Science of the Total Environment 920 (2024) 170980

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

A low-methane rice with high-yield potential realized *via* optimized carbon partitioning

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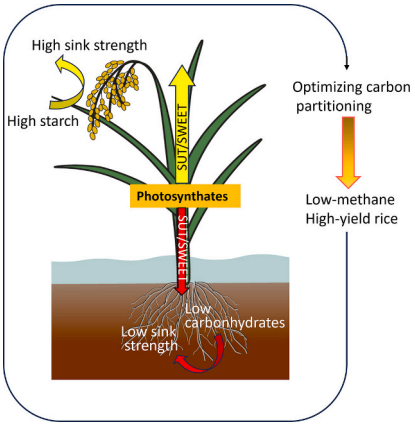
HIGHLIGHTS

- Low-methane rice varieties can reduce methane emissions by 70 %.
- Hybridisation breeding can be used to obtain high-yielding, low-methane rice lines.
- Sugar transport to the areal parts of rice plants increases the yield potential.
- A reduction in rice root exudates contributed to lower methane emissions.
- Primarily, exudated glucose plays a critical role in regulating methane emissions.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Charlotte Poschenrieder

Keywords:
Global warming
Rice cultivars
Carbon partitioning
Methane emission
Agronomic practices
Root exudates

ABSTRACT

Global rice cultivation significantly contributes to anthropogenic methane emissions. The methane emissions are caused by methane-producing microorganisms (methanogenic archaea) that are favoured by the anoxic conditions of paddy soils and small carbon molecules released from rice roots. However, different rice cultivars are associated with differences in methane emission rates suggesting that there is a considerable natural variation in this trait. Starting from the hypothesis that sugar allocation within a plant is an important factor influencing both yields and methane emissions, the aim of this study was to produce high-yielding rice lines associated with low methane emissions. In this study, the offspring (here termed progeny lines) of crosses between a newly characterized low-methane rice variety, Heijing 5, and three high-yielding elite varieties, Xiushui, Huayu and Jiahua, were selected for combined low-methane and high-yield properties. Analyses of total organic carbon and carbohydrates showed that the progeny lines stored more carbon in above-ground tissues than the maternal elite

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https://doi.org/10.1016/j.scitotenv.2024.170980
Received 20 September 2023; Received in revised form 25 January 2024; Accepted 13 February 2024
Available online 17 February 2024

varieties. Also, metabolomic analysis of rhizospheric soil surrounding the progeny lines showed reduced levels of glucose and other carbohydrates. The carbon allocation, from roots to shoots, was further supported by a transcriptome analysis using massively parallel sequencing of mRNAs that demonstrated elevated expression of the sugar transporters *SUT-C* and *SWEET* in the progeny lines as compared to the parental varieties. Furthermore, measurement of methane emissions from plants, grown in greenhouse as well as outdoor rice paddies, showed a reduction in methane emissions by approximately 70 % in the progeny lines compared to the maternal elite varieties. Taken together, we report here on three independent low-methane-emission rice lines with high yield potential. We also provide a first molecular characterisation of the progeny lines that can serve as a foundation for further studies of candidate genes involved in sugar allocation and reduced methane emissions from rice cultivation.

1. Introduction

Due to the anaerobic conditions in paddy soils, which favour the growth of methanogenic archaea, rice cultivation is often associated with considerable methane emissions (Liesack et al., 2000), according to FAO estimates in 2019, corresponding to 24 Tg globally. However, different cultivars are associated with different methane emission rates (Hu et al., 2023), likely caused by variance in carbon allocation, both within a plant and to the surrounding soil rhizosphere (Jiang et al., 2017; Hu et al., 2023; Su et al., 2015; Chen et al., 2021; Kwon et al., 2023).

In rice plants, carbon fixation, *i.e.* photosynthesis, mainly occurs in the leaves, which serve as source tissues. The assimilated carbon molecules are then transported from the leaves to different sink tissues, such as stems, roots and grains, to support their growth and development (White et al., 2016; Liu et al., 2019). Notably, carbon allocation is a dynamic process that decides the ratio between canopy and root growth (Lynch et al., 2012). As a consequence, carbon transport into seeds directly determines seed size and yield, whereas import into the root enhances root elongation (Farrar and Jones, 2000), and affects the growth of surrounding microorganisms (Dennis et al., 2010; Panchal et al., 2022).

While the primary photosynthetic carbon molecule is glucose, carbon is commonly transported through the plant in the form of sucrose (Zimmermann and Ziegler, 1975; Slewinski and Braun, 2010; Julius et al., 2017; Li et al., 2017). It has been demonstrated that SWEET (Sugar Will Eventually be Exported Transporter) efflux proteins take part in the transport of sucrose molecules from the photosynthetic mesophyll cells to the apoplast (Jeena et al., 2019). While SUT (sucrose transporter) transporters (Braun, 2012; Chen et al., 2012; Julius et al., 2017) load sucrose from the apoplast into the cells responsible for the long-distance transport, *i.e.* the phloem cells (Lalonde et al., 2004; Carpaneto et al., 2005; Reinders et al., 2012).

Once the transported sucrose has reached a sink tissue, it is commonly converted back into glucose or to other non-structural carbohydrates, such as fructose and starch. While glucose is an immediate energy source that sustains growth, it may also serve as a signalling molecule. In fact, >2000 plant genes are directly or indirectly regulated by glucose (Sheen, 1990; Price et al., 2004; Xiong et al., 2013; Aguilera-Alvarado and Sanchez-Nieto, 2017). For long-term storage, plants commonly store carbon as starch in sink tissues. Starch is a polysaccharide consisting of 1–4 linked α glucose monomers (Wang et al., 2017) and can be found in the form of linear amylose or branched amylopectin. Amylose is synthesized by ADP-glucose pyrophosphorylase (AGPase) and starch synthase (GBSS). Whereas amylopectin is synthesized by AGPases, a soluble form of starch synthase (SS) and the starch branching enzyme (SBE) (Qu et al., 2018). The deposit capacity of starch in crop seeds usually decides the yield potential (Parida et al., 2022).

After the sugars have been transported to the root, they are converted into different organic carbons that may be stored or used for root growth. However, part of the carbon molecules are also secreted into the soil, mainly in the form of sugars, organic acids and amino acids (Canarini et al., 2019). The released carbon molecules support the growth of surrounding microorganisms. In rice paddies, the dominating anaerobic microorganisms commonly consist of (poly) saccharolytic bacteria and methanogenic archaea, which feed on root exudates (King and Reeburgh, 2002; Lu and Conrad, 2005; Chen et al., 2021) and as a by-product produce methane. It has been estimated that 70 % of the emitted methane from rice paddies is derived from root exudates (Watanabe et al., 1999; Khosa et al., 2010). Based on the substrate and metabolic pathway of methane production, methanogens in rice paddies are mainly separated into two categories: i) acetoclastic and ii) hydrogenotrophic methanogens. Acetoclastic methanogens convert acetate to methane and carbon dioxide (Lueders et al., 2001; Watanabe et al., 2006; Westerholm et al., 2022), whereas hydrogenotrophic methanogens use hydrogen or format to reduce carbon dioxide to methane (Chin et al., 2004; Hashimoto-Yasuda et al., 2005; Berghuis et al., 2019).

Historically, breeding efforts have mainly been focused on increased yields (Evenson and Gollin, 2003). However, if increased yield equals increased total biomass and larger roots (Jiang et al., 2019; Kaysar et al., 2023), this may lead to an increased methane emission potential. However, there is a possibility to increase carbon allocation to the shoot in favour of the root and thereby reduce methane emissions (Su et al., 2015; Kwon et al., 2023). Therefore, optimizing carbon allocation is crucial for obtaining high-yielding rice varieties with reduced methane emissions. Recently the local rice cultivar Heijing 5 was identified as being a low-methane rice variety (Hu et al., 2023). Heijing 5 was identified in a screen for low methane emissions among twenty local varieties/breeding lines. Heijing 5 has favourable growth characteristics that allows it to be grown at high latitudes, but its yield potential cannot compare to most high-yielding commercial varieties (Hu et al., 2023). To increase our knowledge about a possible mechanism for the low methane trait displayed by Heijing 5 and to study if this trait can be combined with the high-yielding traits of commercial elite varieties, we have performed crosses and generated three independent progeny lines that are potentially high yielding and associated with low methane emissions. Using these lines we study differences in carbon allocation, methane emissions, and yield potential. To increase our understanding of how different genes contribute to differences in carbon allocation and, potentially, the low methane trait, we have also performed a transcriptome study using massively parallel sequencing of mRNA samples (RNAseq), derived from the stems of the progeny lines and the parental lines. In summary, the presented results demonstrate that it is possible to breed for high yields and low methane emissions by optimizing the carbon allocation within a rice plant.

