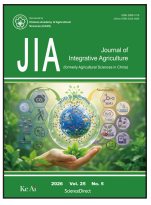
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RESEARCH ARTICLE

Cytokinins redistributing drives nitrogen remobilization from source to sink in wheat under moderate water limitation during grain filling

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Highlights

- Post-anthesis moderate water limitation enhances source-to-sink nitrogen remobilization, significantly boosting grain yield and nitrogen use efficiency in wheat.
- Post-anthesis moderate water limitation promotes the redistribution of cytokinins between source and sink in wheat.
- Redistributing cytokinins from source to sink drives elevates nitrogen distribution in sink organs under moderate water limitation post anthesis.

Abstract

This study examined the involvement of cytokinins in the process by which moderate water limitation (MWL) mediates nitrogen (N) remobilization from source to sink during the grain-filling phase in wheat. Field experiments were performed using N application rates of low (LN), medium (MN), and high (HN). Two soil moisture regimes were implemented for each N rate: conventional well-watered (CWW) and MWL post anthesis. The MWL application optimized N, total free amino acids (FAA), and *trans*-zeatin (Z)+*trans*-zeatin riboside (ZR) reallocation from the source organs (stems and leaves) to the sink organ (spikes) in wheat. Compared to those in the CWW regime, the activities of proteolytic enzymes, including endopeptidase, carboxypeptidase, and aminopeptidase within stems and leaves, and the expression levels of total FAA transporter genes in spikes were significantly elevated in the MWL regime, showing a close correlation with the Z+ZR levels in the spikes. Application of kinetin to stems and leaves significantly inhibited proteolytic enzyme activities, promoting N retention in stems and leaves, decreasing N accumulation in the sink organ, and reducing the N harvest index. In contrast, applying kinetin to spikes significantly upregulated expression levels of FAA transporter genes, reducing N retention in stems and leaves, increasing N accumulation in the sink organ, and raising the N harvest index. Such facilitation induced by the MWL in the remobilization of N from source to sink was greater at HN than at LN or MN. Results demonstrate that post-anthesis MWL can significantly intensify the remobilization of N from source to sink, while also synergistically enhancing grain yield and N use efficiency through strategically redistributing cytokinins (Z+ZR) between source and sink in wheat.

Keywords: cytokinins, moderate water limitation, nitrogen remobilization, nitrogen use efficiency, wheat (*Triticum aestivum* L.)

1. Introduction

Wheat (*Triticum aestivum* L.) is widely recognized as the third most significant cereal crop worldwide, playing a crucial role in maintaining global food security and stability (Guo *et al*. 2024). Nitrogen (N) is a pivotal determinant and a constraining factor in crop productivity. Given the significant yield-boosting effect of N fertilization, its use has gained widespread support in contemporary wheat cultivation in

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recent years, fostering the belief that increasing N fertilizer directly leads to higher grain yields (Huang and Jiang 2019; Bashir *et al.* 2020). Excessive use of chemical fertilizers will exceed plants' ability to absorb them and the soil's capacity to retain nutrients. This approach not only fails to improve yields as expected but also leads to a considerable buildup of chemicals in the soil, causing severe pollution of both soil and groundwater (Amin and Jilani 2024; Srivastav *et al.* 2024). Further investigation reveals that the global N use efficiency (NUE) of cereal crops is only 33%, indicating that over 60% of the applied N is wasted and remains unutilized by these crops (Raun and Johnson 1999; Zhang *et al.* 2022). To enhance the cost-effectiveness and environmental sustainability of farming practices, optimizing NUE is of paramount importance (Hawkesford and Griffiths 2019). Hence, understanding the precise mechanisms that regulate N uptake and utilization, coupled with devising strategies to simultaneously improve NUE and crop yield without relying on excessive N fertilizer, represents a major challenge in agricultural science (Chen *et al.* 2020; Dong and Lin 2020; Zhang and Chu 2020).

NUE is an essential metric for evaluating how effectively cereal crops utilize N, including their ability to absorb, translocate, assimilate, and remobilize N from external sources (Wang *et al.* 2018; Congreves *et al.* 2021). During the reproductive stage of cereal crops, nutrient absorption often declines, triggering the onset of remobilization mechanisms. These processes facilitate the redistribution of crucial nutrients to aid the development and expansion of newly formed nutrient sinks within the plants (Maillard *et al.* 2015). In cereal crops, a substantial amount of N needed for seed development comes from reserves in the vegetative organs (e.g., leaves), which are mobilized and redirected to the seeds to support their growth (Maillard *et al.* 2015; Peng *et al.* 2022; Wei *et al.* 2024). In the vegetative growth phase, plants quickly absorb soil N in the forms of nitrates, ammonium, or free amino acids (FAA), directing it predominantly to the leaves where it aids in protein synthesis; whereas in the seed development stage, the degradation of proteins is an essential process in N remobilization from vegetative organs to sink organs (e.g., seeds) of cereal crops (He *et al.* 2024; Meng *et al.* 2024). Proteins stored in vegetative organs, such as leaves, are rapidly degraded into free amino acids by proteolytic enzymes like endopeptidase (EP), carboxypeptidase (CP), and aminopeptidase (AP), which are then transported through the phloem to sink organs, such as seeds, where they are reconstituted into storage proteins (Zhu *et al.* 2023). In cereal crops, the N that reaches the grains constitutes between 50 and 90% of the total N accumulation in the grains, with this N mainly being redistributed from aging leaves (Asad *et al.* 2024; Zhang *et al.* 2024). During the senescence phase of plants, biochemical processes evolve from nutrient intake to redistribution through mobilization strategies (Wu *et al.* 2022; Estiarte 2023). Consequently, the timing and progression of senescence across the whole plant dictate the efficiency of remobilizing and transporting stored N assimilates from vegetative tissues to grains, influencing the potential NUE of crops like cereals (Borrill *et al.* 2019; Sultana *et al.* 2021; Li *et al.* 2023). Postponing plant senescence may improve NUE; however, overly prolonged senescence can impede the effective transfer of nutrients from vegetative parts to grains, ultimately lowering both NUE and grain yield in cereal crops (Wu *et al.* 2022; Vadez *et al.* 2024). Nevertheless, the mechanisms by which water status affects nutrient remobilization remain insufficiently explored.

