by methanotrophs (Philippot & Hallin, 2011; Rajkishore *et al.*, 2015) (Fig. 2).

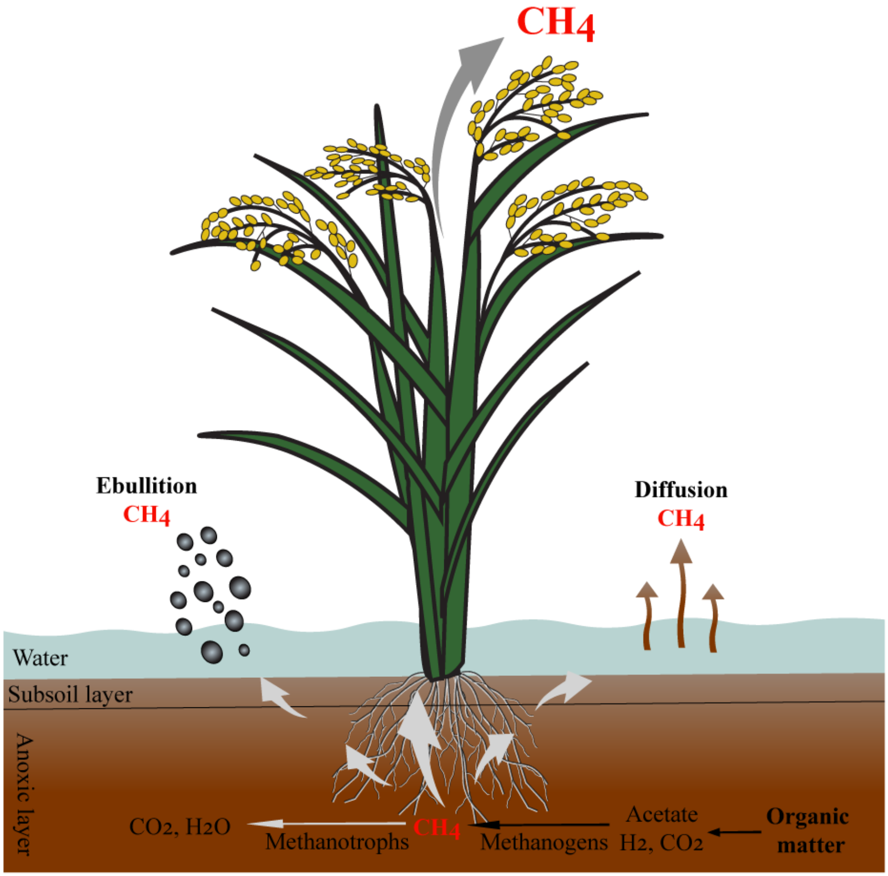

Figure 2. Production, transportation and consumption of methane in the rice rhizosphere. Adapted from Rajkishore *et al*., 2015

In anoxic paddies, methanogenic archaea are the predominant microorganism within the microbial community (Liesack *et al.*, 2000). Based on the substrate and metabolic pathways of methane production, methanogens can be divided into i) acetoclastic, ii) hydrogenotrophic, and iii) methylotrophic types (Lyu *et al.*, 2018). Acetoclastic methanogens dominate in rice soils and comprise two families, Methanosarcinaceae and Methanosaetaceae, which can convert acetate to methane and carbon dioxide (Conrad, 1999; Lueders *et al.*, 2001; Watanabe *et al.*, 2006; Conrad *et al.*, 2012). Hydrogenotrophic methanogens belonging to the orders Methanomicrobiales, Methanobacteriales and Methanocellales dominant in rice root. These Hydrogenotrophic methanogens use hydrogen or formate to reduce carbon dioxide to methane (Grosskopf *et al.*, 1998; Chin *et al.*, 2004; Hashimoto-Yasuda *et al.*, 2005). Methylotrophic methanogens are more common in marine and hypersaline, sulphate-rich sediments, and their contribution to global methane emissions is very small (Lyu *et al.*, 2018).

The carbon sources for methane emissions from rice paddies derived from rice straw and green manure, soil organic matter, and small carbon molecules produced directly by plant photosynthesis. These small carbon molecules account for 70% of methane emissions, predominantly produced after the vegetative stage (Watanabe *et al.*, 1999; Khosa *et al.*, 2010). The composition and amount of carbon compounds secreted from the plant root usually determine the abundance of methanogens, which is further on linked to the methane emissions (King & Reeburgh, 2002; Lu & Conrad, 2005). Many efforts have been made to reduce methane emissions from rice paddies, including 1) oxidising methane to carbon dioxide, 2) supplying competitive substrate, 3) applying biochar-based fertiliser, 4) field drainage and 5) carbon re-allocation.

Oxidising methane to carbon dioxide. The methane produced under anaerobic conditions in flooded paddy soils is partly oxidized in the relative oxygen-rich microzone by aerobic methanotrophs (Groot *et al.*, 2003; Knief, 2015; Strong *et al.*, 2015). The efficiency of these methanotrophs are determined by the physicochemical properties of the soil, soil fauna, vegetation, and climate conditions. It has been found that applying methanotrophs to soil (at a rate of 6.25 kg ha^{-1}) could minimise methane emissions by 60% compared with untreated soil (Davamani *et al.*, 2020). However, the increase in methanotroph activity will result in more carbon dioxide being emitted into the atmosphere from rice paddies, and the consequences for the environment of field application of methanotrophs are unknown.

Supplying competitive substrate. Ferric iron is the terminal electron acceptor for many microorganisms under anaerobic conditions (Banfield *et al*., 2005; Lentini *et al*., 2012). Blast furnace slag, which is iron-rich, has been tested as a silicate fertiliser to reduce methane emissions from rice paddies (Lim *et al*., 2022). The presence of ferric iron results in a shift in electron flow from methanogenesis to ferric iron reduction, which can suppress methane emissions from flooded rice paddies (Frenzel *et al*., 1999). However, the long-term effects of silicate fertiliser application are still unknown. Application of sulphate-containing fertilisers (*e.g*. ammonium sulphate) has also been proposed for methane emissions mitigation in rice paddies (Linquist *et al*., 2012). Since sulphate-reducing bacteria use the same substrate, they compete with methanogens and they also have a high affinity for acetate and rapid growth rate (Goevert & Conrad, 2008; Sela-Adler *et al.*, 2017).

Applying biochar-based fertiliser. Biochar can reduce methane emissions by increasing the biodiversity and abundance of methanotrophic bacteria (Liu *et al.*, 2011; Feng *et al.*, 2012; Qin *et al.*, 2016). However, this effect is gradually lost with biochar ageing, since methanotrophic abundance changes over time following a single biochar application (Wang *et al.*, 2019). The effect of biochar application on methane emissions also depends on the concentration applied. For example, one study found that methane emissions were not affected by addition of 10 Mg ha^{-1} biochar to soil, but when the dose was increased to 40 Mg ha^{-1}, methane emissions were significantly decreased (Qi *et al.*, 2021).

Field drainage. Irrigation-drainage involving a single or multiple drying periods are reported to be an effective management approach to reduce methane emissions, by creating temporary oxidising conditions in the soil (Carrijo *et al.*, 2017; Martinez-Eixarch *et al.*, 2022). An increase in oxygen concentration in paddies improves methane oxidisation by aerobic methanotrophs. It has also been reported that early-season drainage can oxidise the carbon from rice straw leading to a prolonged reduction in methane emissions (Tariq *et al*., 2018).