2. Materials and methods

2.1. Plant growth conditions

2.1.1. Phytotron conditions and screening of low methane rice through cross breeding

Initially, controlled crosses were done between the low methane emission cultivar Heijing 5 (*Oryza sativa* L. *ssp. Japonica*) (Hu et al., 2023) as paternal line and three elite japonica varieties, Xiushui, Huayu and Jiahua as maternal donors, as described by Poehlman and Sleper (Poehlman and Sleper, 1999). The offspring of the crosses were

subsequently inbred for six generations. During all generations the plants were grown in a phytotron with 14 h of light (400 μmol photons m^{-2} s^{-1}) at 30 °C, and 10 h of dark at 21 °C. The humidity in the growth chambers was 80 % for the whole growth period. Each plant was cultivated in a pot (30 cm high with an upper diameter of 29 cm and a bottom diameter of 19 cm). In each generation, methane emissions from individual lines were measured biweekly, starting four weeks after planting. We used gas chromatography to identify and select the lowest methane emission lines in each generation (see below). The resulting F6 progenies with stable low methane emission rates, along with the parental varieties (Heijing 5 (HJ), Xiushui (XS), Huayu (HY), Jiahua (JH)), were used in the subsequent experiments.

2.1.2. Field trial conditions

Field trials were performed in 2021 and 2022 to check the yield-related traits and methane emissions (Fig. S1). In 2021, a field trial was conducted in the province of Nanjing, China (32° N, Fig. S1) from July to October. In Nanjing, the average min/max temperatures during the cultivation period were between 22 °C and 35 °C, with an average of 80 % humidity. F6 Plants were grown in the field with a distance of 40 cm from each other. The cultivation area for each line/variety was 1.4 × 8 m^2. In 2022, two separate field trials were conducted from July to October in the cities of Huanggang (31° N) and Jingzhou (30° N), China. The average min/max temperatures in Huanggang were from 27 °C to 37 °C with 69 % humidity, and the temperature in Jingzhou ranged from 24 °C to 36 °C, with 80 % humidity. The field area for each line/variety in the Huanggang and Jingzhou field trials were 3 × 5 m^2.

2.2. Yield-related trait measurements

Yield performance analyses under phytotron conditions were performed by recording panicle length and tiller number of the F6 progeny lines and corresponding parental varieties. More than five biological replicates were used for each analysis. In the Nanjing field trial phenotyping with respect to plant height, tiller number, panicle length, and dry weight was performed with at least eight individual plants of each progeny line and parental variety. Plant height was measured as the height from the soil surface to the tip of the panicle. The tiller number per plant and panicle length were counted manually. To measure panicle dry weight, panicles were randomly selected and weighed using an electronic balance.

2.3. Methane measurements

Methane emissions of the progeny lines and corresponding parental varieties were detected both in the phytotron and the fields. Methane was collected from phytotron grown plants from 2.00 p.m. at the stages of flowering, 15 days after flowering (DAF) and 25 DAF. In the 2021 field trial in Nanjing methane was collected at the flowering stage starting at 10.00 am each collection day. In the Huanggang and Jingzhou field trials in 2022, methane collection was performed six weeks after planting, at the flowering stage, and 15 DAF, starting at 2 pm each collection day.

Sampling for methane emissions was performed using the same method both in the phytotron and in the field trials. In short, individual rice plants were covered with a sealed plastic cylinder (diameter: 15 cm, height: 95 cm). Gas samples were taken from the headspace of the plastic cylinders by a syringe and pooled in a sealed vial (10 ml). In the phytotron emissions from three plants were sampled from each rice line after covering for 10 min. In the Nanjing field trail in 2021, emissions from at least eight randomly chosen plants were sampled from each rice line after covering for 10 min also. In 2022, emissions from at least three independent plants from each line were sampled *i*) six weeks after planting, *ii*) at the flowering stage, and iii) 15 days after flowering (DAF). In i) and iii), samples were collected two times, one at time zero and one 15 min after the plants had been covered with the plastic cylinder. In ii) methane were collected three times, at time zero, 15 min and 30 min after the plants had been covered with the plastic cylinder.

All samples were analysed by gas chromatography and the concentrations were calculated using a standard curve derived from samples with known methane concentrations (10, 20, 250, 1000 and 5000 ppm). An air methane concentration of 1.8 p.p.m. was used as the background for the calculations of methane emissions in the phytotron and in Nanjing field. Methane flux calculation was done as described by (Su et al., 2015).

2.4. qPCR quantification of methanogens communities

To quantify the abundance of methanogenic communities, Rhizospheric soil samples were collected from pots with rice plants grown in the phytotron. The collection started at 2.00 p.m., and each sample was taken from a soil depth of five cm. Subsequently, total genomic DNA were extracted from 500 mg of fresh rhizospheric soil samples according to protocols described in the FastDNA Spin Kit for soil (MP Biomedicals LLC, USA). Next, quantitative real-time PCR (qPCR) were performed using 20 ng of total genomic DNA as a template and the different methanogenic group-specific primers targeting Methanosaetaceae (MST), Methanosarcinaceae (MSC), Methanobacteriales (MBT), Methanomicrobiales (MMB), Methanocella-specific (MCL) and total archaea (ARC) and methanogens (MET) (Narihiro and Sekiguchi, 2011). The samples were analysed on a Bio-Rad CFX qPCR machine (California, USA). Previously cloned 16sRNA gene fragments from different pure cultures of methanogens were used as standards (Narihiro and Sekiguchi, 2011; Westerholm et al., 2011; Su et al., 2015). Primer sequences are listed in (Table S1a) and qPCR programs in (Table S2).

2.5. Measurement of carbohydrates and total organic carbon contents

Leaves, stems, reproductive tissues, and roots at the flowering stage and 9 DAF of progeny lines and parental plants were collected to measure the carbohydrate composition. Each sample was snap-frozen in liquid nitrogen and subsequently ground into a fine powder using cold mortars. Glucose, sucrose and starch were isolated and analysed as described previously (Sun et al., 2005; Jin et al., 2017), according to protocols provided in the kits from Megazyme (Bray, Co. Wicklow, Ireland). Total organic carbon analysis was performed at Agrilab AB (Sweden) using the SS-ISO 10694 method (Anonymous, 2006). Briefly, all aboveground and underground parts of the rice plants were harvested at week six after planting or the flowering stage. The samples were dried for 6 days at +80 °C and ground into a fine powder. Each sample (0.5 g) was analysed using a LECO CN928 machine (USA) which measures the total carbon content in a sample. Samples were purged in the sealed purge chamber, after which gas was removed. The purged sample was transferred automatically to a furnace operated at 1100 °C. To ensure complete and rapid combustion (oxidation) of the sample, the furnace environment is composed of pure oxygen with a secondary oxygen flow directed to the sample *via* a ceramic lance. The combustion gases are swept from the furnace through a thermoelectric cooler to remove moisture and collected in thermostatically controlled ballast volume. Collected gases were equilibrated and mixed in the ballast before a representative aliquot of the gas was extracted and introduced into a flowing stream of inert gas for analysis. The aliquot gas is carried to non-dispersive infrared (NDIR) cells to detect carbon (as carbon dioxide). Calibration was done by using substances with known carbon values. Control samples were used roughly every 10th sample.

2.6. Nuclear Magnetic Resonance (NMR) analysis

To analyse metabolites from soil samples, 100 mg freeze-dried rhizospheric soil from flowering plants grown in the phytotron was dissolved in 8 ml methanol, vortexed well, and sonicated for 10 min at 50–60 Hz, and then vortexed again. The samples were centrifuged at

3000*g* for 10 min and the obtained supernatant was transferred into a new 15 ml Falcon tube. The liquid was subjected to vacuum centrifugation overnight. Subsequently, 380 μl MilliQ water was added to the dried samples, vortexed well, sonicated for 10 min at 50–60 Hz, and vortexed again. After another centrifugation at 3000*g* for 10 min, 350 μl of the supernatant was transferred into a 1.5 ml Eppendorf tube together with NMR analysis solutions: 50 μl D2O, 30 μl Internal Standard (TPS, 5.8 mM), 20 μl MilliQ water, and 150 μl 0.4 M phosphate buffer (pH 7.0). After mixing, 580 μl of the solution was used for NMR analysis, as described previously (Coulomb et al., 2015; Rohnisch et al., 2018). ^{1}H NMR spectra were acquired using a Bruker Avance III spectrometer. The spectrometer operated at 600 MHz and was equipped with a cryogenically cooled probe and autosampler. NMR spectra were recorded with a zgesgp pulse sequence (Bruker Biospin) (25 °C, 128 transients, 4 s relaxation delay, 65,536 data points and spectral width of 17,942).