The plant's water status plays a critical role in regulating senescence, photosynthetic activity, and the transfer of carbon and N assimilates to the grains (Impa *et al.* 2021; Islam *et al.* 2021). In cereal crops, drought stress triggers early senescence, leading to premature nutrient remobilization from sources to sinks, which significantly reduces photosynthetic efficiency and grain yield (Narayanasamy *et al.* 2020). Conversely, inducing post-anthesis moderate soil drying enhances the remobilization of stored carbon from the stems while alleviating the slight reduction in photosynthetic activity associated with senescence triggered by the drying. This strategy can lead to increased grain yield and an improved harvest index in rice and wheat (Yang ang Zhang 2006; Wang and Zhang 2020). There is scarce information on the effectiveness of post-anthesis MWL in assisting the movement and redistribution of pre-stored N from source tissues to sinks, and on its impact on NUE and grain yield in wheat, particularly in contexts where excessive N fertilizer application delays senescence.

Plant senescence is largely governed by intrinsic factors, particularly phytohormones, and is finely tuned through the coordination and alignment of environmental signals across the plant's complex developmental pathway (Zhao *et al.* 2020; Singh and Roychoudhury 2023). Cytokinins, primarily found as classical phytohormones in the forms of *trans*-zeatin (Z), *trans*-zeatin riboside (ZR), N^6-(Δ^2-isopentenyl)-adenine (iP), and N^6-(Δ^2-isopentenyl)-adenine riboside (iPR), are essential regulators of numerous physiological functions in higher plants (Gu *et al.* 2018; Hosek *et al.* 2020). These are well-established as essential controllers of plants' entire lifecycle, stress responses, and nutrient absorption and utilization, particularly N uptake and utilization (Hudeček *et al.* 2023; Wei *et al.* 2023). Reports indicate that Z+ZR are primarily synthesized in the roots and transported to the shoots through the xylem *via* transpiration, where they play a key role in regulating N partitioning, remobilization, and metabolism. In contrast, the predominant synthesis of iP+iPR occurs in shoots, with subsequent translocation to roots *via* the phloem, effectively modulating N uptake, translocation, and root morphological characteristics in higher plants (Zürcher and Müller 2016; Cárdenas-Aquino *et al.* 2022). A broad range of studies has examined plants' N uptake from soil using different techniques. Nevertheless, whether and how cytokinins mediate MWL's effect on N remobilization from source to sink (grains) in cereals such as wheat, and the precise mechanisms involved, are still uncertain.

This research investigated the role of cytokinins in mediating MWL's impact on N remobilization from source to sink during grain filling in wheat across varying N application rates. The study delved into elucidating the mechanisms underlying N remobilization by investigating alterations in cytokinin, FAA, and N distribution across various organs, alongside assessing

the activity of enzymes integral to protein degradation in wheat. Chemical regulators were employed to investigate how cytokinins influence N remobilization from source to sink. This study provides new strategies for improving grain yield and NUE and offers new insights into the specific mechanisms of N remobilization from source to sink in wheat.

2. Materials and methods

2.1. Plant materials and treatments

The experiment was conducted at the research farm of Yangzhou University in Jiangsu Province, China (32°30′N, 119°25′E, 21 m elevation), spanning two successive wheat growing seasons, from October 2021 to June 2022 and from October 2022 to June 2023. The field soil type was sandy loam (Typic Fluvaquent, Entisol, US classification), containing 24.6 g kg^{-1} organic matter, 107 mg kg^{-1} alkali-hydrolysable N, 35.1 mg kg^{-1} Olsen-P, and 68.4 mg kg^{-1} exchangeable K.

The experiment utilized Yangfumai 8 (YFM-8), a high-yielding wheat variety commonly grown in the region. Wheat was sown on 6 November, 2021 and 5 November, 2022 at a sowing rate of 180 m^{-2} with a row spacing of 0.30 m. The plants were harvested on 25 May, 2022 and 23 May, 2023. Conventional irrigation was carried out according to the habits of local farmers until grain filling, at which point soil moisture regimes were initiated. Essential control measures were implemented for weeds, insects, and diseases to mitigate potential yield losses. During the wheat growth period from 2021 to 2023, the daily average temperature and precipitation close to the experimental site are shown in Appendix A.

