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EFFECTS OF NITROGEN FERTILIZER ON PHOTOSYNTHETIC CHARACTERISTICS, C4 PATHWAY, AND RELATED GENE EXPRESSION OF MAIZE VARIETIES WITH DIFFERENT NITROGEN EFFICIENCY

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Abstract

The regulation mechanism of nitrogen fertilizer on the C4 photosynthetic pathway of different nitrogen-efficient varieties, the Xianyu 335 (XY 335) maize variety of high nitrogen efficient type and the Jingnongke 728 (JNK 728) maize variety of low nitrogen efficient type was investigated. Five nitrogen application levels of 120, 180, 240, 300, 360 kg hm^{-2} (NCK) were set, and nitrogen application in local field production was used as control (NCK). The photosynthetic characteristics, C4 photosynthetic pathway key activities, non-enzymatic substances, and key enzyme genes of different genotypes of maize were examined in response to nitrogen reduction. The results showed that the yield of XY 335 reached a high level when the nitrogen application rate was 240-300 kg hm^{-2}; when the nitrogen application rate was 180-240 kg hm^{-2}, the yield of JNK 728 reached a high level. Under low nitrogen conditions, compared with XY 335, the phosphoenolpyruvate carboxylase (PEPC), NADP-malase (NADP-ME), malate dehydrogenase (NADP-MDH), and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) of JNK 728 maintained high enzyme activity. At elongation stage, PEPC and NADP-ME3 genes of XY 335 were significantly up-regulated. Except for the big bell stage, the Rubisco gene of XY 335 was significantly increased at all growth stages. The PEPCs and NADP-MEs genes of the two varieties were significantly up-regulated from the big bell stage to the filling stage. At the jointing stage, the NADP-MDH6 and PPDK1 genes of JNK 728 were significantly up-regulated. At the filling stage, the NADP-MDH gene of JNK 728 was significantly up-regulated. The photosynthetic response mechanism of different maize genotypes to nitrogen fertilizer regulation differed at different growth stages. In conclusion, the results showed that C4 photosynthetic enzyme activity and key gene expression played an important role in improving the adaptability of plants to nitrogen fertilizer at the photosynthetic physiological level in different nitrogen efficient maize.

Key words: Maize, C4 photosynthetic enzymes, Gene expression, Photosynthetic characteristics.

Introduction

Nitrogen is an essential nutrient element for plant growth and development (Huang *et al*., 2015). Nitrogen fertilizer, as an important exogenous input of agricultural production, has greatly contributed to crop yield and income. However, because of soil leaching, microbial consumption and denitrification, the utilization rate of nitrogen fertilizer decreased significantly (Ju *et al*., 2009; Ghassemi-Golezani *et al*., 2015). It has been reported that the average nitrogen fertilizer utilization efficiency in China is about 30%, and that in western developed countries, is about 50-60% (Luo & Zhou, 2019). The loss of nitrogen fertilizer causes serious damage to the surrounding ecosystem and ultimately affect human health (Williamson, 2011). Rational utilization of nitrogen fertilizer is an important measure to achieve green, efficient, and high-yield corn.

C4 plants, such as maize (*Zea mays*), are highly efficient users of light energy, water, and nutrients (Zhu *et al*., 2010). C4 plants can assimilate NH4+ in both mesophyll cells and vascular bundle sheath cells, synthesize amino acids and proteins, and increase their nitrogen utilization efficiency (Mu & Chen, 2021). Although the C4 pathway requires a variety of enzyme proteins to complete catalysis, high catalytic efficiency of these enzymes does not require a large number of proteins. The C4 cycle allows lower total protein and nitrogen, which improves the utilization efficiency of photosynthetic nitrogen. The absorption, assimilation, and redistribution of nitrogen are regulated and controlled by photosynthetic rate (Pn) to a certain extent. Unless Pn also increases, nitrogen utilization efficiency (NUE) will tend to be stable (Sage *et al*., 2011; Bräutigam *et al*., 2014; Zhang *et al*., 2019). Therefore, how to achieve efficient utilization of C4 pathway photosynthetic enzymes and further improve NUE has become a research focus.

In the two-cell C4 system, CO2 enters the MC cytoplasm and is converted to bicarbonate. Under the catalysis of phosphoenolpyruvate carboxylase (PEPC), the bicarbonate is immobilized to form oxaloacetic acid (OAA). Malic acid and OAA produced from the catalytic dehydrogenation of OAA by malate dehydrogenase (NADP-MDH) enter the tricarboxylic acid cycle (TCA) to enhance respiration and provide rich organic acid and carbon skeleton for amino acid synthesis; the other part of OAA forms aspartic acid through transaminase. However, the OAA generated by the reaction of CO2 and PEP catalyzed by PEPC enters TCA, and OAA and malic acid are transformed into each other to supplement the carbon

skeleton consumed in the synthesis of amino acids in time, thereby promoting the synthesis of amino acids and improving NUE (Senthilkumar *et al*., 2019; Swain *et al*., 2021; Wang *et al*., 2021). Tang *et al*., (2015) showed that C4-PEPC transgenic rice plants had higher activities of photosynthetic enzymes and nitrogen metabolic enzymes under low nitrogen conditions. Zhang *et al*., (2019) transferred the complete maize C4-PPDK gene into indica rice IR64 and found that the nitrogen accumulation in flag leaves of transgenic rice plants was higher than that of the control, suggesting that it promoted the absorption of nitrogen from the soil by plants.

For the accumulation and transportation of nitrogen as well as nitrogen use efficiency of different maize varieties, scientists have carried out considerable research on varieties, genotypes, and genetic characteristics (Chardon *et al*., 2010; Huang *et al*., 2022; Gao *et al*., 2023; Wang *et al*., 2022). However, the photosynthetic characteristics and efficient operation mechanism of C4 pathway in maize varieties with different NUE have not been reported. In this study, Jingnongke 728 (JNK 728), a low-nitrogen efficient maize variety, and Xianyu 335 (XY 335), a high-nitrogen efficient maize variety, were selected as test materials. In field and greenhouse pot experiments, different nitrogen application levels were set, and nitrogen application by farmers was used as control. The photosynthetic characteristics, the evolution characteristics of C4 pathway key enzymes, non-enzymatic substances, and key enzyme gene expression of maize plants were explored under nitrogen reduction conditions. The goals were to analyze the physiological regulation mechanism and genetic characteristics of high photosynthetic efficiency of maize varieties with different nitrogen requirements, and provide a theoretical basis for the cultivation and breeding of maize varieties with high photosynthetic nitrogen utilization and the promotion of fertilizer reduction and efficiency.

Material and Methods

Experimental materials and planting: The experimental materials were Jingnongke 728 (JNK 728), a low nitrogen efficient maize variety, and Xianyu 335 (XY 335), a high nitrogen efficient maize variety, which had been previously screened by our research group (Wang *et al*., 2020).

Field experiment: From 2018 to 2021, the field test was conducted in Changli Experimental Station (40º4'N, 118º95'E) of Hebei Normal University of Science and Technology (HNUST), China. The area is located in the east of Hebei Province, with a warm temperate and semi humid continental climate. The soil type of the test site is medium loam. The results of soil physical and chemical properties are shown in (Table S1). Five nitrogen fertilizer levels were set: 120 kg hm^{-2}, 66.66% less nitrogen than NCK (N1), 180 kg hm^{-2}, 50% less nitrogen than NCK (N2), 240 kg hm^{-2}, 33.33% less nitrogen than NCK (N3), 300 kg hm^{-2}, 16.66% less nitrogen than NCK (N4), and 360 kg hm^{-2}, local production nitrogen rate (NCK). The nitrogen fertilizer was controlled by urea, and the superphosphate (120 kg hm^{-2}) and potassium chloride (45 kg hm^{-2}) required for high yield, were applied together. The fertilizer was applied as base fertilizer. The experiment was conducted in a completely randomized block design with three replicates. Well water irrigation was used to prevent the possible interference of nutrients in the irrigation water. Other methods were the same as high-yield cultivation management, and diseases and insect pests were strictly controlled. After the corn had ripened and harvested, the yield of each plot was calculated by single harvesting, single drying, and single weighing. The area is 36 m2, with a planting density of 60000 plants hm^{-2}. The field experiment was used to determine the photosynthetic characteristics, C4 pathway key enzymes, and non-enzymatic substances.