Carbon re-allocation. Around 20-60% of the carbon in rice plants is allocated to the roots and a substantial portion of this carbon is secreted to the soil through the roots (Marschner, 1995; King & Reeburgh, 2002; Liu *et al*., 2019), in the form of organic exudates (Canarini *et al*., 2019). Exudations rate is lowest at the seedling stage and increases until flowering, but decreases at maturity (Aulakh *et al*., 2001a). In anoxic rice paddies, methanogenic archaea mainly utilise acetate as an energy and carbon source. (King & Reeburgh, 2002; Lu & Conrad, 2005), and hence methane is the final product of anaerobic degradation of organic matter (Liesack *et al*., 2000). There are strong indications that the carbon compounds secreted by rice roots play a vital role in methane production, with high-yielding rice cultivars showing lower root exudation than low-yielding cultivars (Aulakh *et al*., 2001b). In line with this, it has been demonstrated that removing spikelets to reduce the capacity of the plant to store photosynthetically fixed carbon in grains can lead to increased methane emissions (Denier Van Der Gon *et al*., 2002). A later study confirmed the correlation between high starch aboveground and low methane emissions by overexpressing the SUSIBA2 gene from barley (*HvSUSIBA2*) in rice (Su *et al*., 2015). The ectopic expression of *HvSUSIBA2* in rice in that study drove carbon partitioning to the grain, which resulted in reduced belowground biomass. This transgenic rice showed a 90% reduction in methane emissions from rice paddies compared with control plants (Su *et al*., 2015). In conclusion, root exudates are important to methanogens and reducing root exudation can be considered the most viable strategy for reducing methane emissions (Maurer *et al*., 2018). However, the compounds in root exudates that affect methane emissions from rice paddies are still unknown.

1.3 Plant carbon metabolism

1.3.1 Carbohydrate metabolism and allocation

Carbon is an indispensable element for plant growth and is required in large quantities for crop production. Plants primarily obtain carbon through photosynthesis. Photosynthates are partitioned by different loading processes from source tissues (leaves) to different sink tissues, such as stems, roots and grains (Foyer & Paul, 2001), thus supporting their growth and development. Plant-assimilated carbon in rice is first partitioned into the shoots and roots to maintain plant growth in the seedling stage, but as the plant ages more carbon is transported to the harvested parts, mainly to the grains (Kuzyakov & Gavrichkova, 2010; Zang *et al.*, 2019). The amount of carbon sources in roots or stems determines the rate of aboveground and belowground growth. Carbohydrate import into grain directly determines grain size and yield, while carbohydrate import into roots enhances root elongation and increases root exudation into the soil as rhizodeposits (Sosso *et al.*, 2015; Wu *et al.*, 2018). Most carbon sources in the root are transported from aboveground tissues through the phloem to the stem in the form of sucrose (Wang *et al.*, 2017).

Sucrose is a sugar composed of glucose and fructose subunits, and is synthesised from the precursors UDP-glucose and fructose 6-phosphate (Sun *et al.*, 2011; Ruan, 2014). Sucrose synthesis is catalysed by the enzyme sucrose-6-phosphate and occurs primarily in photosynthetic organs, *i.e.* leaves. As the primary carbohydrate is transported long distances in many plant species, sucrose is first loaded into the phloem and then transported distally to the sink tissues (Slewinski & Braun, 2010; Julius *et al.*, 2017). During vegetative growth, stems and roots serve as the primary storage organs of carbohydrates in crop plants. Sucrose is transported into the root and the stem and converted to different non-structural carbohydrates, such as glucose, fructose and starch. After flowering, sucrose is mainly transported into the reproductive organs. In rice, sucrose transport from photosynthetic cells, *e.g.* mesophyll cells, to the apoplast is driven by SWEET efflux proteins, while long-distance sucrose transport via the phloem depends on the combined activity of SWEET and SUT transporters (Braun, 2012; Chen *et al.*, 2012; Julius *et al.*, 2017) (Fig. 3). SUTs are H^+/sucrose symporters that utilise the proton motive force acting across the plasma membrane to load sucrose against its concentration gradient into the phloem (Carpaneto *et al.*, 2005; Reinders *et al.*, 2012). In contrast, members of the SWEET transporter family act as passive uniporters that efflux sucrose and/or hexoses down their concentration gradient across a cell membrane (Braun, 2012; Chen *et al.*, 2015; Latorraca *et al.*, 2017; Jeena *et al.*, 2019) (Fig. 3).

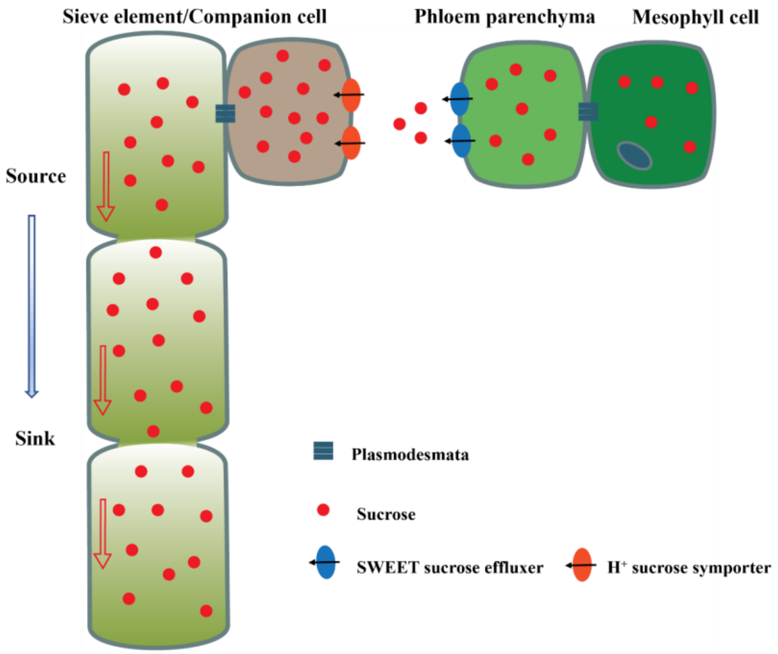

Figure 3. Sugar transport in the plant. Adapted from Jeena *et al*., 2019

Glucose is the most abundant monosaccharide in rice, serving as the primary carbon and energy source for most metabolic processes. It has been demonstrated that more than 2000 genes in plants are regulated by glucose (Sheen, 1990; Jang & Sheen, 1994; Villadsen & Smith, 2004; Price *et al.*, 2004; Xiong *et al.*, 2013). In plant and microorganism interactions, glucose, not sucrose, is the primary carbon energy source transferred (Sutton *et al.*, 1999). This is possibly because glucose is taken up immediately by microorganisms, while sucrose and fructose require time to be metabolised as glucose is active across the cell membrane (Mendgen & Nass, 1988).

Starch is the principal storage carbohydrate and is composed of amylose or amylopectin glucan polymers (Sabelli & Larkins, 2009). The process of starch biosynthesis in crops requires the cooperation of various starch biosynthetic enzymes and is coordinated with other parts of the cell metabolism. Initially, glucose 1-phosphate is converted to ADP-glucose through the action of ADP-glucose pyrophosphorylase (AGPase), which contains a large AGPase unit and a small AGPase unit. Different proteins synthesise amylose and amylopectin. Amylose is synthesised by granule-bound starch synthase (GBSS), while amylopectin is synthesised by the coordinated activity of soluble starch synthase (SS), starch branching enzyme (SBE) and isoamylase (ISA) (Preiss, 2018) (Figure 4). In crops, *e.g.* rice and wheat, at least two types of starch are synthesised, including transitory starch and storage starch. Transitory starch is usually found in the photosynthetic organs, *e.g.* leaves and stems (Stitt & Zeeman, 2012). Storage starch is produced in the amyloplasts of non-photosynthetic sink tissues, *e.g.* rice endosperm, which requires the supply of sucrose and ATP from the source organs (Li *et al.*, 2021).