2.2. Irrigation and N treatments

The experiment followed a factorial completely randomized block design, consisting of six treatments with three N application rates, two irrigation regimes, and three replications. Each plot measured 4.5 m×4.5 m and was delineated by a drainage ditch (0.5 m wide) for isolation. N application treatments consisted of three levels: 120 kg ha^{-1} (low level, LN), 240 kg ha^{-1} (medium level, MN), and 360 kg ha^{-1} (high level, HN). N was applied following a 4:2:2:2 ratio across four stages: as a base application, and at the tillering, jointing, and booting stages.

Two irrigation regimes were conducted from the day post-anthesis (DPA) through to 7 days before harvest, i.e., (1) conventional well-watered (CWW), keeping soil water potential (Ψ_{soil}) ranges from –30 to –40 kPa at 15–20 cm soil depth; (2) moderate water limitation (MWL), keeping Ψ_{soil} ranges from –60 to –70 kPa at 15–20 cm soil depth. Such a soil water potential in the MWL regime was chosen based on the previous work (Yang and Zhang 2018). Five tension meters, provided by the Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China, were installed in each plot to monitor Ψ_{soil} at depths of 15 to 20 cm, allowing for systematic tracking under both the MWL and CWW irrigation treatments. Once the predetermined threshold reading was met, 0.08 m^3 of tap water was added per plot to the CWW plants and 0.02 m^3 to the MWL plants, respectively. A steel-framed structure, covered in plastic, served as a protective rain shelter, shielding the plot from rainfall.

2.3. Sampling and measurements

Determination of leaf water potential and photosynthetic rate Leaf water potential (Ψ_{leaf}) of the flag leaf was recorded every two hours at 20 DPA, using well-lit flag leaves for sampling. Measurements were taken from 6:00 to 18:00 over two consecutive days. A Model 3000 pressure chamber was used, with 10 leaves per treatment. Leaf photosynthetic rate was measured using the LI-6400 portable photosynthetic system (LI-Cor, Inc., Lincoln, NE, USA) between 9:00 a.m. and 11:00 a.m. at various growth stages, including jointing, anthesis, 20 DPA, and maturity. For each plot, the photosynthetic rate was assessed on 3–5 leaves.

Measurement of N content From each plot, 500 main stems or tillers with spikes emerging on the same day were selected and labeled. Out of these, 150 were sampled at 0, 10, 20, 30, and 40 DPA. Roughly one-third of these samples were divided into leaves, stems, and spikes, dried at 70°C to a constant weight for measuring dry matter, and analyzed for N content using the micro-Kjeldahl digestion method of Yoshida *et al.* (1976). Methods for calculating NUE were according to Wang *et al.* (2016), i.e., N partial factor productivity (PFP_N, kg kg^{-1}): grain yield (kg)/the amount of N applied (kg); internal N use efficiency (IE_N, kg kg^{-1}): grain yield (kg)/the total amount of N uptake in aboveground plants at maturity (kg); N harvest index (HI_N, %): N in grains (kg)/N uptake of aboveground plants at maturity (kg)×100. Remaining samples were stored at −80°C for subsequent analysis of cytokinin (Z and ZR) and total FAA contents, proteinase activities (CP, EP, and AP) in stems and leaves, and the expression of key amino acid transporter genes (*TaCAT9*, *TaCAT8*, *TaAAP20*, and *TaLHT8*) in spikes.

FAA contents determination and qRT-PCR analysis Assay kit (Comin Biotechnology Co., Ltd., Suzhou, China) was employed according to the manufacturer's instructions to evaluate the total FAA contents in leaves, stems, and spikes, and results were reported as mg g^{-1} of dry weight (DW).

To quantify key FAA transporter genes (*TaCAT9*, *TaCAT8*, *TaAAP20*, and *TaLHT8*), qRT-PCR was performed using the methods described by Wang *et al.* (2020). These genes were chosen for their strong correlation with amino acid levels in wheat grains, as evidenced by earlier research (Wan *et al*. 2017; Kasia *et al*. 2018). Gene accession numbers and qRT-PCR primer sequences are detailed in Appendix B. Expression levels were normalized to the wheat *actin* gene, with samples analyzed in triplicate.

Determination of soluble protein content and proteolytic enzyme activity Soluble protein content in wheat organs was measured using the Coomassie brilliant blue G-250 colorimetric assay and expressed in units of mg g^{-1} (Xu *et al.* 2023). Frozen leaves and stems were extracted with 0.05 mol L^{-1} Tris-HCl (pH 7.2) phosphate buffer saline and then centrifuged at 3,000×g for 10–15 min. Supernatant assayed for AP, CP, and EP activity by ELISA (Comin Biotechnology, Suzhou, China) per manufacturer's guide. Activities expressed as μg^{-1} g^{-1} DW h^{-1}.