NMR data were exported into Excel with AMIX (version: 3.9.7). During the export process, bucket width was cut into pieces with 0.01 ppm, and positive intensity mode was used to scale to the reference region from left (0.05 ppm region) to the right (−0.05 ppm). Exported data was then analysed with SIMCA (version 14) with the model of principal component analysis (PCA). Data categorization and model validation was analysed first. Then raw data was used to do volcano analysis (https://huygens.science.uva.nl/VolcaNoseR/) based on the values of Log2 foldchange/-Log10(*P*-value < 0.05) to find the significant difference or most enrichment compounds.

2.7. Glucose treatment

Progeny line rice plants grown for six weeks were used in the glucose treatment experiments. One litre of a 5 mM glucose solution was added to each 10 litre rice cultivation pot every second day for one week to treat potted plants with glucose. After the glucose treatments, methane emissions and soil DNA extractions to estimate the abundance of methanogenic communities were performed as described above.

2.8. Gene expression analysis by quantitative PCR (qPCR) and RNA-seq

Stems and reproductive tissues for RNA extractions were collected at the flowering stage and 9 DAF, starting at 2.00 pm. Four biological replicates were sampled from each tissue and time point. Each sample was snap-frozen in liquid nitrogen and stored until further usage. Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. All the samples were treated with Dnase I (Sigma-Aldrich) to remove trace amounts of DNA contamination. cDNA synthesis and qPCR analysis were performed as previously described (Su et al., 2015). Ubiquitin10 was used as the reference gene. All the primers used are listed in Table S1b. Isolated RNA of stems collected at the flowering stage were stored at −70 °C for further RNA sequencing experiments. Briefly, samples with RIN (RNA Integrity Number) value ≥7 were used to synthesize mRNA libraries using the TruSeq stranded mRNA library preparation kit (Illumina, USA). The resulting libraries were sequenced with paired-end 150 bp using the NovaSeq PE150 platform at Novogene UK, resulting in approximately 6 G of raw data per sample. The original sequencing data were defined as raw reads. The clean reads were generated from the raw reads after removing low-quality reads, mismatches and adaptor sequences. All downstream analyses were based on the cleaned data.

The read data were mapped to the rice MSU v.7.0 reference genome. In the Rice Genome Annotation Project (RGAP) using Hisat2. Differential expression analysis was performed using the DESeq2 R package (Love et al., 2014). The resulting *p*-values were adjusted using the Benjamini-Hochberg approach for the control of the false discovery rate (FDR). The genes with a significant threshold FDR < 0.05 were assigned as being differentially expressed. The DEGs were mapped to KEGG pathways in the Plant GeneSet Annotation Database (http://systemsbiology.cau.edu.cn/PlantGSEAv2/). A Hypergeometric test was used to obtain significant KEGG pathways with FDR < 0.05. Gene annotations were performed and determined using the reference genome from the Rice Genome Annotation Project (RGAP) (http://rice.uga.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/). Venn diagrams were performed on the website (https://bioinformatics.psb.ugent.be/webtools/Venn/). VolcaNoseR (https://huygens.science.uva.nl/VolcaNoseR) was used for volcano plot analysis. SRPLOT (http://www.bioinformatics.com.cn/srplot) were used for creating KEGG pathway diagrams.

2.9. Statistical analysis

Biological and technical triplicates were used for all the experiments except the analysis of yield, in which eight plants of each variety were analysed. Simple analyses were carried out with Microsoft Excel. Further analyses, such as one-way ANOVA, were performed with GraphPad Prism 9.0 (GraphPad Software, USA). Differences between maternal rice and corresponding progeny lines were considered statistically significant when $p < 0.05$.

3. Results

3.1. Conventional crosses yielded three independent rice lines associated with reduced methane emissions

To combine the previously established low methane emission properties of Heijing 5 (Hu et al., 2023) with the high yield properties of modern elite rice varieties, we performed controlled crosses using Heijing 5 (HJ) as a pollen donor and the elite varieties Xiushui (XS), Huayu (HY) and Jiahua (JH) as maternal varieties. In the sixth generation (F6), methane emissions were analysed in the phytotron at different growth stages, *e.g.* at the flowering stage, 15 DAF and 25 DAF (Fig. 1a). The paternal line (♂HJ) emitted 1.3, 4.0 and 7.4 mg m^{-2} h^{-1} at flowering, 15DAF and 25DAF, respectively. The maternal line (♀XS) emitted 15.3 mg m^{-2} h^{-1}, 20.2 mg m^{-2} h^{-1}, and 40.7 mg m^{-2} h^{-1} methane at flowering, 15DAF and 25DAF, respectively. Meanwhile, the line derived from the cross between Heijing 5 and Xiushui (♂HJ × ♀XS) had significantly reduced methane emissions by *ca.* 65 % compared to the maternal plants, and emitted 5.5 mg m^{-2} h^{-1} methane at the flowering stage, 9.5 mg m^{-2} h^{-1} at 15DAF, and 8.6 mg m^{-2} h^{-1} at 25DAF (Fig. 1a). Similar results were also observed for the lines derived from the crosses between Heijing 5 and Huayu (♂HJ × ♀HY), as well as between Heijing 5 and Jiahua (♂HJ × ♀JH) (Fig. S2a), with a methane reduction of 55–75 %.

To test if the low methane properties of the progeny lines also are present under outdoor conditions, we conducted field trials in the summer of 2021 in Nanjing, China and in 2022 in Huanggang and Jingzhou, China. In the Jingzhou trials, the methane emissions of the progeny lines ♂HJ × ♀XS from week six to 15DAF were 1.02 mg m^{-2} h^{-1}, 0.51 mg m^{-2} h^{-1} and 0.02 mg m^{-2} h^{-1}, respectively. The corresponding numbers for the maternal lines were 5.13 mg m^{-2} h^{-1}, 1.42 mg m^{-2} h^{-1}, and 0.07 mg m^{-2} h^{-1}, respectively (Fig. 1b). Hence, the reduction in methane emissions from the progeny lines as compared to the maternal lines in the Jingzhou field trial, was *ca.* 72 % (Figs. 1b and S2d). Similar results were also found in the other field trials both in 2021 and 2022 (Fig. S2b-d).

3.2. Methanogenic archaea communities are reduced in low-methane rice lines

Next, we analysed if the low methane properties of the paternal line Heijing 5 and the progeny lines were associated with a shift in the abundance of the methanogenic archaea communities. qPCR analysis of methanogenic communities in the rhizosphere showed that the paternal variety HJ displayed a significantly lower abundance of most methanogenic communities than the high-yielding XS variety at flowering