Pot experiment: In 2022, the pot experiment was conducted in the glass greenhouse of Changli Experimental Station of HNUST, China. The pot experimental treatment setting was the same as that of the field test. Each nitrogen application amount was applied to 10 pots. The pot soil was taken from the Changli Test Station. The soil type is medium loamy soil. Each pot of soil weighed 15 kg. Nitrogen, phosphorus, and potassium fertilizers were added according to the design proportion of the experiment. Leaf samples were taken at jointing stage, big bell mouth stage, silking stage, and filling stage. The sixth leaf was taken at elongation stage (ES), the eighth leaf was taken at flare opening stage (FOS), and the twelfth leaf was taken at tasseling stage (TS) and filling stage (FS). Leaves were frozen in liquid nitrogen and placed in a -80°C refrigerator for the determination of gene expression.

Photosynthetic characteristics: At the ES, FOS, TS, and FS of maize, from 9:00 a.m. to 11:00 a.m. on a sunny day, the photosynthetic parameters of plants were measured with the portable photosynthetic meter LI-COR 6400 (LI-COR Inc., Lincoln, NE, USA). Five plants with uniform growth were selected for each treatment and repeated three times to measure the net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO2 concentration (Ci), and transpiration rate (Tr) of leaves.

C4 pathway-related enzyme activity: The activity of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was determined according to Ku *et al*., (1999). The activity of NADP malate dehydrogenase (NADP-MDH, EC 1.1.1.37) was determined according to the method of Tsuchida *et al*., (2001). The activity of NADP malic enzyme (NADP-ME, EC 1.1.1.40) was determined according to the method described by Wang *et al*., (2021). The activity of ribulose 1,5-diphosphate carboxylase/ oxygenase (Rubisco, EC 4.1.1.39) was detected according to the method of Camp *et al*., (1982). The enzyme activity was determined by spectrophotometry, and the absorbance spectrophotometer was measured at 340 nm with UV2600 (SHIMADZU, Kyoto, Japan). UVProbe software (SHIMADZU) was used for data analysis.

C4 pathway non-enzymatic substances

Malate content: Malate content was determined according to the method of Cui *et al*., (2021). HPLC liquid phase conditions were as follows: Agilent 1100 high performance liquid chromatograph, Kromasil C18 reversed phase column (250 mm × 4.6 mm, 5 μm), mobile phase, A: B = 95: 5 (A: phosphate buffer 13 mm KH2PO4, 1 mm K2HPO4; B: 100% methanol), injection volume 10 μL. The flow rate was 1.0 mL min-1, the column temperature was 25℃, the retention time was 50 min, and the wavelength was 325 nm.

Oxaloacetic acid content: The content of oxaloacetic acid was determined by referring to Wang *et al*., (2021). HPLC conditions: Agilent 1100 high performance liquid chromatography, Kromasil C18 reverse-phase column (250 mm × 4.6 mm, 5 μm), mobile phase, A: B = 95: 5 (A: phosphate buffer 13 mm KH2PO4, 1 mm K2HPO4; B: 100% methanol), injection volume 10 μL. The flow rate was 0.8 mL min-1, the column temperature was 25℃, the injection time was 20 min, and the UV wavelength was 214 nm.

Pyruvic acid content: The pyruvic acid content was determined according to Wang *et al*., (2018). The change in absorbance at 520 nm was recorded.

RNA isolation and real-time RT-PCR: The gene sequence of C4 pathway related enzymes was obtained from www.maizegdb.org, and www.ncbi.nlm.nih.gov/. According to the gene sequence in the databases, Primer Premier 5 software was used to design gene primers, and primer sequence comparison was conducted on NCBI to ensure specificity. The internal reference gene was GADPH (Tables S2 & S3). Taq SYBR® Green qPCR test box was used for qRT-PCR. The reaction system was: 10 μL SYBR, 0.8 μL upstream primer, 0.8 μL downstream primer, 0.4 μL ROX Reference Dye, 6 μL sterilized water, mix the reaction system, and 2 μL of 10-fold diluted cDNA template was added. The reaction procedure was: pre-denaturation at 95℃ for 30 s, denaturation at 95℃ for 5 s, amplification at 60℃ for 30 s, and circulation for 40 times. The relative expression of target gene was calculated by the 2-ΔΔct method.

Statistical analysis

Microsoft Excel 2007 and SigmaPlot 12.5 were used to process and plot experimental data, and SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) was used to perform analysis of variance (ANOVA). and a least significant difference (LSD) test was used to compare mean values at a $p<0.05$ threshold of significance.

Table S1. The content of fundamental nutrients in soil.

Year	Organic matter (g kg –1)	Total nitrogen (g kg –1)	alkaline hydrolyzed nitrogen (mg kg –1)	Available phosphorous (mg kg –1)	Available potassium (mg kg –1)
2018	21. 54	1. 51	134. 67	10. 58	85. 02
2019	19. 08	1. 68	102. 35	23. 59	74.10
2020	15.27	1.41	112.41	16.31	83.21
2021	17.41	1.48	121.31	15.32	77.47

Table S2. List of primers used for the Real-time RT-PCR.

Sequence ID	Forward Primer	Reverse Primer
GRMZM2G083841	TCCTCCAAACGAGCCCTAC	AAACTCCAGAAGCCAGCAGA
GRMZM2G473001	CGAGCGGAGGTATCGACTC	GGATTACCCTTGGCGAACA
GRMZM2G129513	TGTTGGTGCGGACTGTGG	TGCGCTTTCGTTTGGTGA
GRMZM2G068455	TTCTTGACTTCACTGGACCCT	ATTGTAGACACCCTTCTGCTTC
GRMZM2G085019	TCCTCCAGTCCATCAAACCTA	CACGAGCACGGATAATCTTCTA
GRMZM2G122479	TGGTTGGTGGACTCAAAGGT	TACAAGGTTGTCAAGGGCTCA
GRMZM2G306345	CTGTGAAGTAGGGGTCCAAGG	CAAGGTGTACCAGAACGCAAA
GRMZM2G162200	AGCCAGGGCCAAAAGTCT	CTTTGCCTCCCCAGATACC

Table S3. Phosphoenolpyruvate carboxylase, NADP-malic dehydrogenase, NADP-malic enzyme, pyruvate orthophosphate dikinase, and Ribulose-1,5-bisphosphate carboxylase/oxygenase genes of *Zea mays*.