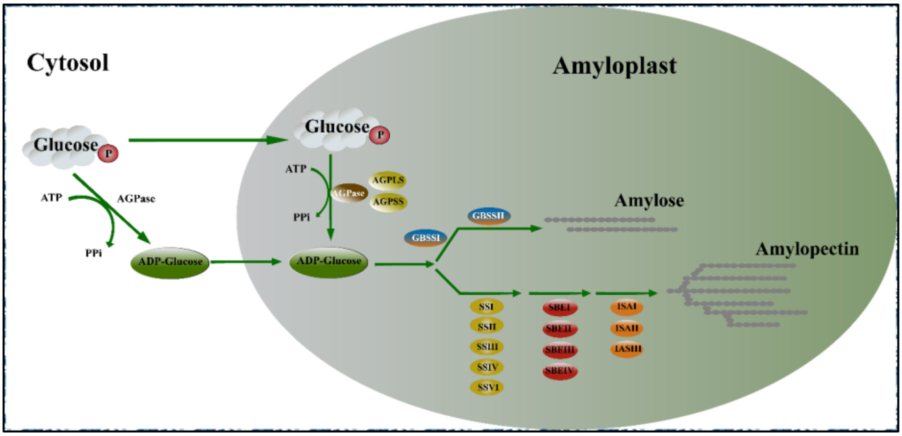

Figure 4. Synthesis of amylose and amylopectin. Adapted from Qu *et al.*, 2018

1.3.2 Krebs cycle in plants and microorganisms

The Krebs cycle, also known as the tricarboxylic acid (TCA) cycle, is the vital biological process of carbon metabolism, as it acts as the energy source for cells and is an integral part of aerobic respiration (Choi *et al.*, 2021). The Krebs cycle is also part of glucose metabolism. Glucose is converted to pyruvate through the glycolysis pathway, and then pyruvate is acetylated to form acetyl-CoA (Cleveland & Morris, 2015). Acetyl-CoA is the primary substrate for the Krebs cycle. The reactants and products of this process are boxed (Fig. 5a). However, if the cells are under stress conditions such as hypoxia, the normal function of the Krebs cycle is affected and a part runs inversely (Ariza *et al.*, 2012), as shown in Fig. 5b.

Many anaerobic organisms use the reverse Krebs cycle to produce carbon compounds from carbon dioxide and water (Wachtershauser, 1990) (Figure 5c). There are three enzymes specific to the reverse Krebs cycle, namely citrate lyase, fumarate reductase and ɑ-ketoglutarate synthase (Bar-Even *et al.*, 2012). It has recently been reported that, under anaerobic conditions, the Krebs cycle intermediate fumarate acts as the terminal electron acceptor in the mammalian electron transport chain (Spinelli *et al.*, 2021). Reduction of fumarate to succinate keeps the electron transport chain active under anoxic conditions. At the same time, fumarate is usually used as an important carbon source for microbial growth (Bronder *et al.*, 1982; Han *et al.*, 2022).

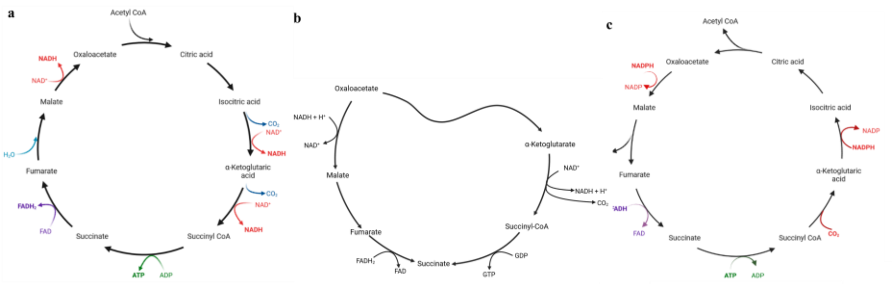

Figure 5. Overview of the citric acid cycle (a) in the aerobic condition, (b) in aenarobic codition, and (c) reverse citric acid cycle. Adapted from Wachtershauser, 1990; Kang *et al.*, 2021; and Kang *et al.*, 2021

1.3.3 Rice straw

Rice straw, an inevitable by-product of rice cultivation, constitutes around 50% of the gross weight of a paddy plant and annual rice straw production on a global scale is roughly 800-1000 million tonnes (Harun *et al.*, 2022). In general, rice straw can be used in agriculture to improve the soil through carbonisation and composting, while it can also be used for production of bioenergy and other industrial materials (Elhelece, 2018; Chivenge *et al.*, 2020; Harun *et al.*, 2022). However, the straw disposal alternative most universally adopted by farmers is open burning, which has great negative impacts on the environment (Sharma *et al.*, 2020), because it requires low labour inputs and low investment.

Lipids are an important carbon source in dicots and are mainly present in the embryo of plants in the form of triacylglycerol (TAG) (Bates *et al.*, 2013;

Troncoso-Ponce *et al*., 2016). In plant cells, part of the glucose is converted to pyruvate in the cytoplasm through glycolysis, and then pyruvate is further converted into acetyl-CoA. The acetyl-CoA is either transported to the endoplasmic reticulum and used for synthesis of fatty acids or to the mitochondria for use in the Krebs cycle. Due to the economic value of food oil, much research effort has been devoted to studying the mechanism of fatty acid synthesis in the seed of dicots (Liu *et al*., 2010; Kong *et al*., 2019; Li *et al*., 2022; Lim *et al*., 2022). However, increasing the lipid content in vegetative tissues of rice could increase its value in bioenergy production. The plant transcription factor WRINKLED 1 (WRI1) is interesting in this regard, as it can activate genes that are important for glycolysis and triacylglycerol synthesis in plants through binding to the AW-box located in the promoter region (Cernac & Benning, 2004). Overexpression of WRI1 in vegetative tissues of rice plants could activate the fatty acid synthesis pathway and thereby increase the energy content of rice straw.

2. Aims of the study

The main aims of this thesis were to breed climate-friendly rice varieties, increase rice straw utilisation so as to reduce the impact of rice cultivation on the environment and increase rice production. Specific objectives of the studies described in Papers I-IV were to:

- Identify the mechanism behind the low methane emissions in SUSIBA2-rice (Paper I)
- Test the adaptability of Heijing 5 to cultivation in high-latitude regions such as Scandinavia (Paper II)
- Breed low-methane, high-yielding rice varieties and study the underlying mechanism for the low methane trait (Paper III)
- Increase the value of rice straw for use in bioenergy production (Paper IV)

3. Results and discussion

3.1 Identification of major root exudates regulating methane emissions from rice paddies

In rice paddies, methane emissions are affected by many factors, such as temperature, soil pH, root exudates, water management and the balance of methanogens and methanotrophs in the rhizosphere (Wang *et al.*, 1999; Malyan *et al.*, 2016). Root exudates are a particularly important factor, as they act as the substrate for methanogenic communities to form methane in the rhizosphere (Waldo *et al.*, 2019). Thus, for regulation of methane emissions from rice cultivation, a detailed understanding of the compounds and metabolic mechanism of root exudates in rice soil is needed. A low-methane rice line, SUSIBA2-rice, has been generated using a transgenic approach (Su *et al.*, 2015), but the exact reason for the low methane trait in SUSIBA2-rice has not been identified. In Paper I, methane emissions from SUSIBA2-rice were measured and analysed. In agreement with previous results (Su *et al.*, 2015), it was found that SUSIBA2-rice emitted significantly less methane than the conventional Nipponbare (Nipp) cultivar at all growth stages (Paper I, Fig. 2a). Analysis of rhizosphere chemical compounds for plants at the middle tillering stage showed that fumarate was the most significantly reduced compound in SUSIBA2-rice compared with Nipp (Paper I, Fig. 2b). Further analysis showed that fumarate concentrations were consistently lower in SUSIBA2-rice rhizosphere soil than in Nipp rhizosphere soil during all growth stages (Paper I, Fig. 2c). Fumarate has been shown to act as both a carbon source and an electron acceptor for microorganisms under anoxic condition (Butler *et al.*, 2006).