Extraction and detection of cytokinins Cytokinins were extracted and purified from leaves, stems, and spikes, and quantified precisely by LC-MS/MS (TSQ Vantage, Thermo Fisher) following Pan *et al.* (2010). The reason for analyzing

only Z+ZR is that research has shown Z+ZR have higher biological activity and are more critical in N metabolism in wheat compared to iP and iPR (Gu *et al.* 2018). Z+ZR content is expressed in pmol g^{-1} DW.

Final harvest Plants from a 2.0 m×1.5 m area (excluding borders) in each plot were harvested at maturity, adjusted to 13% moisture for grain yield assessment. Yield components (i.e., spike number, grain number per spike, and grain weight) were analyzed from random 0.5 m^2 samples (excluding borders) per plot.

Chemical application Beginning at 0 DPA, YFM-8 plants grown in experimental plots under the CWW and MN treatments were treated with either 40 μmol L^{-1} of 6 furfurylaminopurine (KT, a synthetic cytokinin) or 40 μmol L^{-1} of lovastatin (LOV, a cytokinin inhibitor). The solutions were brushed onto either the source organs (stems and leaves) or the sink organ (spikes) of main stems or tillers with spikes that emerged on the same day. Four milliliters of the solution were applied daily for 5 days, either to the whole source organ or individual spikes. Both the source and sink organs received the same volume of deionized water, matching the amounts used for their respective control 1 and control 2 treatments. One hundred main stems or tillers were replicated, and all source and sink organs were labeled for each treatment. *TaCAT9*, *TaCAT8*, *TaAAP20*, and *TaLHT8* expression levels and activities of CP, EP and AP were determined from 60 main stems or tillers at 20 DPA. N content in the aboveground plants was measured from 40 main stems or tillers, using the same methods as previously described.

2.4. Statistical analysis

Using SAS/STAT statistical software (version 9.2, SAS Institute, Cary, NC, USA), variance analysis was carried out, factoring in replication, year (Y), irrigation regime (I), N rate (N), and their interactions (Y×I, Y×N, I×N, and Y×I×N). Plots were created with SigmaPlot 10.0, and R software (corrplot package, version 3.5.1, https://cran.r-project.org) was used to compute and plot the correlation coefficients. Multiple comparisons of each indicator under different treatments were performed using the LSD method with a significant probability level of 0.05. The data in figures and tables were the average of three replicates.

3. Results

3.1. Grain Yield and its components

The highest grain yield was observed in the MN, whereas the lowest yield was attained in the LN under the CWW treatment. However, grain yield showed an upward trend with increasing N application rate under the MWL treatment. MWL increased grain yield by 5.2 to 7.7% at LN, 9.8 to 10.3% at MN, and 17.7 to 19.5% at HN, relative to the CWW treatment (Table 1). A higher grain yield at MWL than at CWW was mainly attributed to a higher grain weight at all N treatments (Table 1). There were no significant differences in the number of spikes per square meter and the number of grains per spike between CWW and MWL under all N treatments (Table 1). These yield components were unaffected because the soil moisture regimes were mild and applied after anthesis. Additionally, the grain yield decreased from MN to HN at CWW by 0.35 to 0.46 t ha^{-1}, but increased at MWL by 0.15 to 0.20 t ha^{-1} (Table 1).

3.2. N uptake and use efficiency

In the same soil moisture regime, N in wheat grains increased with higher N application rates. MWL increased N in grains by 4.47% at LN, 8.90% at MN, and 23.0% at HN, when compared with those in the CWW (Table 2). The NUE (N partial factor productivity, PFP_N; internal N use efficiency, IE_N) and N harvest index (HI_N) decreased as N rates increased, but NUE (PFP_N and IE_N) values were significantly higher under MWL than CWW at the same N rate (Table 2).

3.3. Leaf water potential, photosynthetic rate, and shoot dry matter weight

Higher N rates increased the shoot dry matter weight across all soil moisture conditions. At 10 DPA, the shoot dry matter accumulation under MWL was generally lower than under CWW across various N rates, whereas at 20, 30, and 40 DPA, it was slightly lower but not significantly different (Appendix C). Overall, shoot dry matter accumulation showed no significant difference between MWL and CWW across various N rates (Appendix C).

The Ψ_{leaf} varied from –0.23 to –0.43 MPa at pre-dawn (06:00) and from –0.72 to –1.12 MPa at midday (12:00) under CWW. In contrast, a much sharper reduction in Ψ_{leaf} was observed during the day under MWL, with pre-dawn values ranging from –0.20 to –0.43 MPa and midday

Table 1 Grain yield and its components under multiple soil moisture and nitrogen treatments of Yangfumai 8