Name	Locus name	Location	Chromosome	Transcript length (bp)	Translation length (aa)
PEPC1	GRMZM2G083841	70927112–70932455	9	5344	970
PEPC4	GRMZM2G473001	90705409–91710822	7	5414	967
NADPH-MDH 6	GRMZM2G129513	207808743–207812529	1	3787	432
NADPH-MDH	GRMZM2G068455	86715935–86718298	4	2364	397
NADPH-ME 3	GRMZM2G085019	7184775–7189972	3	5198	636
NADPH-ME 2	GRMZM2G122479	150362627–150375761	6	13135	669
PPDK 1	GRMZM2G306345	157076348–157086887	6	10540	947
Rubisco	GRMZM2G162200	1225439–1227877	4	2439	433

Results

Pn: At the same nitrogen application level, with the development of the growth period, the Pn of high nitrogen efficient variety XY 335 and low nitrogen efficient variety JNK 728 both showed a trend of first increasing and then decreasing. The Pn of both varieties reached the maximum value at the TS, and then began to decline (Table 1). By comparing the Pn of different nitrogen treatments in the same period, the Pn of XY 335 in each growth period reached the maximum value in N4, and was significantly higher than in N1, but not significantly higher than in NCK. The Pn of JNK 728 reached the maximum in N2 at all growth stages and was significantly higher than that in NCK. By comparing the Pn in leaves between both varieties, the Pn of JNK 728 was significantly higher than that of XY 335 at middle and low nitrogen levels. At high nitrogen levels, the Pn of XY 335 was significantly higher than that of JNK 728.

Tr: Under the same nitrogen application level, there were differences in Tr performance among different nitrogen efficient maize varieties (Table 2). Comparing the Tr of different nitrogen treatments at the same period, the Tr of XY 335 reached the maximum in N4 at each growth stage, among which, the Tr of N4 was significantly higher than that of other treatments except NCK at ES and FOS; The Tr difference among treatments did not reach significant level during TS. At the FS, Tr of N4 was only significantly higher than that of N1, and there was no significant difference with other treatments. With increasing nitrogen application, Tr of low nitrogen efficient variety JNK 728 increased first and then decreased at each growth stage. Tr in each growth period of JNK 728 reached the maximum value in N2, and then followed a downward trend. Tr of N2 was significantly higher than that of NCK at ES, FOS, and FS. At the FOS stage, there was no significant difference among the treatments.

Gs: At the same growth period, with increasing nitrogen application, the GS of XY 335 showed a trend of first increasing and then decreasing (Table 3). The maximum value was reached in N4 at each growth stage, which was 9.09%, 25.92%, 44.18%, and 22.22% higher than that of N1 at each growth stage. At the same growth period, with the increase of nitrogen application, the GS of JNK 728 increased first and then decreased. GS of JNK 728 reached the maximum in N2 at ES, FOS, and TS, which were 42.85%, 6.25%, and 11.76% higher than that of NCK, respectively. During the FS, the GS of JNK 728 reached the maximum under N3, which was 26.19% higher than that of NCK.

Ci: At the same nitrogen application level, Ci of XY 335, a high nitrogen efficient variety, decreased first and then increased with the growth period (Table 4). The Ci of XY 335 reached the minimum value in N4 at each growth stage, which was significantly different from the applied amount of nitrogen. With increasing nitrogen application, Ci of low nitrogen efficient variety JNK 728 showed an upward trend in each growth period. The Ci of JNK 728 reached the maximum value in NCK in each growth period. Ci of N2 was significantly lower than that of NCK at ES, TS, and FS; At the FOS stage, the difference among treatments was not significant.

C4 pathway related enzyme activity

PEPC enzyme activity: At the same nitrogen application level, the PEPC activity regulation of the two varieties differed with the growth period (Fig. 1a and b). With increasing nitrogen application, the PEPC activity of XY 335 in each growth period showed an increasing trend. At ES, the PEPC activity of NCK was significantly higher than that of N1 and N2. The PEPC activity of N4 was clearly higher than that of N1 and N2 at the FOS and TS. At FS, the PEPC activity of N3 was significantly higher than that of N1 and had no significant difference with other treatments. The regulation of PEPC activity of low nitrogen efficient variety JNK 728 in different growth stages, the yield of maize was different with different nitrogen application amount. At ES, FOS, TS, and FS, the PEPC enzyme activity of JNK 728 at N2 level was significantly higher than that of NCK by 34.60%, 7.70%, 3.31%, and 9.79%, respectively.

NADP-MDH enzyme activity: At the same nitrogen application level, with the development of the growth period, the NADP-MDH activities of XY 335, a high nitrogen efficient variety, and JNK 728, a low nitrogen efficient variety, showed an upward trend, and reached the maximum at the FS (Fig. 1c and d). The NADP-MDH activity of different nitrogen application levels in the same period were compared. At ES, there was no significant difference in the NADP-MDH activity of XY 335 between different nitrogen treatments. At the FOS stage and FS, XY 335 reached its maximum at N4, which was 3.46% and 10.20% higher than that of NCK, respectively. At the same growth period, with increasing nitrogen application, the NADP-MDH activity of JNK 728 increased first and then decreased. In ES, TS, and FS, the NADP-MDH activity of JNK 728 reached the maxim`um value in N3, which was 34.04%, 31.15%, and 13.22% higher than that of NCK, respectively. At the big bell mouth stage, the NADP-MDH activity of JNK 728 reached the maximum in N2 and was significantly higher than that of NCK.

NADP-ME enzyme activity: At the same nitrogen application level, with the development of the growth period, the NADP-ME activities of XY 335, a high nitrogen efficient variety, and JNK 728, a low nitrogen efficient variety, showed an upward trend, and the NADP-ME activities of both varieties reached the maximum at the FS (Fig. 2a and b). A comparison of the NADP-ME activity of different nitrogen treatments at the same growth stage showed that there was no significant difference in the NADP-ME activity of XY 335 among different nitrogen treatments at the ES. The NADP-ME activity of N4 and NCK was significantly higher than that of N1 at the TS and FS. The NADP-ME activity of JNK 728 showed great difference in the late growth period. The NADP-ME activity of NCK was significantly higher than that of other treatments during TS, and was higher than that of N1 during FS.

Table 1. Effect of nitrogen application rate on net photosynthetic rate of maize with different nitrogen efficiency (μmol m− 2 S− 1).

Variety	Treatment	Elongation stage	Flare opening stage	Tasseling stage	Filling stage
XY 335	N1	29.97 ± 0.61c	32.50 ± 0.73c	32.03 ± 1.13c	25.75 ± 0.82b
	N2	31.67 ± 0.17b	32.76 ± 0.52c	33.34 ± 0.49bc	26.51 ± 0.85b
	N3	32.29 ± 0.50ab	34.78 ± 1.09b	35.27 ± 1.30ab	27.89 ± 2.28b
	N4	33.11 ± 0.27a	37.46 ± 0.16a	37.56 ± 0.54a	35.78 ± 0.60a
	NCK	32.64 ± 0.36a	36.23 ± 0.63a	36.27 ± 0.44ab	34.15 ± 0.49a
JNK 728	N1	33.03 ± 1.63ab	32.34 ± 0.99b	33.51 ± 3.28b	31.84 ± 3.28a
	N2	35.44 ± 1.01a	37.11 ± 1.87a	38.21 ± 0.78a	32.88 ± 0.78a
	N3	30.61 ± 1.35bc	32.54 ± 0.65b	34.67 ± 0.82ab	27.26 ± 0.82b
	N4	28.32 ± 3.03cd	31.84 ± 1.08ab	32.78 ± 0.65b	26.25 ± 0.65bc
	NCK	25.98 ± 1.00d	30.84 ± 1.03b	31.11 ± 1.16b	25.26 ± 1.16c

Different small letters in the same column indicate significant differences among treatments of the same variety ($p<0.05$)

Table 2. Effect of nitrogen application rate on transpiration rate of maize with different nitrogen efficiency [mmol (H2O) m− 2 s− 2].