The results in Paper I indicated that fumarate released from rice plants most likely directly influences the level of methane emissions. To test the role of fumarate in methane emissions from rice paddies, methane was collected after adding exogenous fumarate to experimental pots with SUSIBA2-rice and Nipp plants. The results demonstrated that fumarate addition significantly increased methane production (Paper I, Fig. 2d). In parallel, fumarate addition to anaerobic cultures with fresh rhizosphere samples from Nipp rice pots used as inoculum resulted in a continuous increase in methane production (Paper I, Fig. 2e). These results indicate that fumarate regulates methane emissions possibly by influencing the activity of microbial communities in rhizosphere soil. To further investigate this, next-generation sequencing (NGS) of bacterial 16S rRNA genes was performed. The sequencing data showed that *Geobacter* (recently renamed to *Citrifermentans*; Waite *et al*., 2020) and *Propionivibrio* accumulated after external addition of fumarate (Paper I, Fig. S5a), suggesting that members of these bacterial genera participate in the metabolic process of fumarate conversion. *Geobacter* is a representative member of the soil microbial community and is reported to be able to use fumarate as both energy source and carbon source (Yang *et al*., 2010). *Propionivibrio*, a fermentative bacterial species, can convert fumarate to propionate (Albertsen *et al.*, 2016). *Geobacter* and *Propionivibrio* can both use fumarate to produce acetate as an end-product. During anaerobic culture of rhizosphere soil with fumarate addition in Paper I, propionate and butyrate were found as two dominant intermediates, later converted to acetate and methane (Paper I, Figs. 3 and S9). This is in agreement with findings in previous studies (Asanuma *et al*., 1999; Ungerfeld *et al*., 2007). In the rice root, conversion of fumarate was different to that in the soil and fumarate was reduced to succinate and propionate, later converted to acetate (Paper I, Figs. 4 and S11). This difference in intermediate products indicates that fumarate is converted via different metabolic processes in root and soil. As mentioned, in an anoxic environment part of the Krebs cycle can run in reverse (Ariza *et al.*, 2012; Lecomte *et al.*, 2018), and fumarate can be reduced to succinate, which shows enrichment in the root. To further evaluate the role of fumarate in methane emissions, oxantel, a fumarate reductase inhibitor (Ge *et al*., 2000), was added to rice growing in pots and to anaerobic cultures with rhizosphere soil. The results showed that oxantel significantly inhibited methane emissions in both pots and cultures (Paper I, Figs. 2f and S10), demonstrating a clear link between fumarate and methane emissions. This suggests that reducing fumarate secretion by rice plants or inhibiting fumarate reductase activity by treatment with oxantel could be a good strategy to reduce methane emissions in the field.

In both the anaerobic culture and pot experiments, there was a difference in the level of methane emissions between SUSIBA2-rice and Nipp plants in response to fumarate treatment (Paper I, Fig. 5a). SUSIBA2-rice emitted significantly less methane than Nipp, even though similar concentrations of fumarate were present in the samples (Paper I, Figs. 5a and S12a). This indicates that an additional compound that inhibits methane production may be present in SUSIBA2-rice. Analysis of root secretions showed that ethanol concentrations were significantly elevated in SUSISA2-rice growing in pots (Paper I, Fig. 5b). To test whether ethanol influences methane emissions, it was added to the pots and the anaerobic *in vitro* cultures, where it significantly decreased methane emissions in both cases (Paper I, Figs. 5c, e, S14). Scrutiny of the methanogens under the microscope revealed aberrant clump-like structures and chains of microbial cells in samples to which ethanol had been added (Paper I, Fig. 5d), possibly explaining the decreased activity observed for methanogenesis. The effect of ethanol on methane emissions is reported to be concentration-dependent (Liu *et al.,* 2019). A high ethanol concentration has been shown to decrease methane production and alter archaeal community composition, while at a lower concentration ethanol can serve as a source of methane production via syntrophic growth, with bacteria using ethanol as the substrate (Thiele *et al*., 1988; Yang *et al*., 2019). An ethanol concentration of 10 mM was used in Paper I, and was apparently sufficient to exert an inhibitory effect. In addition, the expression level of *SDH1* (succinic dehydrogenase 1), which is the fumarate synthesis gene, was found to be significantly repressed after ethanol addition (Paper I, Fig. S15). This suggests that production of ethanol in rice roots leads to less fumarate synthesis. In an anoxic environment, rice roots produce ethanol through the metabolic pathway of alcohol fermentation, and the higher ethanol level in SUSIBA2-rice roots compared with Nipp roots shows more efficient alcohol fermentation capacity in SUSIBA2-rice. Taken together, the above results show that fumarate and ethanol are important compounds in the exudate from rice roots and that they exert a synergistic effect in influencing methane emission from rice paddies. In particular, low fumarate and high ethanol concentrations in paddy soils of SUSIBA2-rice contribute to the low methane emissions (Paper I, Figs. 1a, b). In field experiments conducted in 2021 and 2022 based on these findings, external addition of oxantel and ethanol to the soil reduced methane emissions from rice paddies by 60% (Paper I, Fig. S22).

3.2 Characterisation of Heijing 5 in a high-latitude region

In Paper I, fumarate and ethanol secreted by rice roots were shown to play a vital role in regulating methane emissions from low-methane emissions SUSIBA2-rice. However, methane emissions from conventional cultivars still needed to be explored. In screening of more than 20 landrace cultivars, Heijing 5 was identified as a potential low-methane emissions cultivar (Paper II, Fig. S1). Heijing 5 is commonly grown in northern parts of China (latitude 50°N), typically in Heilongjiang province. Initial monitoring of methane emissions during cultivation in a phytotron showed that Heijing 5 emitted significantly lower levels than the control line, Nipp, in all growth stages (Paper II, Fig. 1a). To further characterise Heijing 5, field trials were performed in Uppsala, Sweden, in 2020 and in Nanjing, China, in 2021. Measurements of methane emissions from these two field trials showed that Heijing 5 emitted significantly less methane than control plants, including the wild grass species *Acorus calamus* L. in Sweden and the commercial rice cultivar Suxiangjing in China (Paper II, Figs. 1c and d). Thus the initial screening and the subsequent field trials demonstrated that Heijing 5 is a low-methane rice variety. Methanogenesis is the terminal process in fermentation of organic matter under anoxic conditions and it is performed by methanogens in paddy soils (Lyu *et al.*, 2018). During field cultivation of Heijing 5, methanogen abundance in rhizosphere soil were analysed by qPCR. The results showed significantly lower abundance in the rhizosphere soil of Heijing 5 plants compared with that of grass plants growing close by (Paper II, Fig. 1e), which might explain the low methane missions associated with Heijing 5 cultivation. Interestingly, methane emission rates were lower in the field trials conducted in Uppsala than in the pot experiments in the phytotron or in the field trials conducted in Nanjing. One possible explanation for the low emission rates at Uppsala could be that the ambient temperature influences methane emission rates, since it is well known that low temperatures can affect the activity of methanogens and reduce methane emissions (Peng *et al.*, 2008). The relatively higher methane emissions from plants growing in the phytotron were likely due to the higher temperature (21/30°C night/day temperature), which provided good conditions for methanogenic activity. In the field trial at Uppsala, temperatures were lower than those in the phytotron, especially at night, which might have suppressed methanogenic activity and, as a consequence, reduced the methane emissions from the rice paddy. The good performance of Heijing 5 cultivated in the field at Uppsala without the addition of extra fertiliser indicates that the nutrients from the water of river Fyris is sufficient to sustain normal growth of this cultivar (Paper II, Figs. 2d-f). Reduced concentrations of nitrogen and potassium in outflow water from the paddy compared with inflow water confirmed this (Paper II, Fig. 2g).