Year	Treatment[1)]	Spike number (×10^4 ha^{-1})	Grain number per spike	1,000-grain weight (g)	Grain yield (t ha^{-1})
2021–2022	LN-CWW	584 c	31.6 c	42.3 b	6.31 e
	MN-CWW	681 b	33.5 b	40.5 c	7.57 b
	HN-CWW	745 a	34.1 a	37.5 d	7.22 c
	LN-MWL	589 c	31.7 c	43.3 a	6.64 d
	MN-MWL	701 b	33.5 b	42.4 b	8.35 a
	HN-MWL	735 a	34.3 a	40.7 c	8.50 a
2022–2023	LN-CWW	546 c	31.2 c	42.3 b	6.10 e
	MN-CWW	663 b	34.0 b	40.5 c	7.74 b
	HN-CWW	730 a	35.6 a	37.7 d	7.28 c
	LN-MWL	550 c	30.9 c	43.4 a	6.57 d
	MN-MWL	685 b	34.1 b	42.3 b	8.50 a
	HN-MWL	736 a	35.4 a	40.6 c	8.70 a
Analysis of variance					
Year (Y)		12.0**	8.1**	NS	NS
Irrigation regime (I)		NS	NS	542**	344**
Nitrogen (N)		276**	196**	643**	542**
Y×I		NS	NS	NS	NS
Y×N		NS	14.2**	NS	NS
I×N		NS	NS	46.9**	37.1**
Y×I×N		NS	NS	NS	NS

[1)] LN, MN, and HN stand for low, medium, and high nitrogen levels, respectively. CWW refers to conventional well-watered conditions, while MWL stands for moderate water limitation during grain filling.
** indicates significance at P<0.01, while NS denotes non-significance at P=0.05. Different letters within the same year and column indicate significant differences at the P=0.05 (n=3).

Table 2 Nitrogen uptake and utilization efficiency during grain filling under multiple soil moisture and nitrogen treatments in wheat[1)]

Year	Treatment[2)]	N uptake (kg ha^{-1})	N in grains (kg ha^{-1})	IE_N (kg kg^{-1})	PFP_N (kg kg^{-1})	HI_N (%)
2021–2022	LN-CWW	86.3 c	62.6 e	73.2 b	52.6 b	72.6 b
	MN-CWW	130 b	75.3 d	58.5 d	31.5 d	58.1 e
	HN-CWW	166 a	92.7 b	43.5 f	20.1 f	55.8 f
	LN-MWL	85.8 c	65.4 e	77.5 a	55.4 a	76.2 a
	MN-MWL	127 b	82.0 c	65.9 c	34.8 c	64.7 d
	HN-MWL	163 a	114 a	52.2 e	23.6 e	70.0 c
2022–2023	LN-CWW	90.0 c	66.8 e	67.8 b	50.9 b	74.2 b
	MN-CWW	145 b	86.4 c	53.3 d	32.3 d	59.5 e
	HN-CWW	183 a	100 b	39.7 f	20.2 f	54.7 f
	LN-MWL	90.2 c	70.9 d	72.9 a	54.8 a	78.6 a
	MN-MWL	147 b	101 b	57.7 c	35.4 c	68.6 d
	HN-MWL	185 a	133 a	47.0 e	24.2 e	71.6 c
Analysis of variance						
Year (Y)		515**	332**	98.8**	NS	39.6**
Irrigation regime (I)		NS	506**	132**	111**	1,215**
Nitrogen (N)		6,580**	1,720**	846**	3,155**	1,016**
Y×I		8.17**	31.3**	NS	NS	15.1**
Y×N		65.2**	27.2**	NS	NS	7.08**
I×N		NS	129**	NS	NS	166**
Y×I×N		NS	5.53*	NS	NS	NS

[1)] IE_N, internal nitrogen use efficiency; PFP_N, nitrogen partial factor productivity; HI_N, nitrogen harvest index.
[2)] LN, MN, and HN stand for low, medium, and high nitrogen levels, respectively. CWW refers to conventional well-watered conditions, while MWL stands for moderate water limitation during grain filling.
** indicates significance at P<0.01, while NS denotes non-significance at P=0.05. Different letters within the same year and column indicate significant differences at the P=0.05 (n=3).

values ranging from –0.77 to –1.32 MPa. When Ψ_{leaf} was measured at afternoon (18:00) at 20 DPA, MWL was significantly lower than CWW at the same N rate, and they showed no significant difference between MWL and CWW at predawn (06:00) (Appendix D). Under the same soil moisture condition, the leaf photosynthetic rate increased with increasing N rates. Although MWL had a lower leaf photosynthetic rate than CWW, no significant difference was observed between MWL and CWW (Appendix E).

3.4. N translocation

Overall, with the increase of N rates, the N content and accumulation in different organs (stem, leaf, and spike) were significantly enhanced at the same soil moisture treatment. Although the source organs (stems and leaves) had lower N content and accumulation in MWL than in CWW, the sink organ (spikes) showed significantly higher N content and accumulation in MWL compared to those in CWW across all N rates (Fig. 1). Furthermore, the extent of the decrease in N accumulation from CWW to MWL in the source organs under the HN treatment was much greater than that observed under the MN and LN treatments. Similarly, the extent of the increase in N accumulation from CWW to MWL in the sink organ was more significant under HN than under LN or MN (Fig. 1).