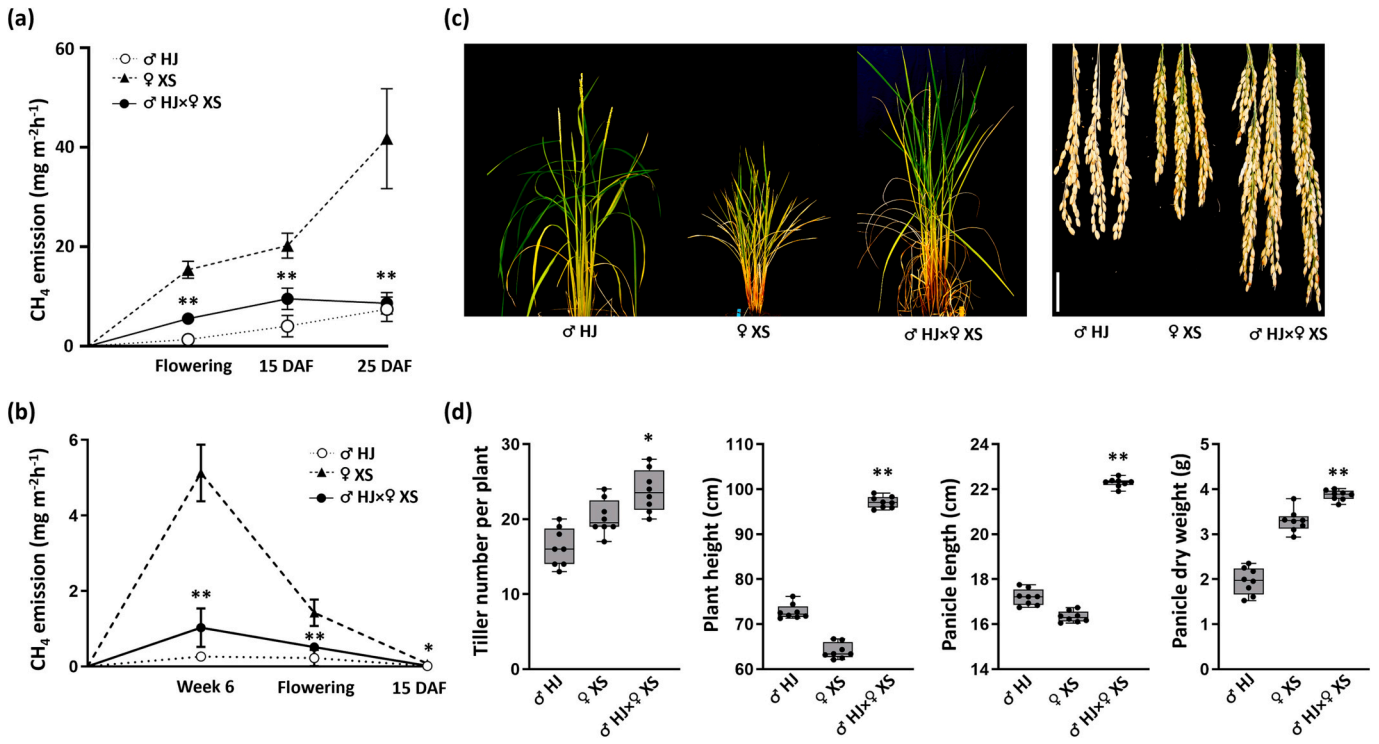


Fig. 1. Methane emissions and yield traits of ♂ HJ × ♀ XS. The micrographs (a-b) show the methane emissions of ♂ HJ × ♀ XS grown in a phytotron (a) and in the field trail of Jingzhou in 2022 (b). The pictures in (c) show the overall growth properties of leaves and tillers in phytotron of the parental lines (♂ HJ and ♀ XS) and the progeny line (♂ HJ × ♀ XS). The micrographs in (d) show the results of the measurements of tiller numbers, plant height, panicle length and panicle dry weight from the field of Nanjing in 2021. One-way ANOVA was used. Error bar represent standard error ($n = 3$ for pot experiment, $n \geq 3$ for methane collection from field and $n = 8$ for yield experiment from field). Bar = 3 cm in c. * and ** indicate significant differences between ♂ HJ × ♀ XS and ♀ XS at $p < 0.5$ and $p < 0.01$, respectively.

and 25DAF (Fig. S3a). The corresponding number for the progeny line ♂HJ × ♀XS was a reduction in most of the analysed methanogenic groups. Similar results were also obtained for the progeny lines between Heijing 5 and Huayu or Jiahua (Fig. S3b and S3c).

3.3. Low methane trait in the progeny lines correlates with an increased yield potential

To estimate if the low methane emission properties also were associated with any changes in the yield potential of the progeny lines, the lines' tiller numbers, plant length, panicle length and panicle dry weight were measured (Figs. 1d and S4). The progeny lines showed a remarkable variation in overall growth and architectural features compared to maternal lines at both the flowering stage and mature stages (Fig. 1c). For ♂HJ × ♀XS from the field of Nanjing in 2021, we detected significant differences in the tiller number, plant height, panicle length and panicle dry weight as compared to the maternal line (Fig. 1d). Similar results were also obtained for the other two progeny lines, except for the average plant height of ♂HJ × ♀HY, which was significantly ($p < 0.01$) reduced compared to the maternal line (Fig. S4a). We also measured tiller number and panicle length in phytotron-grown plants, which displayed similar relative growth characteristics as the field-grown plants, *i.e.,* tiller number and panicle length were all significantly increased in the different progeny lines compared with corresponding maternal varieties (Fig. S5). This indicates that the progeny lines can be used as prebreeding material to generate high-yielding rice cultivars associated with low methane emissions.

3.4. Reduced glucose levels in the soil contribute to reduced methane emissions

Metabolomic NMR analysis were performed to identify potential rhizospheric compound differences of the progeny lines and their corresponding parental lines. Principal component analysis (PCA) of the resulting rhizospheric metabolite profile showed that the paternal, maternal and progeny lines grouped into three distinct clusters (Figs. 2a, S6a and S6d). By comparing the different metabolome profiles, we could identify many compounds with significantly lower levels in the progeny lines than the maternal lines (Figs. 2b, S6b and S6e). This indicates that the overall carbon content was reduced in the belowground of the progeny lines.

The most significantly reduced compounds in the progeny line ♂HJ × ♀XS as compared to the maternal line ♀XS were format, sucrose and glucose. The corresponding compounds in ♂HJ × ♀HY were format and glucose, and in ♂HJ × ♀JH, sucrose and glucose. Hence, glucose levels were consistently lower in all three progeny lines, with a median concentration of 5.6 mM/g as compared to 10.5 mM/g in the maternal lines (Figs. 2c and S6).

To test if external addition of glucose could affect methane emissions, we added 5 mmol glucose to experimental rice pots every second day. After glucose addition, pots with ♂HJ × ♀XS plants emitted methane at a significantly higher rate (3.0 mg m^{-2} h^{-1}) than corresponding pots without glucose treatment (0.5 mg m^{-2} h^{-1}) (Fig. 3a). Furthermore, the methanogenic communities were also significantly increased in glucose treated pots (Fig. 3b). Among the analysed methanogenic groups, genus Methanocellales (MCL) and family Methanosarcinaceae (MSC) were more responsive to glucose (Fig. 3b).

3.5. The progeny lines accumulated organic carbon in leaves, stems and grains

To examine if the parental and the progeny lines differed in their allocation of carbon within a plant, we analysed the total organic carbon in belowground and aboveground tissues six weeks after planting or at