Variety	Treatment	Elongation stage	Flare opening stage	Tasseling stage	Filling stage
XY 335	N1	3.66 ± 0.18b	4.15 ± 0.12c	4.55 ± 0.11a	5.04 ± 0.02b
	N2	4.10 ± 0.07a	4.52 ± 0.23bc	4.85 ± 0.30a	5.22 ± 0.02ab
	N3	4.19 ± 0.08a	4.60 ± 0.14bc	5.10 ± 0.04a	5.30 ± 0.07ab
	N4	4.28 ± 0.02a	4.98 ± 0.02a	5.14 ± 0.08a	5.39 ± 0.04a
	NCK	4.26 ± 0.17a	4.70 ± 0.12ab	5.11 ± 0.07a	5.19 ± 0.08ab
JNK 728	N1	2.93 ± 0.56ab	3.74 ± 0.13a	3.90 ± 0.56c	5.07 ± 0.40b
	N2	3.44 ± 0.18a	4.08 ± 0.19a	6.30 ± 0.01a	6.74 ± 1.08a
	N3	2.67 ± 0.26ab	4.00 ± 0.07a	4.88 ± 0.23b	5.89 ± 0.32ab
	N4	2.39 ± 0.24ab	3.80 ± 0.33a	3.96 ± 0.43c	5.74 ± 0.05ab
	NCK	2.09 ± 0.78b	3.54 ± 0.17a	3.40 ± 0.30c	5.40 ± 0.28b

Different small letters in the same column indicate significant differences among treatments of the same variety ($p<0.05$)

Table 3. Effect of nitrogen application rate on stomatal conductance of maize with different nitrogen efficiency [mol (H2O) m− 2 s− 1)].

Variety	Treatment	Elongation stage	Flare opening stage	Tasseling stage	Filling stage
XY 335	N1	0.20 ± 0.02b	0.20 ± 0.02c	0.24 ± 0.02b	0.42 ± 0.00c
	N2	0.20 ± 0.01b	0.21 ± 0.02c	0.30 ± 0.07b	0.48 ± 0.05bc
	N3	0.20 ± 0.01b	0.22 ± 0.03bc	0.38 ± 0.02a	0.50 ± 0.01ab
	N4	0.23 ± 0.01a	0.27 ± 0.01a	0.43 ± 0.00a	0.54 ± 0.02a
	NCK	0.22 ± 0.01ab	0.25 ± 0.01ab	0.43 ± 0.02a	0.51 ± 0.01ab
JNK 728	N1	0.19 ± 0.02a	0.33 ± 0.02a	0.33 ± 0.02b	0.42 ± 0.00c
	N2	0.20 ± 0.01a	0.34 ± 0.02a	0.38 ± 0.01a	0.47 ± 0.02b
	N3	0.19 ± 0.01a	0.31 ± 0.00a	0.35 ± 0.02ab	0.53 ± 0.00a
	N4	0.18 ± 0.05a	0.32 ± 0.02a	0.34 ± 0.01ab	0.47 ± 0.00b
	NCK	0.14 ± 0.04a	0.32 ± 0.01a	0.34 ± 0.01ab	0.42 ±0.00c

Different small letters in the same column indicate significant differences among treatments of the same variety ($p<0.05$)

Table 4. Effect of nitrogen application rate on intercellular CO2 concentration in maize with different nitrogen efficiency [μmol（CO2) mol −1].

Variety	Treatment	Elongation stage	Flare opening stage	Tasseling stage	Filling stage
XY 335	N1	265.47 ± 10.19a	273.60 ± 8.64a	278.97 ± 8.80a	255.57 ± 2.32a
	N2	248.42 ± 5.29ab	270.20 ± 5.46a	274.55 ± 5.39ab	244.63 ± 1.13ab
	N3	235.77 ± 6.46c	265.25 ± 3.29a	266.66 ± 4.28abc	247.37 ± 1.31a
	N4	215.69 ± 9.74c	224.41 ± 16.37b	256.66 ± 8.18c	236.74 ± 6.59b
	NCK	225.08 ± 3.42bc	230.47 ± 10.85b	257.14 ± 9.39bc	244.74 ± 2.36a
JNK 728	N1	228.68 ± 14.03b	240.13 ± 7.23a	264.38 ± 0.35bc	250.92 ± 5.50b
	N2	228.95 ± 2.04b	242.42 ± 1.15a	255.47 ± 1.55cd	257.02 ± 2.10b
	N3	229.39 ± 9.61b	242.15 ± 6.81a	255.53 ± 2.40d	248.51 ± 4.78b
	N4	243.37 ± 14.26ab	255.48 ± 12.64a	274.44 ± 2.61b	252.23 ± 4.79b
	NCK	264.15 ± 2.55a	266.51 ± 14.34a	290.09 ± 9.76a	275.10 ± 4.59a

Different small letters in the same column indicate significant differences among treatments of the same variety ($p<0.05$)

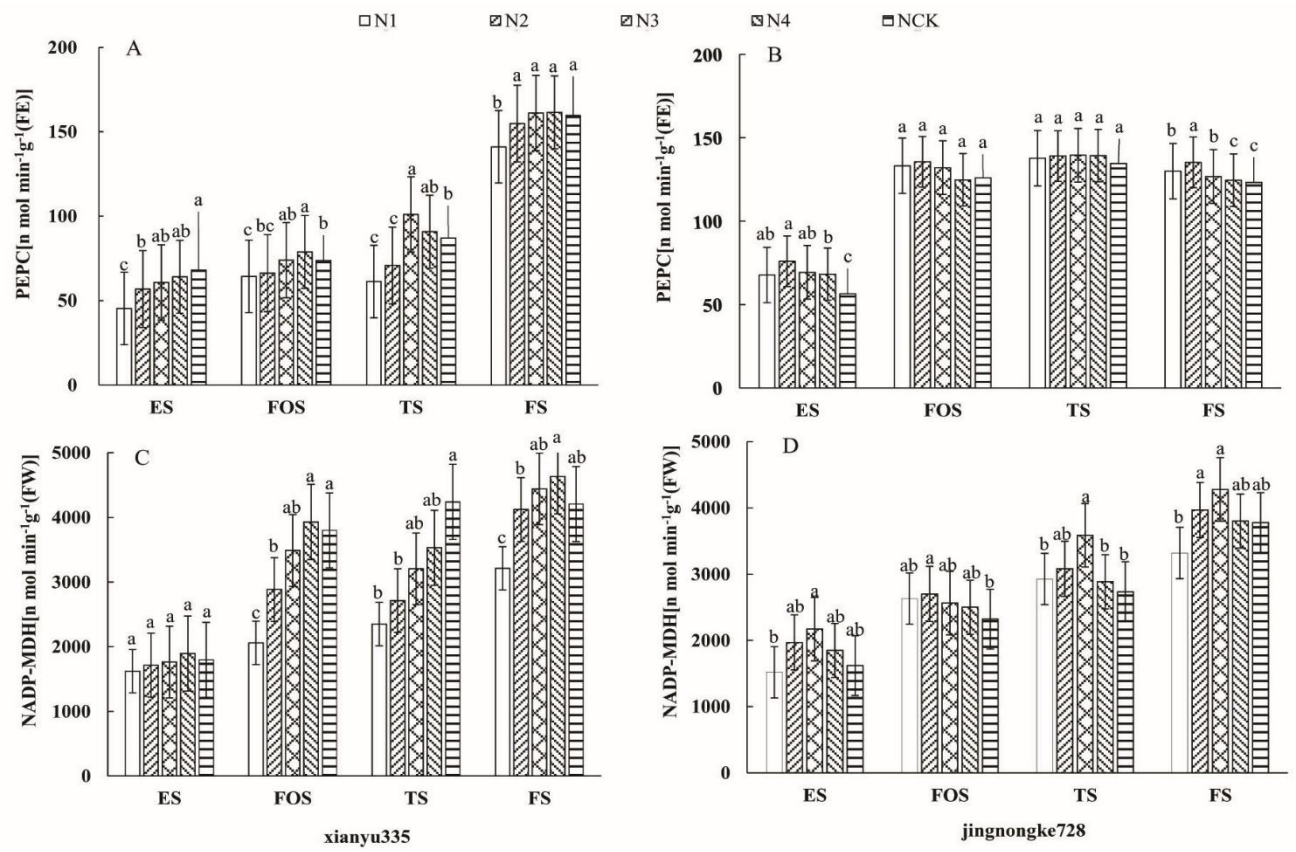

Fig. 1. Effect of nitrogen application rate on the phosphoenolpyruvate carboxylase (PEPC) (A, B) and NADP-malic dehydrogenase (NADP-MDH) (C, D) in leaves of sweet maize seedlings. Vertical bars represent the SE (n = 3). Small letters (a,b) indicate differences between values obtained on different days after nitrogen treatment ($p<0.05$) according to a least significant difference (LSD) test.