Compared with those in CWW, the N translocation amount and translocation rate from the source organs (stems and leaves) to the sink organ (spikes) were significantly lower in MWL. N translocation rate from the source organ to the sink organ decreased from MN to HN treatments in CWW by 4.3 to 8.0% but increased in MWL by 3.0 to 4.3% (P<0.01) (Table 3). N contribution of source organs to N in grains significantly decreased with the increase in N rates, in either CWW or MWL treatments. Overall, the MWL treatment markedly increased the N contribution from source organs to N in grains when compared with the CWW treatment across all N rates. Additionally, the contribution rate of N stored in leaves to N in grains was higher than that of N stored in stems to N in grains (Table 3).

3.5. Total FAA contents and soluble protein content

Total FAA contents increased as N rates were elevated across all irrigation regimes in source organs (stems and leaves). In contrast, total FAA contents were significantly lower in MWL than in CWW, while levels in the sink organ (spikes) were markedly higher in MWL compared to those in CWW (Fig. 2). Under the HN condition, the MWL treatment caused a significantly greater decrease in total FAA contents in source organs and a greater increase in total FAA contents in sink organs compared to the CWW treatment. These effects were more pronounced than those observed under the LN and MN conditions (Fig. 2).

The higher N rates contributed to higher soluble protein content in various organs under the same soil moisture condition. In MWL, soluble protein content in the source organs (stems and leaves) was significantly lower compared to that in CWW, whereas it was significantly higher in the sink organs (spikes) under the same N rates (Appendix F). Furthermore, under HN conditions, the decrease in soluble protein in source organs and the increase in soluble protein in sink organs in MWL were more pronounced compared to under LN and MN conditions in CWW (Appendix F).

3.6. Proteolytic enzyme activities and expression levels of amino acid transporter genes

Under the same soil moisture condition, proteolytic enzyme activities (CP, EP, and AP) in the source organs (stems and leaves) decreased with increasing N rates. Across all N rates, these enzyme activities were significantly higher in MWL than in CWW (Fig. 3). Similar to the total FAA contents in the sink organ (spikes), the relative expression levels of *TaCAT8*, *TaCAT9*, *TaAAP20*, and *TaLHT8* in the sink organ increased with rising of N rates in all irrigation regimes and were significantly higher in MWL than in CWW at the same N rate (Fig. 4).

3.7. Cytokinins

Corresponding to the total FAA contents, the Z+ZR contents in stems, leaves, and spikes increased with higher N application rates in the consistent irrigation regime. At the same N rate, the Z+ZR contents in the stems and leaves were significantly lower in MWL than in CWW, whereas in the spikes, they were notably higher in MWL compared to CWW (Fig. 5). Compared to the CWW treatment, the MWL