To characterise grain quality of Heijing 5 grown in Uppsala, the concentrations of arsenic, lead and cadmium in the grain were analysed. The results demonstrated that the grain contained relatively low concentrations of all three elements compared with rice grain from other countries (Paper II, Table1). This is probably not an inherent trait of the Heijing 5 rice cultivar, but most likely a result of the low arsenic, lead and cadmium concentrations in Uppsala soils (Paper II, Table2). Production of rice with low concentrations of heavy metals would improve global food safety, since heavy metal contamination of soils suitable for rice production is a problem in many rice-producing countries (Zeng *et al.*, 2015; Ali *et al.*, 2020; Giao, 2020; AL-Huqail *et al.*, 2022).

Heijing 5 rice plants grown in the Uppsala field trials showed greater plant height, higher numbers of grain per panicle, higher grain filling rate and higher thousand-kernel weight than reported for rice grown in field trials in Heilongjiang, China (Yang *et al.*, 2011). Moreover, Heijing 5 rice grown in Uppsala showed no difference in number of tillers, plant height and panicle length compared with rice grown at the field site in Nanjing (Paper II, Fig. 2d). These results suggest that Heijing 5 can be cultivated with acceptable yield at high latitudes, *i.e.* even in Scandinavia. Scandinavia experiences relatively cold summers and long days compared with most rice cultivation areas. In fact, the optimal temperature for most rice cultivars is between 25 °C and 35 °C (Hussain *et al.*, 2019). According to World Weather Online (https://www.worldweatheronline.com), mean night/day temperatures at Uppsala during the field trial period (June-August 2020) was 12 °C/22 °C. The average day length in Uppsala in the period June-August is around 17 hours (Dateandtime.info), which exceeds the normal requirement for light for rice growth as rice is a short-day plant (Izawa, 2007). To study the genetic mechanism underlying the observed adaptation of Heijing 5 to high-latitude regions, transcriptome analysis was performed. This involved sequencing mRNA samples derived from leaves of Heijing 5 and Nipponbare collected at the late tillering stage in the Uppsala field trial, from plants growing in the open air and plants cultivated in a tent. The resulting data showed that 15 genes in the GO-term “response to cold” (GO:0009409) were enriched in open-air cultivated Nipponbare plants, while no genes in this GO-term were found in the corresponding Heijing 5 plants (Paper II, Fig. 4d). This indicates that Heijing 5 was more adapted to the relatively cold Uppsala summer temperatures. The GO-term “photoperiodism, flowering” was also enriched with four genes in Heijing 5, both in plants cultivated in open air and in the tent (Paper II, Fig. 4d). These genes encoded the proteins flowering locus T-like1(FTL1), rice flowering locus T 1 (RFT1), heading date 3A (HD3A), and homoserine kinase (Paper II, Table S5). FTL1, RFT1 and HD3A are proteins that regulate rice flowering during short-day conditions and promote early flowering when overexpressed (Kojima *et al.*, 2002; Komiya *et al.*, 2008). However, previous studies have shown that knockout of RFT1 significantly delays flowering in long-day conditions, whereas HD3A knockout only results in a mild delay (Komiya *et al.*, 2009; Brambilla & Fornara, 2013). These results indicate that RFT1 plays a vital role in rice flowering in long-day conditions. Upregulation of the genes for FTL1, RFT1 and HD3A in Heijing 5 indicates that it is an early-flowering cultivar responding to the increase in day length, and that these flowering time genes contribute to the adaptation of Heijing 5 to high-latitude conditions.

3.3 Effect of carbon partitioning on methane emissions

Paper II showed that Heijing 5 is an excellent candidate for expanding rice cultivation areas and reducing greenhouse gas emissions. However, the yield potential of Heijing 5 is not at the same level as in many elite rice varieties cultivated commercially today. In plant breeding work to overcome this, Heijing 5 (HJ) was used as a paternal line to cross with four elite rice varieties: Xiushui (XS), Jiahua (JH), Huayu (HY), Wuchang (WC). These four varieties are currently commercially grown in different provinces of China. The segregating progeny of the four crosses were allowed to self-fertilise for five generations. Lines derived from HJ crossed with JH and with WC were used in Paper I, and lines derived from HJ crossed with XS, JH and HY were used in Paper III. (Note that the line derived from the HJ x JH cross was used in both studies.)

To validate the findings in Paper I in practice, fumarate and ethanol concentrations in root exudate from Heijing 5, which was shown in Paper II to be a naturally occurring low-methane rice variety, were analysed. Low fumarate levels and high ethanol levels were detected in rhizosphere soil of Heijing 5 plants (Paper I, Fig. S17). In an attempt to transfer the low-methane trait to elite rice varieties, HJ was used as a paternal pollen donor in crosses with two elite varieties (JH and WC). It was found that the low-methane progeny lines derived from these crosses had low fumarate and high ethanol concentrations in their rhizosphere soils compared with the corresponding maternal line (Paper I, Figs. 6a-f). The low methane emissions character of the progeny lines was confirmed in field trials in 2021 and 2022, where an average decrease in methane emissions of more than 70% compared with the maternal line was detected (Paper I. Figs, 1d and S20). These results demonstrate that it is possible to transfer the low-methane properties of Heijing 5 to high-yielding elite varieties using conventional crosses and that the low-methane trait is not necessarily linked to yield penalties. Importantly, the low-fumarate and high-ethanol properties are also inherited from the paternal lines to the progeny lines, suggesting that fumarate and ethanol can be used as markers for further low-methane rice breeding.

After five generations of screening, three promising progeny lines from the initial cross between HJ and the three elite lines XS, JH and HY were identified. These three independent progeny lines were found to be associated with low methane emissions during the entire growth period of the rice plants (Paper III, Figs. 1a and S3a). Since the initial screenings for low methane properties were performed in pot experiments under greenhouse conditions, field trials were performed in two consecutive years, in Nanjing in 2021 and in Huanggang and Jingzhou, China, in 2022. In these field trials, both methane emissions and phenotypic growth traits related to yield potential were measured. Cultivation of all three progeny lines was associated with a significant decrease in methane emissions compared with the corresponding maternal line (Paper III, Figs. 1b and S3b-d). Further, yield measurements showed that the progeny lines had higher number of tillers, plant height, panicle length and dry weight than the maternal lines (Paper III, Figs. 1d and S5), indicating high yield potential. A negative correlation between the capacity of rice plants to store photosynthetic carbon in grain and methane production in soil has been established previously (Denier Van Der Gon *et al.*, 2002). Jiang and co-workers found that removing the spikelets from rice plants caused a significant reduction in methane emissions in flooded rice paddies. They also demonstrated that improving harvest index could reduce methane emissions by decreasing soil biomass and reducing root exudates and residues accumulated in the soil (Jiang *et al.*, 2019).