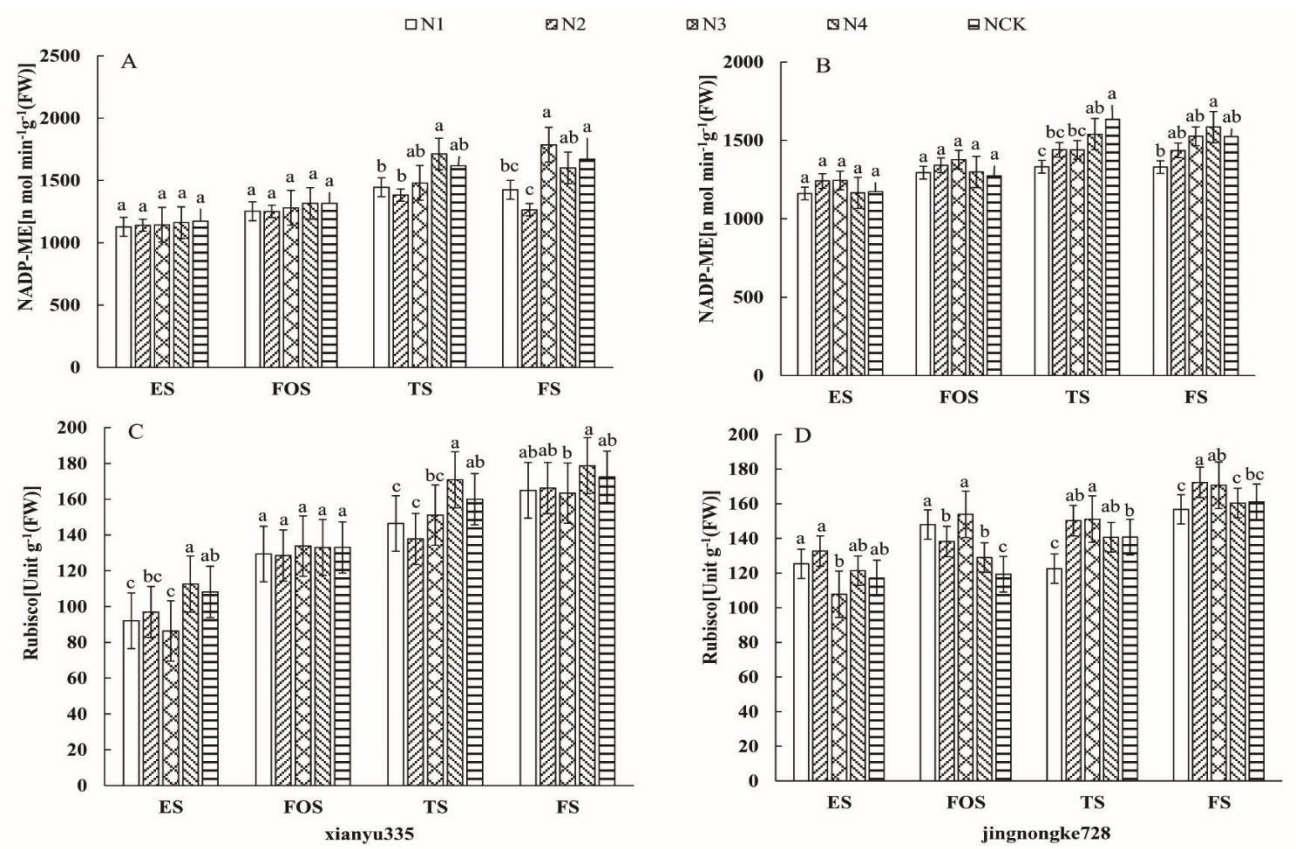

Fig. 2. Effect of nitrogen application rate on the NADP-malic enzyme (NADP-ME) (A, B) and Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (C, D) in leaves of sweet maize seedlings. Vertical bars represent the SE (n = 3). Small letters (a,b) indicate differences between values obtained on different days after nitrogen treatment ($p<0.05$) according to a least significant difference (LSD) test.