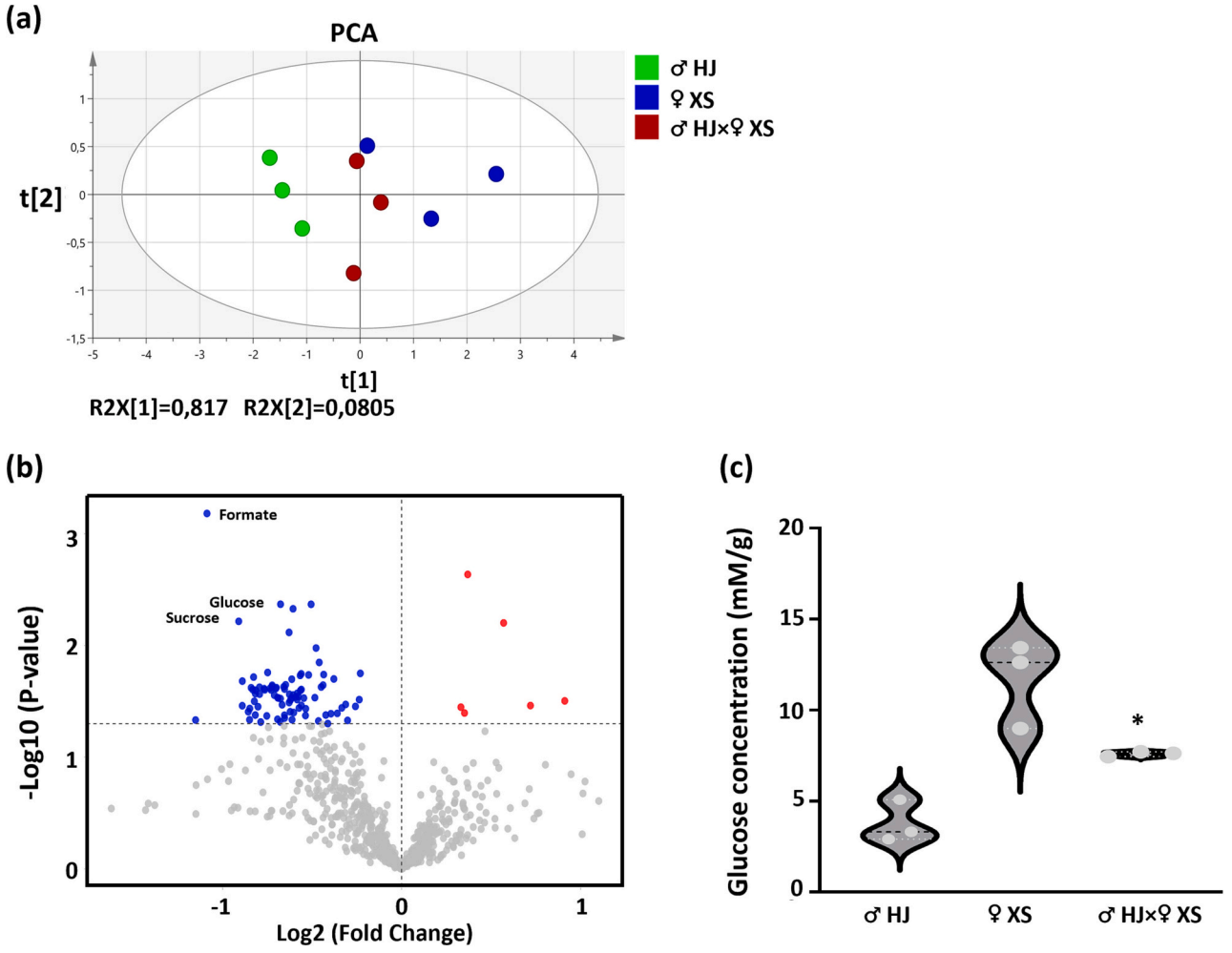


Fig. 2. NMR analysis of metabolic compounds in the rhizosphere of ♂ HJ × ♀ XS. The micrographs show the principal component anaiysis (PCA) plot of the root exudates in (a) and a Volcano plots for all chemical compounds concentration in (b), and glucose concentrations in (c). Each point in (b) represents a compound, blue, red, and grey points show lower, higher and unchanged compound concentrations, respectively, in ♂ HJ × ♀ XS compared to ♀ XS. One-way ANOVA was used (Error bars show SD) in (c). Triplicates were performed in (a), (b) and (c) (n = 3). *p*-value < 0.05 were set up in (b). * and ** indicate significant differences between ♂ HJ × ♀ XS and ♀ XS at $p < 0.5$ and $p < 0.01$, respectively.

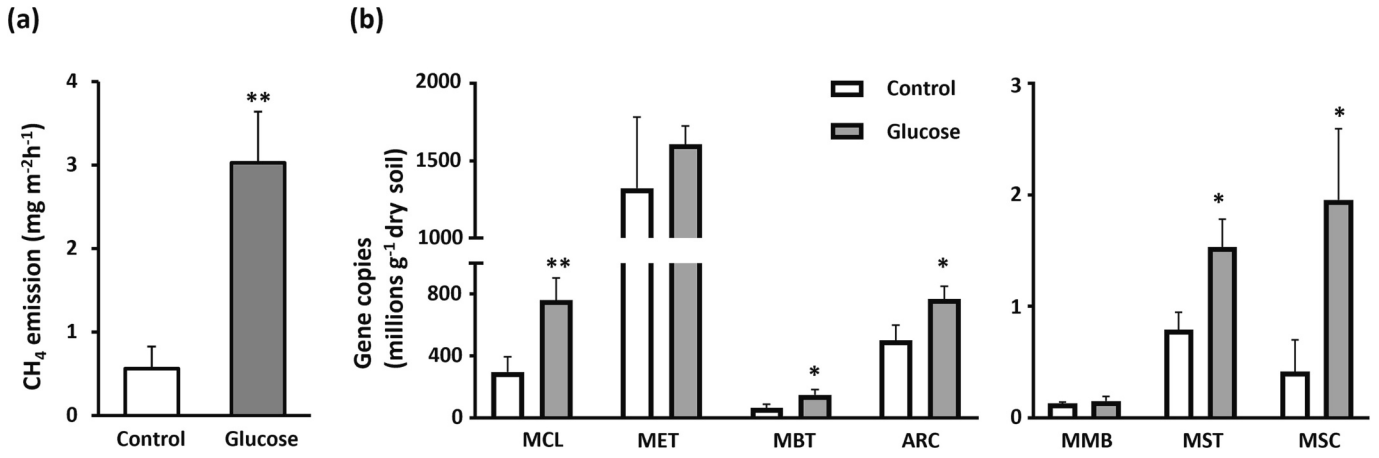


Fig. 3. Quantification of methane emissions (a) and methanogens (b) after glucose treatment of ♂ HJ × ♀ XS pots. Quantification was performed with primesr for total archaea (ARC) and methanogens (MET), families Methanosaetaceae (MST), Methanosarcinaceae (MSC) and genus Methanobacteriales (MBT), Methanomicrobiales (MMB) and Methanocella (MET). One-way ANOVA was used (Error bars show SD). Analysis were peformed in triplicate (n = 3). * and ** indicate significant differences between plants with glucose treatmet and with water at $p < 0.5$ and $p < 0.01$, respectively.

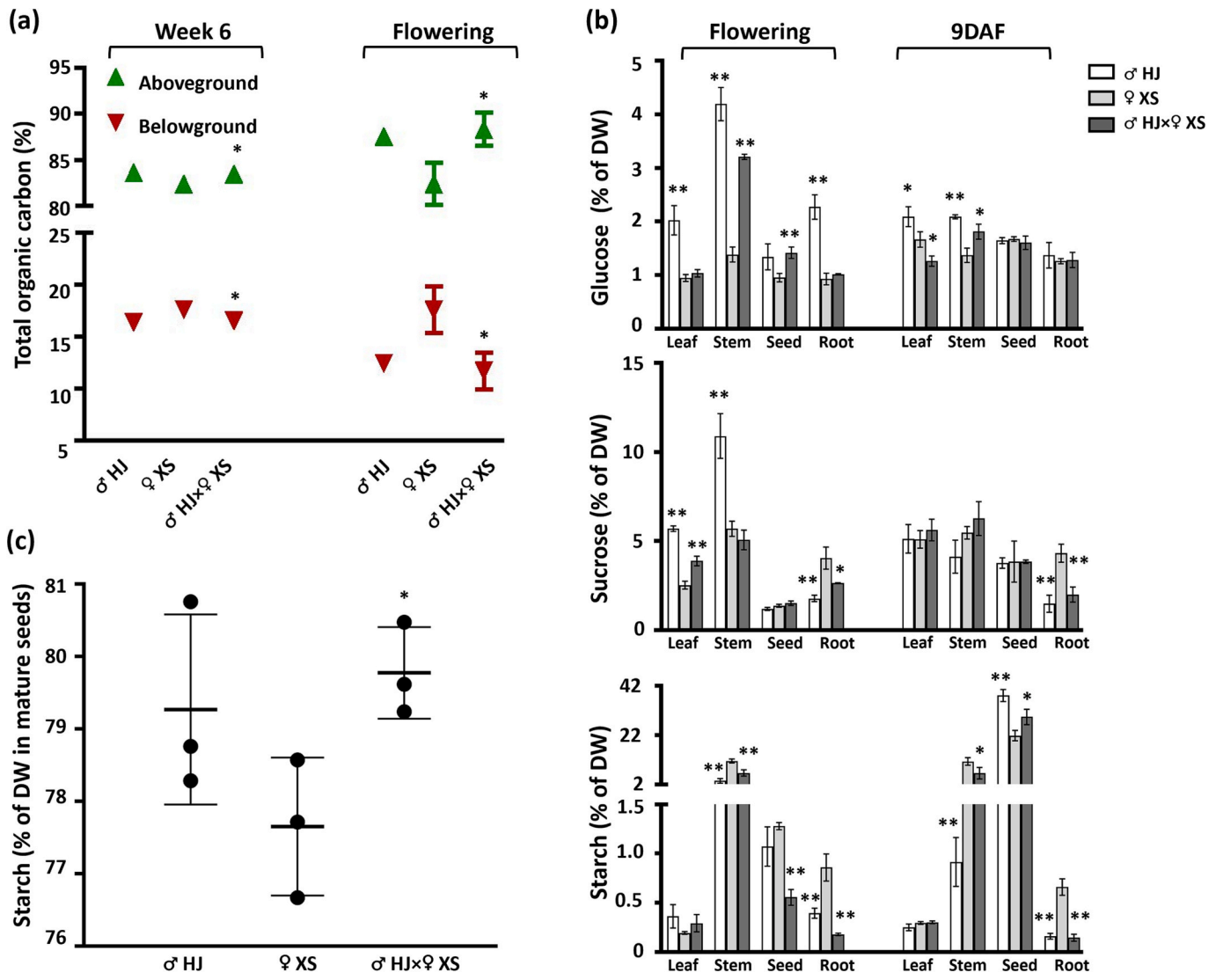


Fig. 4. Measurements of total organic carbon content and carbohydrates in ♂ HJ × ♀ XS plants. The micrographs show the percentage of total organic carbon in (a) and carbohydrates (glucose, sucrose and starch) of ♂ HJ × ♀ XS in aboveground tissues and belowground tissues during flowering and 9DAF in (b). The micrograph in (c) shows the starch content in mature seeds (c). One-way ANOVA was used (Error bars show SD). Triplicates were performed (n = 3). DW indicates dry weight. * and ** indicate significant differences between ♂ HJ × ♀ XS and ♀ XS, ♂ HJ and ♀ XS at $p < 0.5$ and $p < 0.01$, respectively.

the flowering stage (Fig. 4a). In all lines, the aboveground tissues contained >80 % of the total organic carbon (Fig. 4a). In the paternal line (♂HJ) and the progeny line ♂HJ × ♀XS the percentage of total organic carbon allocated to aboveground tissues increased as the plants reached the flowering stage (Fig. 4a). Consequently, the carbon allocated to the roots was reduced compared to the maternal line (Fig. 4a). In contrast, the maternal line, ♀XS, had a stable distribution of total organic carbon between aboveground and belowground tissues over time. More importantly, the progeny line constantly stored more organic carbon in aboveground tissues than the maternal line in week 6 and during flowering (Fig. 4a). Similar trends were also observed in the other two progeny lines (Fig. S7a and S7d).