It has previously been shown that glucose, galactose and sucrose are the major components of water-soluble sugars in the rice rhizosphere (Kimura *et al.*, 1977). As mentioned in Chapter 2 of this thesis, the amount of root exudates from rice roots and the specific molecules that are secreted may influence methane formation. Therefore chemical compounds in rhizosphere soil of the parent and progeny lines grown in greenhouse pot experiments were analysed using nuclear magnetic resonance (NMR). The NMR results showed that the paternal, maternal and progeny lines formed three distinct groups based on their root exudate profile (Paper III, Figs. 2a and S6a, S6d). Interestingly, the profiles of the progeny lines were more similar to the paternal (Heijing 5) line than the corresponding maternal lines. The NMR results also revealed that the concentrations of most compounds secreted by roots were lower in the rhizosphere soil surrounding the progeny lines compared with the maternal lines. In particular, glucose, sucrose and formate concentrations were significantly lower (Paper III, Figs. 2b and S6b, S6e). As mentioned above, glucose is the primary source for acetate production in rice soils, supporting approximately 50-80% of methane production from acetate (Krumbock & Conrad, 1991; Chidthaisong *et al.*, 1999).

To further examine the relationship between glucose concentrations in rhizosphere soil and methane emissions, progeny line plants growing in pots were treated with glucose and methane emissions were measured. It was found that the progeny line plants treated with glucose emitted more methane than plants in untreated pots (Paper III, Figs. 2c and S6c, S6f). Analysis of the gene copies of seven methanogenic archaea communities demonstrated that the progeny lines contained a lower abundance of methanogens in their rhizosphere soil than the maternal lines (Paper III, Figs. S4), and that more methanogens accumulated in soils with glucose treatment (Paper III, Fig. 3). These results indicated that high sugar levels in the rhizosphere could increase methane production from rice.

Up to 70% of the methane emitted from rice fields derives from plant photosynthates (Watanabe *et al.*, 1999). Thus, carbon partitioning after photosynthesis may play a vital role in methane production from rice paddies. Analysis of total carbon content in different tissues revealed that the progeny lines accumulated more carbon in aboveground tissues and less carbon in their roots compared with the corresponding maternal lines. This trend was particularly prominent at the flowering stage (Paper III, Figs. 4a and S7a, S7d). In addition, the main carbohydrates, glucose and sucrose, were found to have accumulated in the stem both at flowering and 9 days after flowering (DAF) (Paper III, Figs. 4b, and S7b, S7e). Starch content was significantly higher in grain of the progeny line at 9 DAF and during maturation (Paper III, Figs. 4c and S7). In contrast, sucrose and starch concentrations in the roots were consistently lower in the progeny lines compared with the maternal lines, both at flowering and at 9 DAF (Paper III, Fig. 4c). These results suggest that more photosynthates in the progeny line were allocated to aboveground tissues to promote growth, especially during grain formation at the reproductive stage. In contrast, relatively more photosynthates were transported to the roots in the maternal lines and ended up as a carbon source for methanogens.

To explore the molecular mechanism underlying carbon partitioning, transcriptome analysis was performed by sequencing mRNA samples derived from stem tissues at the flowering stage. The reason for choosing stem tissues was because this allowed the activity of genes involved in carbon transport to be studied, as carbon is transported through the phloem in the stem (Zimmermann & Ziegler, 1975). By comparing the transcriptomes of the progeny lines with the corresponding maternal line, it was possible to identify upregulated genes involved in carbon metabolism in the progeny, *i.e.* genes involved in glycolysis and the Krebs cycle (Paper III, Fig. S9). Interestingly sugar transport-related genes were upregulated in the progeny lines, whereas starch synthesis-related genes were downregulated (Paper III, Fig. 6). In long-distance sugar transport in plants, SUT transporter(s) play an indispensable role since very few plasmodesmata exist between the sieve element-companion cell complexes and surrounding cells (Geiger *et al.*, 1973). Knockout of SUT2 reduces the number of tillers, plant height and grain weight of rice plants (Eom *et al.*, 2011; Siao *et al.*, 2011). The SWEET sugar transporters also facilitate diffusion of sugars across cell membranes down concentration gradients (Chen *et al.*, 2012). The elevated expression levels of SUTs and SWEETs observed in the progeny lines suggest that long-distance sugar transport is activated. Based on the phenotypic and carbon content results, the sugars produced are mainly transported to the grain. The lower expression levels of starch biosynthesis-related genes in the stem and higher glucose levels in different tissues in the progeny lines imply that carbon allocation is more dynamic and flexible in these lines. Taken together, the results in Paper III suggest that sugar transporters mediate accumulation of photosynthetic carbon to aboveground sink tissues (*e.g.* seed) in the progeny lines, while less carbon is allocated to the roots. As a result, the progeny lines have high yield capacity and are also associated with low methane emissions.

3.4 Effects of Arabidopsis WRI1 (AtWRI1) expression in rice on lipid levels and bioenergy value

Rice straw comprises a large quantity of non-food biomass that could be harvested and used as a source of bioenergy (Harun *et al.*, 2022). At present, the majority of rice farmers worldwide prefer to dispose of rice straw through open burning, instead of using it as a feedstuff, fuel or fertiliser (Launio *et al.*, 2015). However, open-air burning of rice straw contributes to global warming by emitting greenhouse gases, *e.g.* methane, carbon dioxide and nitrous oxide (Romasanta *et al.*, 2017). Using the straw for bioenergy generation would be a more sustainable management method. In this context, it is likely that increasing the lipid content in rice straw could improve the bioenergy value and promote its use for methane production. Arabidopsis WRI1 is a plant-specific Apetala2/ethylene responsive transcription factor

(AP2/ERF) involved in triacylglycerol metabolism. To check the function of Arabidopsis WRI1 in lipid synthesis and the activity of the first AP2 domain, a full-length cDNA and a truncated form lacking the first AP2 domain (ΔWRI1) (Paper IV, Fig. S1) were cloned from *Arabidopsis thaliana*. The two clones were first transiently expressed from the CaMV 35S promoter in tobacco leaves. Gas chromatography analysis of the tobacco leaves demonstrated that both full-length and truncated AtWRI1 could increase triacylglycerol (TAG) levels, but that the full-length AtWRI1 had a stronger (17-fold) effect than the truncated ΔWRI (5-fold effect) (Paper IV, Figs. 1b and 1c).

The function of WRI1 in regulating TAG biosynthesis has been well studied in dicots (Focks & Benning, 1998; Cernac & Benning, 2004; Maeo *et al*., 2009; Kong *et al*., 2020), but not in monocots. Therefore, to test whether AtWRI1 could increase TAG levels when ectopically expressed in a monocotyledonous plant species, transgenic rice lines that either expressed the full-length AtWRI1 or the truncated ΔWRI1 form were generated from barley starch branching enzyme IIB (HvSBEIIb) promoters pSBE: AtWRI1 and pSBE: ΔWRI1 (Sun *et al*., 1998). The constructs were used to transform the indica rice cultivar MH86 and the japonica rice cultivar Nipponbare. Phenotypic characterisation indicated that expression of AtWRI1 in rice lines did not change any of the yield-related traits (Paper IV, Fig. S4). Molecular characterisation of different tissues (leaves, stems, roots, grain) of both types of transgenic plant demonstrated that expression levels of genes involved in fatty acid synthesis, *e.g.* pyruvate kinase cytosolic isozyme (*OsPK-cyto*), pyruvate kinase isozyme g chloroplast (*OsPK-chlor*), acetyl-coa carboxylase (*OsACC*), diacylglycerol transferase 1 (*OsDGAT1*) and *OsWRI1*, were generally higher in transgenic lines than in the corresponding wild type (Paper IV, Figs. 2b, 4b, S6a, S8a). This increased gene expression was confirmed to lead to increased protein levels in the case of OsPK-cyto (E.C. 2.7.1.40) and OsACC (E.C.6.4.1.2) (Paper IV, Figs. 2c, 4c, S6b, S8b). Consistent with the molecular results, TAG levels were also significantly increased in different tissues of the transformants (Paper IV, Figs. 3, 5a-c, S7, S9a, b). Together, the molecular and metabolic analyses demonstrated that both the full-length and truncated versions of AtWRI1 were able to increase lipid biosynthesis in transgenic rice plants when expressed under the control of the *HvSBEIIb* promoter. The lower stimulatory effect of the truncated AtWRI1 on lipid biosynthesis in both tobacco and in rice suggests that the first AP2 domain in WRI1 is important for full activity of WRI1.