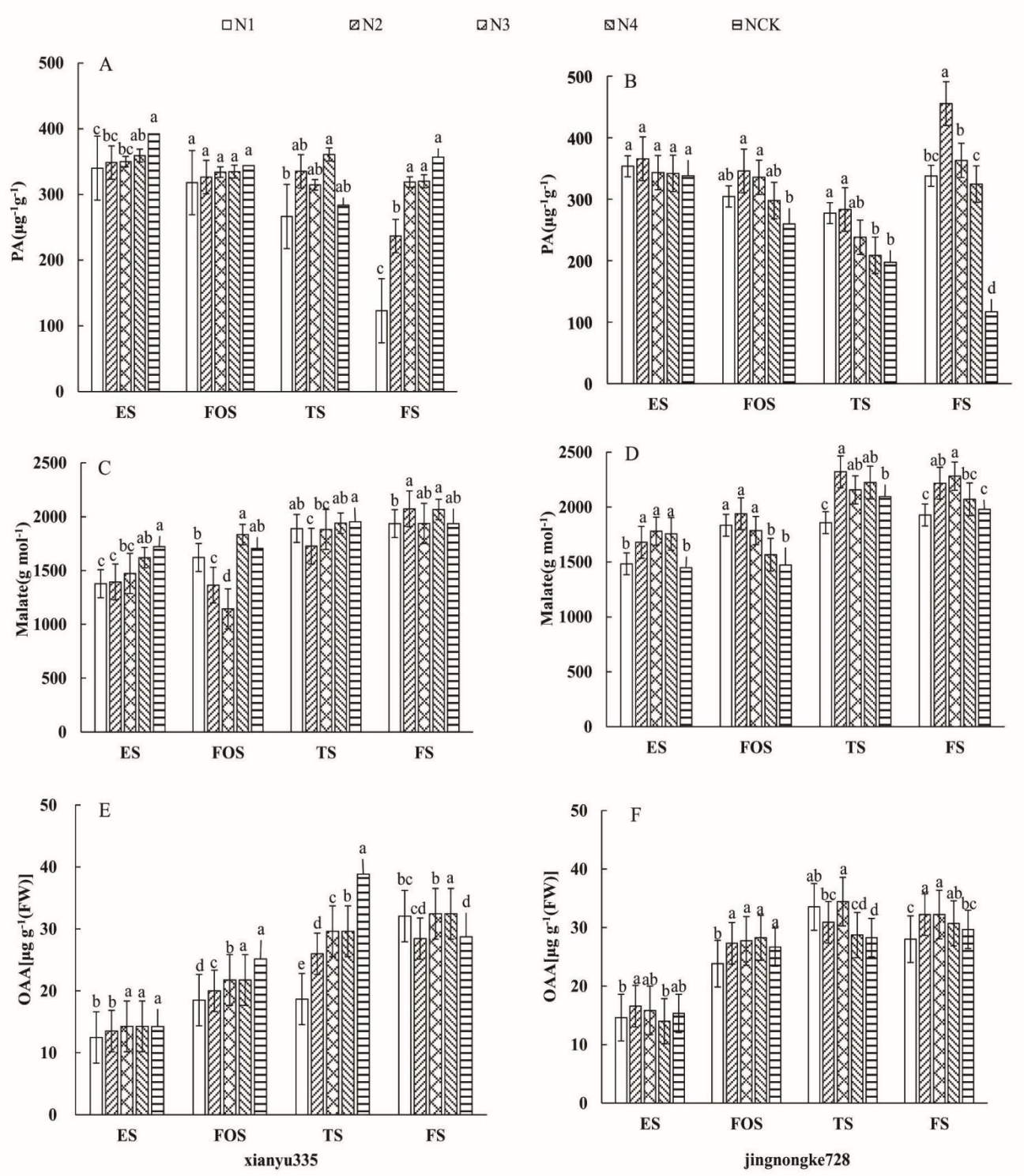

Fig. 3. Effect of nitrogen application rate on the pyruvate (PA) (A, B), malate (C, D), and oxaloacetic acid (OAA) (E, F) in leaves of sweet maize seedlings. Vertical bars represent the SE (n = 3). Small letters (a,b) indicate differences between values obtained on different days after nitrogen treatment ($p<0.05$) according to a least significant difference (LSD) test.

Rubisco enzyme activity: At the same nitrogen application level, the Rubisco activity of the two varieties showed an increasing trend with the growth period and reached the maximum value at FS (Fig. 2c and d). The Rubisco activities of different nitrogen treatments at the same growth stage were compared. Except for the FOS, the Rubisco activities of XY 335 were significantly different among nitrogen application rates. The Rubisco activities of N4 at ES, TS, and FS were significantly higher than those of N1 by 22.36%, 16.66%, and 8.37%, respectively. There were significant differences in nitrogen regulation on Rubisco activity at different growth stages of JNK 728. The activity of Rubisco of JNK 728 reached the maximum at N2 and was significantly higher than that of NCK at ES and FS. The activity of Rubisco of JNK 728 reached the maximum at N3, which was significantly higher than that of NCK by 29.00% and 7.38%, respectively, at FOS and TS.

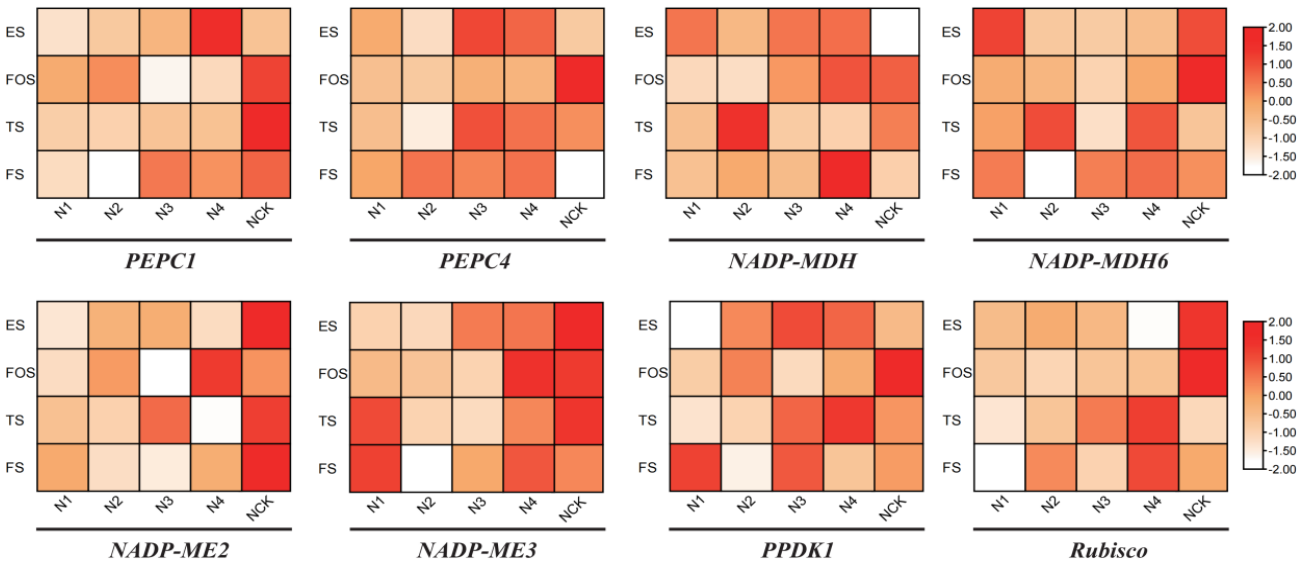

Fig. 4. The transcriptional level of C4 pathway enzymes in XY335 leaves under different nitrogen rates. ES, Elongation stage; FOS, Flare opening stage; TS, Tasseling stage; FS, Filling stage.

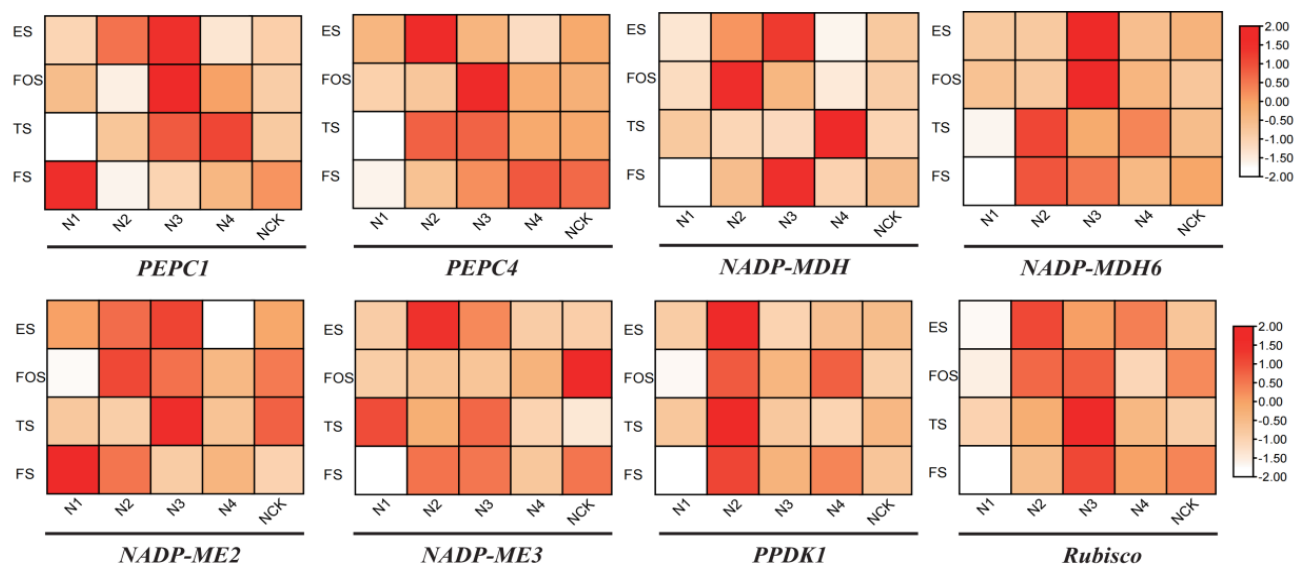

Fig. 5. The transcriptional level of C4 pathway enzymes in JNK 728 leaves under different nitrogen rates. ES, Elongation stage; FOS, Flare opening stage; TS, Tasseling stage; FS, Filling stage.