Additional chemical analysis of the leaves, stems, seeds and roots of the different lines showed that glucose, sucrose and starch mainly accumulated in the stems at flowering (Figs. 4b, S7b and S7e). Interestingly, at this stage, both the paternal line ♂HJ and the progeny line ♂HJ × ♀XS accumulated significantly higher glucose content in stems and seeds as compared to the maternal line. In contrast, less sucrose and starch accumulated in roots (Fig. 4b). At a later stage, 9DAF, the progeny line showed a similar trend as during flowering with higher glucose and lower starch levels in the stems compared to the maternal line. Also, the starch content was significantly increased in the seeds of the progeny line as compared to the maternal line. Whereas sucrose and starch content in progeny line roots were still reduced compared to the maternal line (Fig. 4b). We also noted that the starch content in mature seeds of the progeny line ♂HJ × ♀XS was significantly higher than that in the seeds of the maternal line, 79.7 % and 77.6 % respectively (Fig. 4c). Levels of total organic carbon and carbohydrates followed a similar pattern also in the two other progeny lines ♂HJ × ♀HY and ♂HJ × ♀JH (Fig. S7).

3.6. *Differential expression of genes involved in starch synthesis and sucrose transport*

To further explore the mechanisms underlying the carbon partitioning, we performed a transcriptome analysis using massively parallel sequencing of RNA samples (RNAseq). In the comparison between ♂HJ × ♀XS and ♀XS, a total of 4336 differentially expressed genes (DEGs) were upregulated, whereas 3783 DEGs were downregulated. The corresponding numbers for ♂HJ × ♀HY *vs* ♀HY and ♂HJ × ♀JH *vs* ♀JH, were

5217/1730 upregulated DEGs, and 4571/1779 downregulated DEGs, respectively (Source data 1). Based on the number of DEGs among three comparisons, we found that more genes were differentially expressed in ♂HJ × ♀HY *vs* ♀HY than in the other two comparisons (Fig. S8).

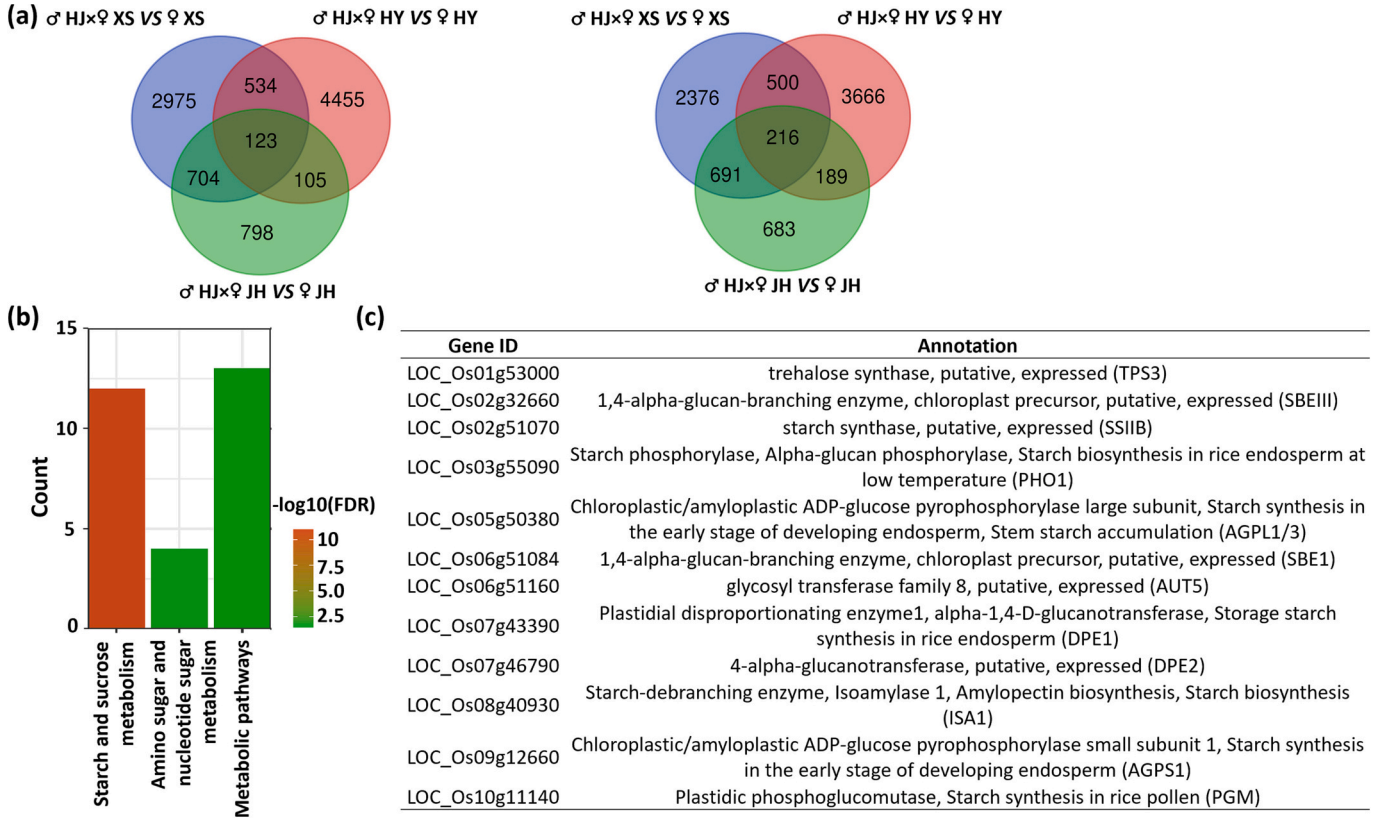
Analysis of KEGG pathways showed that in ♂HJ × ♀XS *vs* XS the pathways of pyruvate metabolism, citrate cycle, butanoate metabolism, glycolysis/gluconeogenesis and glyoxylate and dicarboxylate metabolism were significantly enriched (Fig. S9a). In ♂HJ × ♀HY *vs* ♀HY and ♂HJ × ♀JH *vs* ♀JH, the results showed that pathways related to amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, pentose and glucuronate interconversions, and citrate cycle were enriched in ♂HJ × ♀HY (Fig. S9b), whereas, the pathways related to glycolysis/gluconeogenesis and pyruvate metabolism were enriched in ♂HJ × ♀JH (Fig. S9c). Thus, in all three progeny lines pathways related to organic carbon metabolism were enriched.

Further analyses of the data showed that 123 and 216 genes were commonly upregulated or downregulated in progeny lines' stems, in the three different comparison groups (Fig. 5a and Source data 2). No KEGG pathways were significantly enriched among the 123 genes. However, among the 216 commonly downregulated genes, several KEGG pathways related to carbohydrate and amino acid metabolism were significantly enriched (Fig. 5b). In total, twelve genes related to starch synthesis were identified in this category (Fig. 5c).

Analysis of known genes involved in regulating the carbon flow within a plant (Fig. S10) supported the KEGG pathway analyses and illustrated that genes related to starch synthesis (*e.g. SSs*, *AGPs* and *SBEs*) were down-regulated in stems at the flowering stage of all three progeny lines as compared to the corresponding maternal lines (Fig. 6a). In contrast, the sucrose transport genes (*SUTs* and *SWEETs*) were up-regulated (Fig. 6a). These results were supported by control experiments using quantitative PCR (Figs. 6b and S11). In ♂HJ × ♀XS, seven starch synthesis genes were expressed at low levels in the stem at the flowering stage. In contrast, the same genes were expressed at elevated levels in the reproductive tissues during flowering and 9DAF. At 9DAF, three of the starch synthesis genes (*SS1*, *SBE1* and *ISA1*) were expressed at low levels in stems of ♂HJ × ♀XS, whereas the remaining four (*AGPS2*, *GBSSII*, *PGM* and *HXK2*) were expressed at comparably higher levels. Also, the sugar transport gene *SUT4* was expressed at elevated levels in ♂HJ × ♀XS stems at flowering and 9DAF, while the expression of *SWEET* was slightly decreased at 9DAF (Fig. 6b). The genes involved in regulating the carbon flow showed a similar expression pattern in the comparisons between the two other progeny lines, ♂HJ × ♀HY *vs* ♀HY and ♂HJ × ♀JH *vs* ♀JH (Fig. S11), *i.e.* genes related to starch synthesis expressed at lower levels in the stem before flowering but at elevated in reproductive tissue at flowering and 9DAF. While sucrose transport genes had a higher expression in the stem at the flowering stage.