Since expression of AtWRI1 could increase TAG levels in vegetative tissues, the next step in the work was to investigate whether ectopic expression of AtWRI1 could increase the fatty acid level in rice straw after grain harvest. The results showed that TAG levels were significantly higher in the transformant lines than in the wild type, almost two-fold higher in both the indica and japonica varieties (Paper IV, Fig. 6a). The level of total fatty acids was also significantly increased (Paper IV, Fig. 6a). The higher levels of fatty acids in transgenic rice straw indicated that the straw could have a higher value for bioenergy production. To investigate this possibility, straw from the Indica rice variety was used as a substrate for biogas production. Anaerobic digestion of AtWRI1 rice straw produced significantly more methane than anaerobic digestion of wild-type straw. The mean difference in methane production between the digestions of transgenic straw with high TAG levels and wild-type straw was approximately 20% (Paper IV, Figs. 7a, 7b). However, analysis of methane production from rice husks, an additional by-product from rice cultivation, revealed that methane concentrations generated from those parts of the rice plant were considerably lower than those generated from the rice straw (Paper IV, Figs. 7c, 7d). Taken together, these results indicate that the increased concentrations of lipids and TAG in WRI1 rice straw increase the levels of readily available carbon sources for anaerobic bacteria and consequently improve methane production potential for bioenergy purposes. Rice straw is mainly composed of cellulose, hemicellulose, lignin, ash and silica (Vivek *et al.*, 2019). During anaerobic digestion, microorganisms must degrade these complex organic compounds into soluble organic molecules, *e.g.* sugars, amino acids and fatty acids, and then continue with further fermentation. However, the complex structure of hemicellulose and lignin impedes microbial activity, resulting in relatively slow degradation with low efficiency and low biogas yield (Mothe *et al.*, 2021). In contrast, presence of fatty acids in rice straw makes it easier for microbes to degrade, which explains the increased degradation efficiency and higher bioenergy yield seen for transgenic rice straw.

4. Future perspectives

Many studies have shown that root exudates are linked to methane emissions (Sigren *et al*., 1997; Paterson, 2003; Mitra *et al*., 2005; Waldo *et al*., 2009). This thesis attempted to characterise the typical root exudate affecting methane emissions from rice paddies. The results demonstrated that fumarate and ethanol are the two main compounds secreted from SUSIBA2-rice plants that affect methane production. In line with this, expression levels of genes related to fumarate and ethanol synthesis, *e.g. SDH* (succinic dehydrogenase), *PDC1* (pyruvate decarboxylase 1), *ADH1* (alcohol dehydrogenase 1) and *ADH2* (alcohol dehydrogenase 2), were shown to be affected in SUSIBA2-rice plants. SUSIBA2 is a transcription factor that mediates photosynthate allocation (Sun *et al*., 2003). Ectopic expression of SUSIBA2 may regulate the genes involved in the carbon metabolic pathway and thereby affect carbon allocation. Further experiments are need to identify the genes that are the direct targets of the SUSIBA2 transcription factor. This thesis showed that oxantel, an inhibitor of fumarate reductase, can reduce methane production in rice paddies by inhibiting the oxidation of acetate to methane, but the underlying mechanism for this inhibition is unknown. While the mechanism need to be identified, based on the results in this thesis it can be concluded that methane emissions are affected by root exudates (*i.e.* fumarate and ethanol), as confirmed in three years of field trials. The discovery that fumarate is critical for methane emissions from paddy rice cultivation opens up different venues to breed other low-methane rice varieties and to change management practices that could further reduce methane emissions. For instance: 1) External addition of oxantel to rice paddies could be used to reduce available fumarate levels, which would lead to an immediate reduction in methane emissions. Use of oxantel in agricultural applications must be approved by competent authorities for this to be a viable option, but oxantel is currently used as a drug against intestinal worms in both humans and animals (Palmeirim *et al*., 2021), which indicates limited adverse effects. 2) Targeted breeding methods, *e.g.* CRISPR/Cas9, that do not need to introduce exogenous genes could be used to increase SUSIBA2 expression or specifically knock out the genes involved in fumarate synthesis. This would lead to specific high expression of the endogenous SUSIBA2 gene in rice or a specific reduction in fumarate secretion from rice roots, which could drastically reduce methane emissions from rice paddies.

In independent studies, this thesis showed that low fumarate and high ethanol levels reduce methane emissions. Selected progeny lines obtained in crosses between a naturally occurring low-methane emissions variety and high-yielding elite rice varieties also displayed low fumarate and high ethanol levels. In addition, these progeny lines showed carbon reallocation, with a higher proportion of carbon accumulated in aboveground tissues and less carbon accumulated in the root. The progeny rice lines showed low fumarate and high ethanol concentrations in rhizosphere soil, but the dominant compounds affecting methane production in the progeny rice lines were carbohydrates, typically glucose and sucrose. It is therefore likely that different mechanisms regulate carbon partition in SUSIBA2-rice and the progeny lines. This thesis showed that sugar transport genes, *e.g*. SUTs and SWEETs, are expressed at elevated levels in the progeny lines. One possible way to identify the mechanism in progeny lines would be to generate transgenic plants with sugar transport genes and observe carbon allocation and methane emissions. Determination of the relationship between fumarate and glucose could help understand the mechanism by which root exudates influence methane production. This relationship could be identified by culturing the same rice soil sample in anaerobic conditions, adding fumarate or glucose and assessing the degradation rate and accumulated compounds in each culture.

The field work in Paper II tested the possibility of expanding rice cultivation to high-latitude locations to increase total rice production and, at the same time, cultivate rice associated with low methane emissions. Unlike indica rice varieties, japonica varieties can be cultivated in temperate regions. Hence, to meet future demand for rice, one possibility could be to expand the rice cultivation area further north. This would increase net rice production and possibly offer another cash crop for farmers in high-latitude countries. In this thesis, the variety Heijing 5 was tested in field trials in China and in Sweden. The results showed that cultivation of Heijing 5 was associated with relatively low methane emission levels and that the yield potential was equally high in both China and Sweden. However, the yield traits were compared with published data from studies performed in 2012 and further field trials are needed to confirm the results. Many commercial rice varieties are either short-day plants or adapted to growth in regions with relatively long nights (Maclean *et al*., 2013). Heijing 5 is typically cultivated between latitudes 43°N and 50°N in China. The latitude of Uppsala, Sweden, is 59.8°N, and the average daily temperature from June to August is 12-22°C, with very long day length (up to 17 hours of light per day). Transcriptome analysis in this thesis showed that key flowering time regulators are upregulated in Heijing 5 relatively early in the growing season, when the nights in Uppsala are still very short. This indicates that Heijing 5 is either day length-neutral or a long-day rice variety. Transcriptome analysis also identified two types of differentially expressed genes related to cold tolerance that may play an essential role in the high-latitude adaptation of Heijing 5 plants. The candidate genes identified in this work can be used to further study the genetic mechanism that causes early flowering in Heijing 5 plants. It would also be interesting to study whether the progeny lines obtained in Paper III from crosses between Heijing 5 and elite varieties are also early-flowering, since these lines have excellent properties as regards methane emissions and yield, which could be promoted in the future. Currently, the highest recorded latitude for commercial rice cultivation worldwide is 53°N (northern China) (CABI International, 2022). Extending rice cultivation to higher latitudes would provide greater possibilities to meet the increasing demand for food. The discovery of the molecular mechanism by which Heijing 5 adapts to high latitude lays the foundation for future breeding of new rice varieties adapted to high-latitude regions. Future studies should continue screening and breeding for rice varieties adapted to high latitudes and also associated with reduced methane emissions.