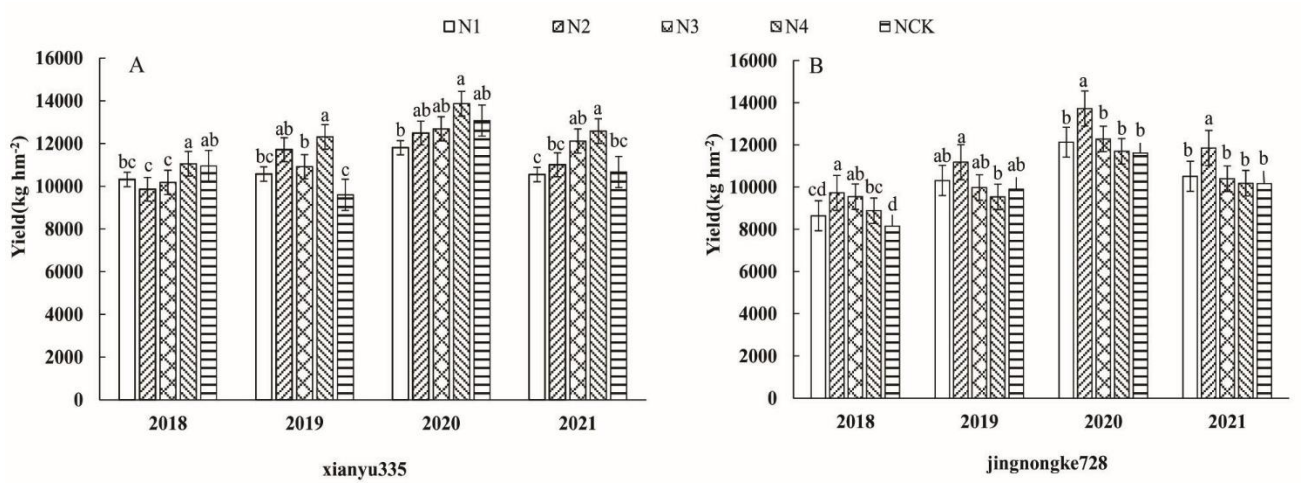

Fig. 6. Effect of nitrogen application on corn yield. Vertical bars represent the SE (n = 3). Small letters (a,b) indicate differences between values obtained on different days after nitrogen treatment ($p<0.05$) according to a least significant difference (LSD) test.

Non-enzymatic substance content

Pyruvate content: The regulation of nitrogen on PA content of the two varieties was different (Fig. 3a and b). Under the same nitrogen application rate, with the development of growth period, the PA content of high nitrogen efficient variety XY 335 showed a downward trend, and the PA content of low nitrogen efficient variety JNK 728 also showed a downward trend. The PA content of Different nitrogen treatments in the same growth period were compared. Except for the FOS, there are obvious differences in PA content among the nitrogen application rates of XY 335. The PA content of N4 was significantly higher than that of N1 at ES, TS, and FS. At ES, there was no difference in PA content of each nitrogen application rate of JNK 728. At FOS and TS, the PA content of N2 was markedly higher than that of NCK, and at FS, the PA content of N3 was significantly higher than that of NCK.

Malate: The regulation of nitrogen application rate on malate content of XY 335 in different growth periods was different (Fig. 3c). At TS, the malate content of NCK was noticeably higher than that of N2; During the ES and FOS periods, the malate content of N4 was significantly higher by 16.16% and 34.44% compared to N2, respectively. With increasing nitrogen application, the malate content of JNK 728 generally increased first and then decreased at each growth stage (Fig. 3d). At ES and FS, the malate content of JNK 728 at N2 level significantly higher than that of NCK by 15.93% and 12.00%, respectively. At FOS and TS, the malate content of JNK 728 at N3 level significantly higher than that of NCK by 21.33% and 3.00%, respectively.

OAA: The regulation of nitrogen application rate on OAA content of XY 335 in different growth periods differed (Fig. 3e). At ES, the OAA content of N4 was significantly higher than that of N1 and N2; the OAA content of N4 was significantly higher than that of N1 at FOS and FS; the OAA content of NCK was significantly higher than that of other treatments during TS. With increasing nitrogen application, the OAA content of JNK 728 generally increased at first and then decreased at each growth stage (Fig. 3f). At ES, FOS, and FS, the OAA content of JNK 728 was higher at N2 level, which was 8.04%, 2.44%, and 8.57% higher than that of NCK, respectively.

Gene expression: The expression levels of key enzyme genes of C4 photosynthetic pathway were determined (Fig. 4 and 5). The results showed that the relative expression levels of PEPC1, NADP-ME3, and Rubisco genes of XY 335 were significantly upregulated during ES. At the FOS, PEPC4, NADP-ME2, NADP-ME3, and PPDK1 of XY 335 were significantly upregulated. During TS, PEPC4, NADP-MDH6, NADP-ME2, PPDK1, and Rubisco genes of XY 335 were significantly upregulated. During the FS, PEPC4, NADP-MDH6, PPDK1, and Rubisco genes of XY 335 were significantly upregulated. NADP-MDH was only significantly upregulated in the FOS.

The relative expressions of NADP-MDH6, PPDK1, and Rubisco genes in JNK 728 were significantly upregulated throughout the growth period. The relative expressions of PEPC1, NADP-ME2, NADP-ME3 genes in JNK 728 were significantly up-regulated at the FOS. Other genes were significantly downregulated during this period; PEPC1 and PEPC4 genes of JNK 728 were significantly upregulated during TS. During the FS, PEPC4, NADP-MDH, NADP-ME3 genes of JNK 728 were significantly upregulated.

Yield: In 2018-2021 (Fig. 6), the yield of low-nitrogen high-efficiency variety JNK 728 was between 8158.22-13724.12 kg hm^{-2}, and that of high-nitrogen high-efficiency variety XY 335 was between 9898.81-13865.16 kg hm^{-2}. In 2018-2021, the yield of XY 335 reached the maximum at the N4, which was 7.15%, 14.84%, 17.46%, and 19.29% higher than that of N1, respectively. In 2018-2021, the yield of JNK 728 reached the maximum at the N2, which was 19.21%, 11.19%, 18.18%, and 16.59% higher than that of NCK.

Discussion

In modern crop production systems, nitrogen plays an important role in crop yield formation; It is the largest mineral element required by plants, and also a nutrient element that limits crop growth (Castro-Rodríguez *et al*., 2017; Luo *et al*., 2019). Understanding the characteristics of nitrogen demand of different nitrogen efficient varieties will help to better conduct nitrogen fertilizer field management for different nitrogen efficient varieties and reduce excessive nitrogen fertilizer application. Our previous research results showed that the nitrogen content of XY 335, a high nitrogen efficient variety, was higher at N3-N4 levels in each growth stage, and the nitrogen content of each organ of the plant decreased as nitrogen application continued to increase to NCK level. The results of this study showed that the yield of XY 335 reached the maximum at the level of N3-N4 in 2018-2021, and then declined. This shows that under high nitrogen level, especially in the late growth stage, XY 335 can fully exert its absorption and utilization capacity of nitrogen fertilizer to achieve high yield. The yield of JNK 728, a low nitrogen efficient variety, reached the maximum value at the level of N2-N3, and the nitrogen content of the plants in each growth stage was high. This shows that under the condition of nitrogen reduction, JNK 728 has better adaptability to low nitrogen.

Nitrogen in leaves is mainly distributed in the photosynthetic protein complex, thus affecting photosynthesis (Han, 2011; Wu *et al*., 2019). Photosynthesis is also very sensitive to various environmental changes, especially to the availability of nitrogen, because about 75% of nitrogen in leaves is located in chloroplasts and is used to synthesize compounds related to photosynthesis (Wang *et al*., 2015; Onoda *et al*., 2017). In this study, at the level of N4, the Pn, Gs, and Tr of high nitrogen efficient variety XY 335 were palpably higher than those of other treatments, while Ci was remarkably lower than those of other treatments.