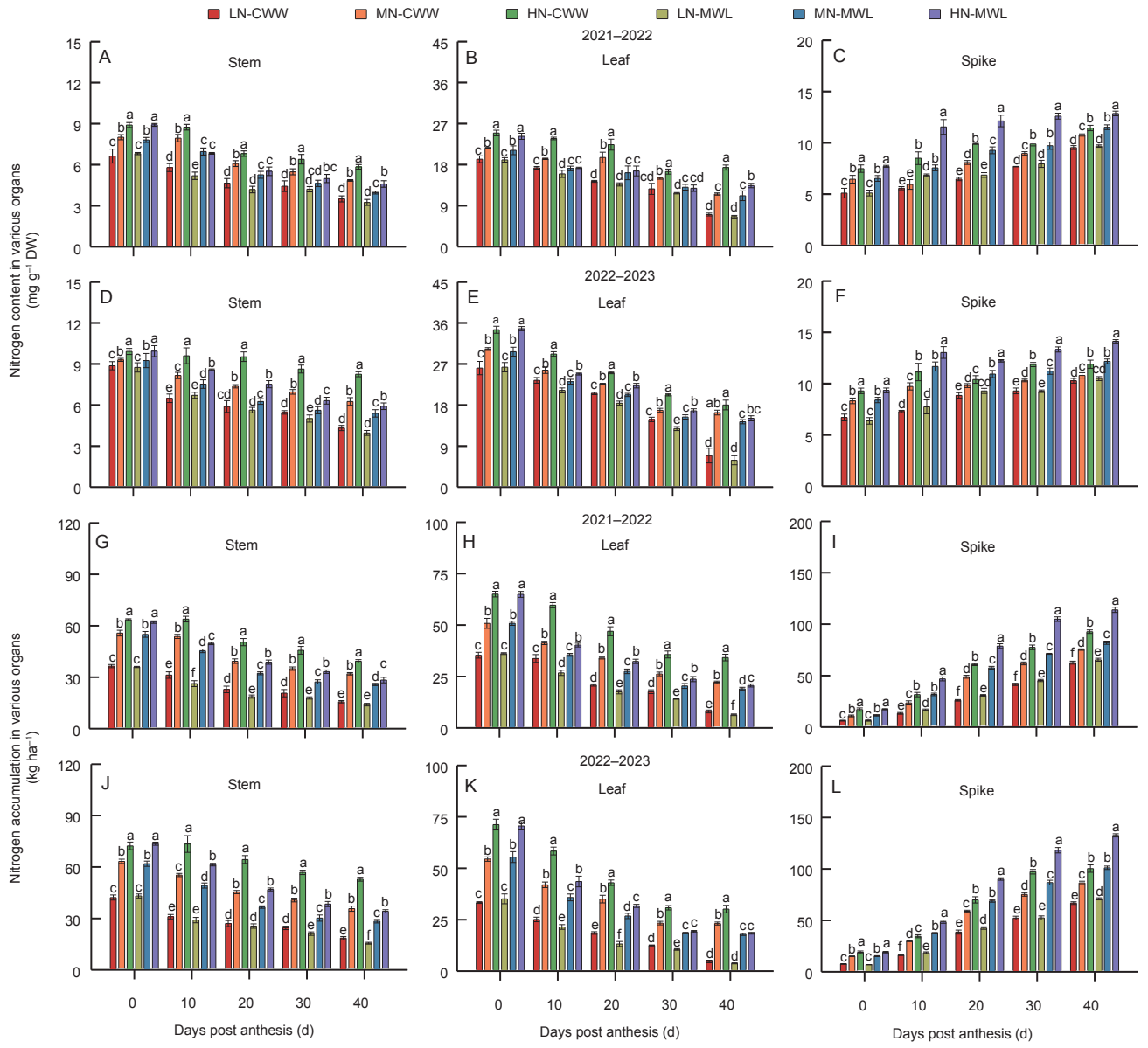


Fig. 1 Changes in nitrogen content (A–F) and accumulation (G–L) in various organs during grain filling under multiple soil moisture and nitrogen treatments in wheat. LN, MN, and HN stand for low, medium, and high nitrogen levels, respectively. CWW refers to conventional well-watered conditions, while MWL indicates a moderate water limitation during the grain filling phase. Bars are mean±SD (n=3). Differences indicated by lowercase letters represent the least significant difference at P=0.05 for the same measurement date.

treatment under HN conditions led to a significantly larger decrease in Z+ZR content in the stems and leaves and a greater increase in Z+ZR content in the spikes than observed under LN and MN conditions (Fig. 5).

3.8. Exogenous cytokinin application

Compared to the control group treated with deionized water, applying 6-furfurylaminopurine (KT, a synthetic cytokinin) to the entire source organs (stems and leaves) or administering lovastatin (LOV, a cytokinin inhibitor) to the sink organ (spikes) significantly reduced the activities of CP, EP, and AP in source organs. This treatment also led to a notable increase in N retention in the source organs, accompanied by a significant decrease in N accumulation in spikes and HI_N (Fig. 6-B; Appendices G and H). Conversely, when KT was applied to the sink organ or LOV to the source organs, there was a marked upregulation of *TaCAT8*, *TaCAT9*, *TaAAP20*, and *TaLHT8* expression levels in spikes, along with a significant reduction in N retention in the source organs. In contrast, these treatments resulted in a substantial increase in N accumulation in spikes and HI_N compared to the control (Fig. 6-B; Appendices G and I). Notably, the total N uptake in aboveground plants remained consistent across KT treatments, LOV treatments, and the control group (Fig. 6-A).

3.9. Correlations of the change extent in physiological parameters in MWL relative to those in CWW

Pearson's correlation analysis disclosed dual trends: notable positive correlations existed between changes (Δvalues) in Z+ZR content and those in N accumulation, total FAA

Table 3 Nitrogen transport rate during grain filling and contribution rate of nitrogen from source organs to the grain under multiple soil moisture and nitrogen treatments in wheat[1)]

Year	Treatment[2)]	N_{TRA} in source organ (kg ha^{-1})	N_{TRR} in source organ (%)	N_{CR} from source organ to grain (%)		
				Leaf	Stem	Total
2021–2022	LN-CWW	54.4 e	69.7 b	44.4 a	33.2 ab	77.6 a
	MN-CWW	63.1 c	53.8 e	38.0 b	31.5 bc	69.5 c
	HN-CWW	72.1 b	49.5 f	33.3 c	26.1 d	59.4 d
	LN-MWL	58.0 d	74.0 a	45.5 a	33.6 ab	79.1 a
	MN-MWL	72.4 b	61.8 d	38.8 b	35.7 a	74.5 b
	HN-MWL	95.3 a	66.1 c	38.9 b	29.7 c	68.5 c
2022–2023	LN-CWW	52.3 d	69.2 b	42.8 ab	35.4 b	78.3 b
	MN-CWW	59.0 c	50.1 e	36.3 c	32.3 c	68.2 c
	HN-CWW	60.5 c	42.1 f	40.9 ab	19.4 e	60.3 d
	LN-MWL	59.0 c	75.4 a	44.3 a	38.9 a	83.2 a
	MN-MWL	71.1 b	60.5 d	36.9 c	33.8 bc	70.9 c
	HN-MWL	91.6 a	63.5 c	39.3 bc	29.8 d	69.1 c
Analysis of variance						
Year (Y)		35.2**	41.1**	NS	NS	NS
Irrigation regime (I)		544**	946**	63.3**	NS	92.5**
Nitrogen (N)		509**	882**	125**	43.7**	254**
Y×I		14.0**	17.9**	13.0*	NS	NS
Y×N		11.6**	18.5**	17.6**	8.29**	6.54**
I×N		115**	127**	9.94**	NS	10.86**
Y×I×N		NS	NS	7.93**	NS	NS