4. Discussion

The concept of a low-methane, high-yielding rice was suggested already at the beginning of this century (Denier Van Der Gon et al., 2002). Furthermore, it has been shown that higher harvest index varieties are associated with lower methane emissions compared to lower harvest index varieties (Jiang et al., 2017; Kwon et al., 2023). Moreover, we previously reached proof of concept for the transgenic SUSIBA2 rice, a low methane rice variety that accumulated high starch levels in aboveground tissues (Su et al., 2015). However, this transgenic rice has not been commercialized due to a hindering legislative framework in China and Europe. Here, we utilized a naturally occurring low-methane rice cultivar, Heijing 5 (Hu et al., 2023), in a cross with high-yielding elite rice varieties. The resulting progeny lines were associated with approximately 70 % reduction in methane emissions, and a high yield



Gene ID	Annotation
LOC_Os01g53000	trehalose synthase, putative, expressed (TPS3)
LOC_Os02g32660	1,4-alpha-glucan-branching enzyme, chloroplast precursor, putative, expressed (SBEIII)
LOC_Os02g51070	starch synthase, putative, expressed (SSIIB)
LOC_Os03g55090	Starch phosphorylase, Alpha-glucan phosphorylase, Starch biosynthesis in rice endosperm at low temperature (PHO1)
LOC_Os05g50380	Chloroplastic/amyloplastic ADP-glucose pyrophosphorylase large subunit, Starch synthesis in the early stage of developing endosperm, Stem starch accumulation (AGPL1/3)
LOC_Os06g51084	1,4-alpha-glucan-branching enzyme, chloroplast precursor, putative, expressed (SBE1)
LOC_Os06g51160	glycosyl transferase family 8, putative, expressed (AUT5)
LOC_Os07g43390	Plastidial disproportionating enzyme1, alpha-1,4-D-glucanotransferase, Storage starch synthesis in rice endosperm (DPE1)
LOC_Os07g46790	4-alpha-glucanotransferase, putative, expressed (DPE2)
LOC_Os08g40930	Starch-debranching enzyme, Isoamylase 1, Amylopectin biosynthesis, Starch biosynthesis (ISA1)
LOC_Os09g12660	Chloroplastic/amyloplastic ADP-glucose pyrophosphorylase small subunit 1, Starch synthesis in the early stage of developing endosperm (AGPS1)
LOC_Os10g11140	Plastidic phosphoglucomutase, Starch synthesis in rice pollen (PGM)

Fig. 5. Transcriptome analysis. The Venn diagrams in (a) show the number of differentially expressed genes (DEGs) in the comparisons between the three progeny and the corresponding maternal lines. The micrograph in (b) shows the enriched KEGG pathways of downregulated DEGs that overlap among three groups (b). The table in (c) shows gene-IDs and annotations of genes related to starch and sucrose metabolism in b. The filter condition of the Venn diagram and KEGG pathway analysis was FDR < 0.05. The source for the annotation was from the Rice Genome Annotation Project (RGAP).

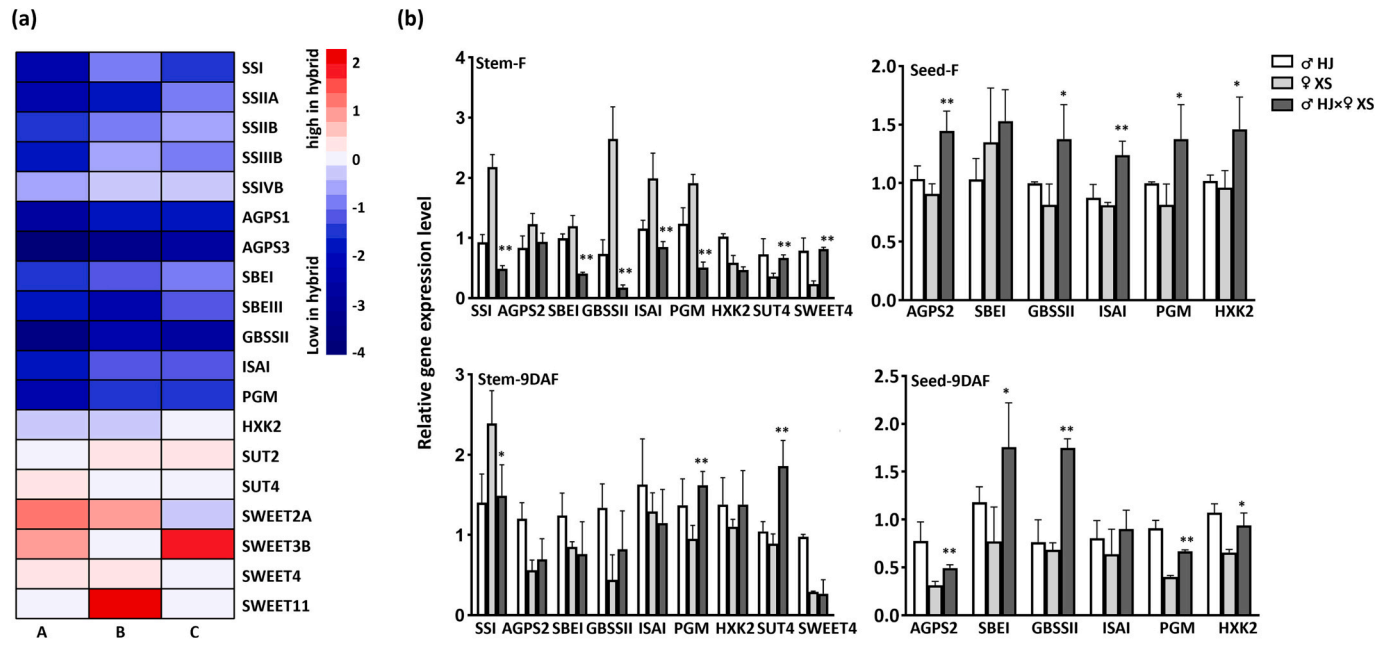


Fig. 6. Expression patterns of genes related to starch synthesis and sucrose transport. The heatmap in (a) shows the relative expression of starch and sucrose genes based on RNAseq data (a) in three progeny lines (A-C). The micrographs in (b) shows the expression levels of the same set of genes estimated using quantitative real-time (qPCR). A, ♂ HJ × ♀ XS *VS* ♀ XS; B, ♂ HJ × ♀ HY *VS* ♀ HY; C, ♂ HJ × ♀ JH *VS* ♀ JH. F, flowering; DAF, day after flowering. One-way ANOVA was used. Error bar represent standard error (n = 3). * and ** indicate significant differences between ♂ HJ × ♀ XS and ♀ XS at $p < 0.5$ and $p < 0.01$, respectively.

potential, compared to the elite varieties. According to FAO, global methane emissions from rice paddies in 2019 contributed to approximately 24 Tg of methane being emitted into the atmosphere (http://www.fao.org/faostat). A theoretical calculation on the possibility of reducing methane emissions using low-methane lines such as the ones presented here indicates that it would be possible to decrease methane emissions by 16.8 Tg annually and that the calculated annual reduction in methane emissions would be equivalent to 420 Tg CO_2 emissions in climate impact terms (Sobanaa et al., 2023).

Controlled crosses between two distinct parental genotypes followed by repeated backcrosses can combine wanted traits from the parental lines into a new inbred and non-segregating progeny line (Hayes and Paroda, 1974; Fei et al., 2022). Here, we aimed to combine the low methane trait displayed by Heijing 5 with the high-yielding capacity of the elite cultivars Xiushui (XS), Huayu (HY) and Jiahua (JH). The resulting inbred F6 progeny lines displayed a high yield potential and were all associated with a reduction of methane emissions by approximately 70 % as compared to the high yielding elite cultivars. Although we cannot, in this study, pinpoint the exact loci responsible for the reduced methane emissions, phenotyping of whole plant grow characteristics, measurements of carbon allocation and transcriptome studies can indicate which physiological processes and mechanism that contribute to the reduction in methane emissions in the progeny lines. Also, when comparing methane emissions the yield potential has to be taken into account since the relevant performance comparator is not methane emission rates per plant but per kg produced rice grain. Therefore, yield parameters such as panicle length, tiller number, starch content, and root architecture which may affect the agricultural performance, have to be evaluated when selecting a progeny line for further breeding activities.