As mentioned above, the low-methane rice lines studied in this thesis had higher biomass content in aboveground tissues than the parental lines. A hallmark of the bioeconomy is to reduce waste by using side-streams as substrate for generating new materials or bioenergy. As in many other cereals, grains are the main product in rice, but other parts of the plant could also be utilised to a greater extent. Molecular breeding tools can facilitate breeding for multiple targets, *e.g*. increased grain yield and altered molecular composition of the straw. As proof of concept, the rice lines produced in Paper IV accumulate lipids (especially triacylglycerol) in the straw, increasing the energy content and making the straw suitable for bioenergy production. In future work, it would be interesting to combine the low-methane, high-yield progeny lines developed in Paper III with the bioenergy traits introduced in Paper IV.

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Popular science summary

According to IPCC in 2014, anthropogenic methane emissions have contributed to 16% of the Earth's warming since pre-Industrial times. Rice paddies generate methane emissions due to frequently occurring anaerobic conditions, high temperatures and an abundance of nutrients, which are favourable for growth of methane-producing microorganisms. Around 100 million tonnes of methane are produced from rice paddies every year and the level is expected to increase in future as a result of increasing demand for food driven by global population growth. Although strategies to reduce methane emissions from rice paddies have been studied for decades, knowledge is still lacking about the underlying mechanism for methane emissions from rice paddies. Strategies developed to mitigate methane emissions have therefore not been very efficient.

Plants fix carbon from the air through photosynthesis. About 20% of the fixed carbon is transported to plant roots, partly to support root growth but also to support microorganisms that live in the soil surrounding the roots. Carbon molecules are transported from the roots to the soil through a process called exudation. Exudation of carbon molecules from rice roots has been well studied and is suggested to contribute to methane emissions. This thesis explored the possibility of breeding for rice varieties that are associated with reduced methane emissions. It also examined the effects of different root exudates on methane emissions from rice paddies. The experimental work carried out was mainly based on conventional breeding methods, such as cross-breeding followed by selection, in combination with carbon and carbohydrate measurements, metabolomics analyses and molecular analyses.

The plant breeding work was successful in producing rice lines associated with high yields and also 70% lower methane emissions. Other data obtained indicated that increased allocation of carbohydrates to aboveground tissues, *i.e.* leaves, stems and grain, was partly responsible for the lower methane emissions. This is because there is competition between roots and aboveground plant tissues for available carbon sources. When more carbon is partitioned into aboveground tissues, rice lines with high yield and low methane emissions are obtained. This thesis showed that fumarate in exudate from rice roots directly influences methane emissions in rice paddies. Fumarate is derived from glucose through glycolysis and the Krebs cycle, indicating the importance of glucose availability for methane emissions.

In field trials, a low-methane rice variety was successfully cultivated in Uppsala, Sweden. This variety has an early-flowering phenotype, allowing it to complete its growing cycle in the relatively cold and short summers in central Sweden. As confirmation, genes involved in the regulation of flowering time and cold acclimatisation were identified as being differentially expressed in the low-methane rice variety. Taken together, the results in this thesis increase current knowledge of the compounds that affect methane emissions in rice paddies. The work also yielded unique and well-characterised plant material, adapted to growing conditions in both northern Europe and China, that can be used to breed for high-yielding rice varieties associated with low methane emissions. This would increase rice production to meet growing future demand and, at the same time, substantially reduce global emissions of greenhouse gases from rice production.

Populärvetenskaplig sammanfattning

Enligt beräkningar från IPCC in 2014 har metanutsläpp från antropogena källor bidragit till 16 % av den globala uppvärmningen sedan förindustriell tid. Höga temperaturer i kombination med en syrefattig miljö och rikligt med näring gynnar förekomsten av metanproducerande bakterier i risfält vilka årligen producerar cirka 100 miljoner ton metan. Metanutsläppen från risfält förväntas dessutom öka som ett resultat av ökande efterfrågan på mat pådriven av den globala befolkningstillväxten. Trots att stora ansträngningar gjorts under de senaste decennierna för att minska metanutsläppen från risfält saknar vi fortfarande både kunskap om mekanismen bakom metanutsläppen, samt effektiva metoder för att minska på utsläppen av metan.

Växter tar upp kol från atmosfären genom fotosyntesen, vilket huvudsakligen sker i växternas blad. Cirka 20% av det fixerade kolet transporteras därefter till roten. En stor del av kolet som transporteras till roten används för rotens egen tillväxt, men en betydande del utsöndras även till den omgivande jorden och främjar där marklevande mikroorganismer. I denna avhandling studerar vi hur sammansättningen och mängden kolföreningar som avges från risplantornas rötter påverkar mikroorganismerna och i förlängningen avgången av metan. Studierna är inriktade på att ta fram rislinjer associerade med låga metanutsläpp. Vi använder oss främst av konventionella korsningar och karaktäriserar dessa med kemiska metoder för kol- och kolhydratmätning, metabolommätningar samt molekylära metoder för att studera genuttryck.

Genom konventionella förädlingsmetoder har vi tagit fram fyra oberoende linjer där metanutsläppen i genomsnitt är reducerade med 70% och som samtliga bedöms ha ökad eller åtminstone bibehållen skördepotential.

Genom att mäta hur den totala mängden kol samt olika kolhydrater fördelar sig mellan de delar av plantorna som växter ovanjord (stam, blad, blomma och frö) och roten visar vi på en korrelation mellan höga metanutsläpp och en relativ omfördelning av andelen kol till roten. Omvänt gäller att när mer kolhydrater fördelas till de delar av plantorna som växter ovan jord så får vi rislinjer med hög skördepotential och låga metanutsläpp. Utsöndring av glukos från risplantornas rötter verkar vara speciellt viktig för produktionen av metan. I jorden tas glukos lätt upp av bakterierna och omvandlas i risfältens syrefattiga miljö till fumarat, som därefter förbränns av bakterierna till metan, som avges till atmosfären.

Inom ramen för projektet har vi även genomfört fältförsök i Uppsala med en rissort som härstammar från norra delen av Kina. I en genomgång av flera olika rissorter har den visat sig vara associerad med låga metanutsläpp. Sorten har en tidigt blommande fenotyp, vilket gör att den kan fullfölja sin växtcykel under de relativt kalla och korta somrarna i Mellansverige. Som bekräftelse på detta identifierades gener involverade i regleringen av blomningstid som differentiellt uttryckta i rissorten. Sammantaget ökar resultaten i denna avhandling nuvarande kunskap om de kolföreningar som påverkar metanutsläppen i risfält. Inom ramen för avhandlingen har vi även tagit fram ett unikt och välkarakteriserat växtmaterial, anpassat till växtförhållanden i både norra Europa och Kina, och som kan användas för att vidare förädla högavkastande rissorter förknippade med låga metanutsläpp. Detta skulle kunna öka risproduktionen och samtidigt avsevärt minska på utsläppen av växthusgaser från risproduktionen.

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Methane emission from rice paddies contributes a lot to global greenhouse gas emission. In this thesis, we found the typical compounds that affect methane production in rice paddies. We successfully breed the low-methane and high-yield rice with conventional breeding methods. We also describe increasing lipid levels can improve the bioenergy yield of rice straw. The work in this thesis addresses several issues of rice cultivation and contributes sustainability of rice production.

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