Under N2 level, Pn, Gs, and Tr of low nitrogen efficient variety JNK 728 reached the maximum, which was conspicuously higher than other treatments, Ci reached the minimum, and the yield was significantly higher than in other treatments. This suggests that under the optimal nitrogen application level, different nitrogen efficient maize varieties are more conducive in improving the photosynthetic rate of maize, thereby improving dry matter accumulation and yield (Wu *et al*., 2019). The change trend of Pn and Ci is opposite, which may be because under the optimal nitrogen application level, CO2 is effectively absorbed and used for carbon assimilation when the photosynthetic rate is high, while CO2 accumulated in maize treated with NCK with low photosynthetic rate is not effectively absorbed and used to produce photosynthetic products, which also indicates that the appropriate nitrogen application rate plays a positive role in the regulation of the photosynthetic rate (Mu *et al*., 2016; Wei *et al*., 2016). For JNK 728, a low nitrogen efficient variety, reducing nitrogen application can delay leaf senescence, reduce heat dissipation, improve the transfer rate of photosynthetic capacity and the operating efficiency of photosynthetic apparatus, thus ultimately improving grain yield.

The PEPC enzyme is the primary carboxylase of C4 plants, which catalyzes the carboxylation of PEP using bicarbonate to produce OAA (Lepiniec *et al*., 2003; Gao *et al*., 2018; Huang *et al*., 2013; Ludwig, 2013). This study showed that the PEPC enzyme activity of low nitrogen efficient variety Jingnongke 728 increased first and then decreased, reaching the maximum at the level of N2-N3. PEPC enzyme activity of XY 335, a high nitrogen efficient variety, showed an upward trend and reached the maximum at N4 level. At the same time, the expression level of the PEPC1 gene in XY 335 was significantly upregulated at ES. Except in ES, PEPC4 of XY 335 was significantly upregulated in other stages. The expression level of PEPC gene of JNK 728 was significantly upregulated in FOS; PEPC1 and PEPC4 genes of JNK 728 were significantly upregulated during TS. During FS, the PEPC4 gene of JNK 728 was significantly upregulated. A previous study showed that excessive production of PEPC had a positive impact on photosynthesis and yield of transgenic plant lines. C4-PEPC transgenic plants are more tolerant to low nitrogen levels and have high level parameters related to the function of photosystem II (PSII) and relatively more stable PSII structure (Tang *et al*., 2018). Wei *et al*., (2013) showed that C4-PEPC transgenic plant lines were more tolerant to low nitrogen, and relevant parameters of PSII were significantly improved, making the structure of PSII more stable. In addition, C4-PEPC transgenic plants have higher levels of carbon and nitrogen enzyme activity under low nitrogen field conditions (Tang *et al*., 2018). The results of the present study showed that the upregulated expression of the PEPC gene enhanced the enzyme activity, thereby improving the photosynthetic capacity of the variety under the optimal nitrogen level, thus achieving high yield.

NADP-MDH catalyzes the conversion of OAA to malate acid (Doubnerova & Ryslava, 2011; Crecelius *et al*., 2003). In this study, NADP-MDH activity and gene expression of JNK 728 were significantly increased at the N2 level in each growth period. At the N4 level, the NADP-MDH activity and relative gene expression of XY 335 increased significantly in each growth stage. Thus, the transformation of OAA to malic acid under the optimal nitrogen application level was effectively improved. The increase of NADP-MDH activity can effectively improve the photosynthetic capacity of C4 plants. It has been reported that there is a significant correlation between PEPC and NADP-MDH activities and soybean yield (Huang *et al*., 2013). The present research shows that under the optimal nitrogen application level, NADP-MDH activities of the two varieties are significantly positively correlated with Pn, and ultimately effectively improve corn yield. In addition, under various abiotic stresses, enhancing NADP-MDH activity and gene expression can effectively increase the OAA content in plants (Zhang *et al*., 2019; Wang *et al*., 2021). The results of this study indicate that under the optimal nitrogen application level, increasing the NADP-MDH activity of both varieties effectively improves the content of OAA, thus promoting the transformation of OAA to malate, maintaining a high electron transport rate and NADPH/NADP+ ratio, and improving photosynthesis.

NADP-ME is also one of the key enzymes of the C4 pathway, which can catalyze the decarboxylation of malic acid, and the released CO2 is enriched around Rubisco, thus promoting the carboxylation reaction of Rubisco, reducing its oxygenation reaction, and improving the photosynthesis efficiency (Ryšlavá *et al*., 2007). Ku *et al*., (2001) suggested that the activity of the NADP-ME gene was negatively correlated with the rate of photorespiration. Transferring the NADP-ME gene into C3 plants may be an effective way to reduce photorespiration of C3 plants and improve their photosynthetic efficiency. The NADP-ME activity was significantly regulated by nitrogen fertilizer, especially in the middle and late stages of wheat growth. Under the appropriate nitrogen application level, wheat NADP-ME activity significantly increased (Bachir *et al*., 2017; Zhang *et al*., 2019). This study showed that the NADP-MEs gene expression and NADP-ME enzyme activity of the two varieties increased significantly under the appropriate nitrogen application level. This showed that under the suitable nitrogen application level, the different nitrogen efficient varieties effectively increased the NADP-ME activity and further improved the plant photosynthetic efficiency. This is consistent with research by the WHO, which showed that an increase of NADP-ME enzyme activity by 2 to 3 times can improve the photosynthetic efficiency and chlorophyll accumulation of transgenic rice.

In C4 plants, Rubisco is a bifunctional enzyme; it both catalyzes the carboxylation and oxygenation of ribose-1,5-diphosphate (RuBP) and regulates the relationship between them (Patel & Berry, 2008). In this study, the Rubisco enzyme activity of low nitrogen efficient variety JNK 728 reached the maximum at the level of N2-N3, and the Rubisco gene was significantly upregulated at ES, TS, and FS. The PEPC enzyme activity of XY 335, a high nitrogen efficient variety, showed an upward trend and reached the maximum at the N4 level. The Rubisco gene was significantly upregulated at all growth stages. Gutiérrez *et al*., (2013) showed that the

Rubisco activity of wheat significantly increased under the optimal nitrogen application level. Low concentration of CO2 in the air may reduce the activity of Rubisco, but sufficient supply of nitrogen fertilizer can compensate for the effect of CO2 reduction, promote the high expression of the Rubisco gene in leaves, and maintain the high activity of Rubisco in leaves, thus increasing the Pn of leaves. These results demonstrate that the expression level and activity of the Rubisco gene in leaves could be effectively regulated under the optimal nitrogen application level of different nitrogen-efficient varieties, to maintain a high level of photosynthetic efficiency.

Conclusion

Differences were found in nitrogen uptake and utilization among different nitrogen efficient maize varieties. The yield of XY 335, a high nitrogen efficient variety, reached the maximum when the nitrogen application level was 240-300 kg hm^{-2}. The yield of JNK 728, a low nitrogen efficient variety, reached the maximum when the nitrogen application rate was 180-240 kg hm^{-2}. Optimizing the nitrogen distribution in leaves of different nitrogen efficient maize may be an adaptive mechanism to maximize the nitrogen utilization efficiency of photosynthesis. Under the optimal nitrogen application level, both varieties maintained high Pn, Gs, and Tr. The gene expression level and enzyme activity of PEPC, NADP-MDH, NADP-ME, and Rubisco, key enzymes of the C4 pathway and nitrogen storage banks, were significantly increased, thus enhancing the photosynthetic capacity of both varieties. It is suggested that the key enzymes of the C4 photosynthesis pathway regulated by nitrogen play an important role in enhancing plant photosynthesis.

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