Methane emissions associated with rice cultivation are affected by many factors apart from the plant genotype, *e.g.*, water and soil properties such as salinity and pH, as well management practices (Neue et al., 1996; Mitra et al., 2002; Chandrasekaran et al., 2022). This is reflected in the observed differences in methane emissions rates between plants of the same genotype grown in the phytotron and in the fields. In addition, the timing of methane measurements may affect the emission levels since root exudates are not released uniformly throughout the life of a rice plant. In line with this reasoning, Hou et al. (Hou et al., 2020) and Jiang et al. (Jiang et al., 2022) discovered maximum methane flux at the later tillering stage, whereas others have reported maximum emission rates at the grain-filling stage (Su et al., 2015). Comparing absolute methane rates across experimental sites may therefore be misleading if appropriate controls are not included in the experiments. In this study, all the progeny lines emitted less methane and had a lower abundance of methanogens in the rhizosphere than the maternal lines in both the phytotron and the field experiments, suggesting that the transferred low-methane emission trait displays in all three genotypes irrespectively of cultivation regimes.

The levels and composition of rice root exudates mainly determine methane emissions from rice paddies (Conrad, 2007; Maurer et al., 2018). In the rhizosphere, exudated molecules, such as carbohydrates, are metabolized by anaerobic methanogens into simpler compounds, *e.g.* alcohols, organic acids, CO_2 and H_2 through hydrolysis, fermentation and acetogenesis — the byproduct of the anaerobic metabolism is methane (Krumbock and Conrad, 1991; Chidthaisong et al., 1999; Rajkishore et al., 2015). Interestingly we observed a general reduction of metabolite levels in the rhizospheric soil of the progeny lines compared to the maternal lines, indicating a lower root exudate profile. Specifically, the levels of carbohydrates such as sucrose and glucose were reduced in the rhizosphere of the progeny lines, suggesting that less substrate will be available for the methanogenic communities. In support of this hypothesis, we detected elevated methane production and higher copy numbers of methanogenic communities in pots treated with exogenous glucose. The results presented here align with previous studies, showing that glucose is the preferred substrate for methane production when incubated under anaerobic conditions compared to, *e.g.* acetate (Luo et al., 2022).

During plant growth and development, plants allocate the available carbon source to either the aboveground shoot or the belowground root (Farrar and Jones, 2000; Lynch et al., 2012). The observed reduced levels of root exudates in the rhizosphere of the progeny lines suggest that less carbon was allocated to the root, and as a consequence, more carbon would be available for shoot growth. In turn, this would be

reflected in higher carbon content in the harvested parts of the plant, *i.e.* the grains (Kuzyakov and Gavrichkova, 2010; Ge et al., 2012; Pausch and Kuzyakov, 2018). In our study, we detected elevated levels of total organic carbon in the aboveground tissues of the progeny lines. Analysis of individual polysaccharides demonstrated that glucose levels were elevated in stems and starch was elevated in grains, Indicating that an enlarged pool of glucose eventually leads to increased starch assimilation in the grains, as also suggested by (Wu et al., 2008). Taken together, we demonstrate that the progeny lines allocate more carbon to aboveground tissues than to the roots, which likely leads to the observed reduction of methane emissions and higher yield potential. It is currently debated whether high-yield cultivars provide a feasible way to decrease methane emissions from rice paddy soils (Jiang et al., 2017; Jiang et al., 2019) and meta-analyses have indicated a limited reduction ranging from four to ten % depending on cultivars. In contrast, we report a significant reduction of methane emissions by 70 % in our progeny lines. This stems from the fact that we have used a different breeding strategy that affects the carbon allocation within the progeny lines rather than just focusing on a general yield increase.

However, it is possible that the allocation of carbon tissues in our progeny lines could impact the root performance, especially under stress conditions since the carbon allocation may also affect the growth properties of the roots (Hunt et al., 1990; Lynch et al., 2012) *e.g.*, root length, root number, and the ability to take up nutrients (Zhang et al., 2019; Li et al., 2022). While this merits further studies we have not observed any such issues with the low methane paternal line Heijing 5, which has been commercially cultivated without any reports of reduced root performance (Fei et al., 2020; Hu et al., 2023). Also, based on the calculation of the average yield traits and overall above-ground plant architecture, we can conclude that the root performance of progeny lines is good enough to support plant growth and development, even though the roots of the progeny lines had lower total organic carbon content compared to the maternal varieties.

In our transcriptome analysis, we detected more active processes related to glucose metabolism in the stem, *e.g.* glycolysis, pyruvate metabolism and the citric cycle. The enrichment of genes related to glucose metabolism pathways in the progeny lines supports the notion of a high-yield potential (Nakamura et al., 1989; Sakamoto et al., 2006; Okamura et al., 2021; Lee et al., 2022). It has previously been demonstrated that the cooperation between sugar transport genes and starch synthesis genes increases carbon assimilation to starch in rice grains (Su et al., 2015; Sun et al., 2022). Furthermore, the importance of sucrose transporters (SUT and SWEET) in modulation carbon partitioning has been known to affect source-sink/sink-sink dynamics (Chen et al., 2012; Yang et al., 2018). Therefore, a possible interpretation of the elevated expression of genes involved in sugar transport in the stems and starch synthesis genes in the grains, is that the progeny lines have an altered carbon partitioning with higher strength to push carbohydrates to the grains and there synthesize starch. As a result the progeny lines will reduce starch storage in the root and stems, and increase starch accumulation in the grains. However, if only a greater sugar transport in stem is realized without increasing stach synthesis ability in the grain, assimilated carbon will be accumulated in both stem and root, resulting in higher correspondiong biomass, as reported by Babst et al. (Babst et al., 2021). We note that also Heijing 5 showed the same high sugar transport, and starch synthesis ability as the progeny lines, which would indicate that Heijing 5 also have a high yield potential. However, Heijing 5 is normally grown in the northern parts of China in the temperate region. The adaptation to northern latitude areas requires the rice plants to flower early and reach maturity early, which may contribute to reduced yield traits (Zhao et al., 2018; Hu et al., 2023).

5. Conclusion

We have produced three rice lines with a high yield potential that are associated with low-methane emissions using conventional crosses and selection methods. The rice lines' performance has been tested in field trials for two consecutive years, and we estimate that the lines can reduce methane emissions by *ca.*70 % with sustained yields. Furthermore, here we confirm a negative correlation between grain yield and methane emission, which provides an alternative strategy to breed for a low-methane and high-yield rice variety that can be used in commercial rice cultivation. Since we have used conventional breeding methods in this work, we expect that the low-methane and potentially high-yielding rice lines could be readily used in further breeding programmes globally to reduce methane emissions from rice cultivation and mitigate climate change.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170980.

CRediT authorship contribution statement

Jia Hu: Conceptualization, Data curation, Methodology, Writing – original draft, Funding acquisition, Investigation. **Mathilde Bettembourg:** Investigation. **Lihong Xue:** Investigation. **Ronggui Hu:** Investigation. **Anna Schnürer:** Supervision, Writing – review & editing, Investigation. **Chuanxin Sun:** Funding acquisition, Supervision, Investigation. **Yunkai Jin:** Conceptualization, Methodology, Writing – review & editing, Investigation. **Jens F. Sundström:** Supervision, Writing – review & editing, Conceptualization, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work report in this paper.

Data availability

All data generated or analysed during this study are included in this published article or its supplementary information. The materials are available on request from the corresponding author. The RNAseq raw sequencing data is accessible *via* the National Center for Biotechnology Information (NCBI) database under BioProject ID PRJNA1006562 upon publish.

Acknowledgments

We thank Prof. Dianxing Wu, Prof. Wenfu Chen, Prof. Zhengjin Xu, Prof. Huaibing Li and Prof. Qingyao Shu for providing the rice varieties/lines to screen low-methane rice and elite rice for breeding; Simon Isaksson for methane analysis; Ai Zhang for the asistance of bioinformatic analysis; Ali A Moazzami for the asistance of metabolomic analysis.

Fouding sources

This work was funded by Mr. Zheng Fang, Beijing Xianhe Transportation Technology Co. Ltd.; Trees and Crops for the Future (TC4F); the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS; project no. 2020-01327); Jia Hu obtained a stipend from the China Scholarship Council (file no. 201708430115).

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