[1)] N_{TRA}, amount of nitrogen transported; N_{TRR}, nitrogen translocation rate; N_{CR}, contribution rate of nitrogen.
[2)] LN, MN, and HN stand for low, medium, and high nitrogen levels, respectively. CWW refers to conventional well-watered conditions, while MWL indicates a moderate water limitation during the grain filling phase.
Different letters within the same year and column indicate significant differences at the P=0.05 (n=3). * and **, significances at P<0.05 and P<0.01, respectively; NS, non-significance at P=0.05.

levels, and soluble protein content in source organs (stems and leaves), contrasted by significant negative correlations between Δ(Z+ZR) content in the sink organ (spikes) and identical parameters in source organs. Intriguingly, while the Δ(Z+ZR) content in the sink organ showed a significant or extremely significant negative correlation with ΔN accumulation, Δtotal FAA levels, and Δsoluble protein content in source organs, it also demonstrated a positive correlation of equal significance with ΔN accumulation, Δ total FAA levels, Δsoluble protein content, Δactivities of proteolytic enzymes (AP, CP, and EP) in source organs, and Δexpression levels of key amino acid transporter genes (*TaCAT8*, *TaCAT9*, *TaAAP20*, and *TaLHT8*) specifically in the sink organ (Fig. 7).

4. Discussion

4.1. Effects of post-anthesis MWL on grain yield and NUE under various N rates

Prior research focused on how varying soil moisture status and N rates influence N uptake in wheat (Liu *et al.* 2023). In comparison, there has been less attention to understanding the processes and mechanisms of N remobilization from source organs to sink organs in cereal crops such as wheat. We observed that during the grain filling, the MWL regime, which maintains Ψ_{soil} between –60 and –70 kPa at the depth of 15 to 20 cm, not only efficiently promoted N remobilization from source organs to the sink organ, but also significantly increased the grain N accumulation and HI_N, while synergistically enhancing grain yield and NUE (PFP_N and IE_N) compared to CWW regime under LN, MN, or HN conditions (Tables 1, 2, and 3). Interestingly, as the N application rate increased, the positive effects of MWL on grain yield, NUE (PFP_N and IE_N), and HI_N became even more pronounced. Results imply that the MWL regime could not only alleviate the grain yield loss caused by LN stress but also significantly amplify the positive impact of high N rates (e.g., MN and HN) on grain yield, NUE (PFP_N and IE_N), and HI_N in wheat (Tables 1 and 2).

Notably, it has been reported that despite significant variations in soil water content across different soil types, the water status in plants remains consistent when the soil water potential is the same (Zhang *et al.* 2021). Additionally, it has been observed that a mid-day leaf water potential above –1.55 MPa does not significantly impact photosynthesis in wheat (Yang and Zhang 2018). Similarly, our results demonstrated no inhibition of biomass production and accumulation (including leaf photosynthetic rate, shoot dry matter weight, and N accumulation) in the MWL regime, with

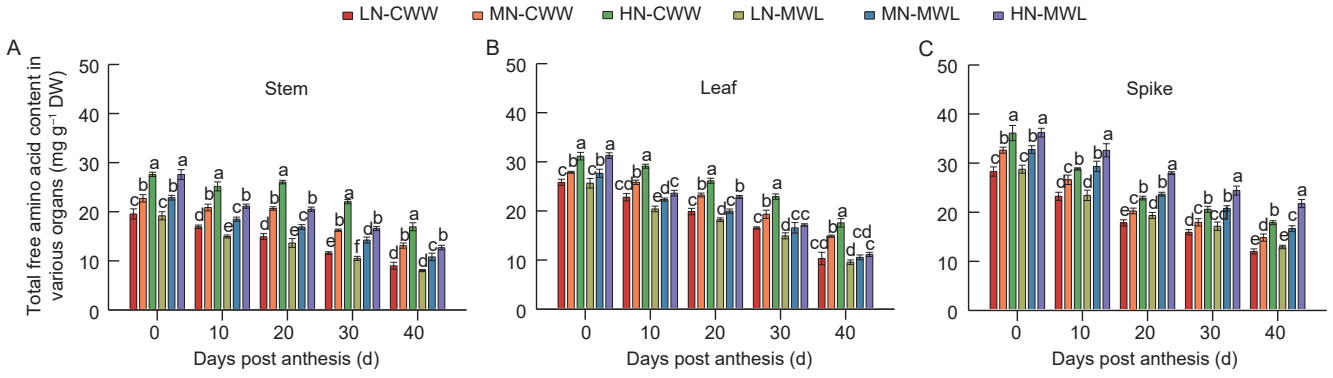


Fig. 2 Total free amino acid content in various organs during grain filling under multiple soil moisture and nitrogen treatments in wheat. LN, MN, and HN stand for low, medium, and high nitrogen levels, respectively. CWW refers to conventional well-watered conditions, while MWL indicates a moderate water limitation during the grain filling phase. Bars are mean±SD (n=3). Differences indicated by lowercase letters represent the least significant difference at P=0.05 for the same measurement date.

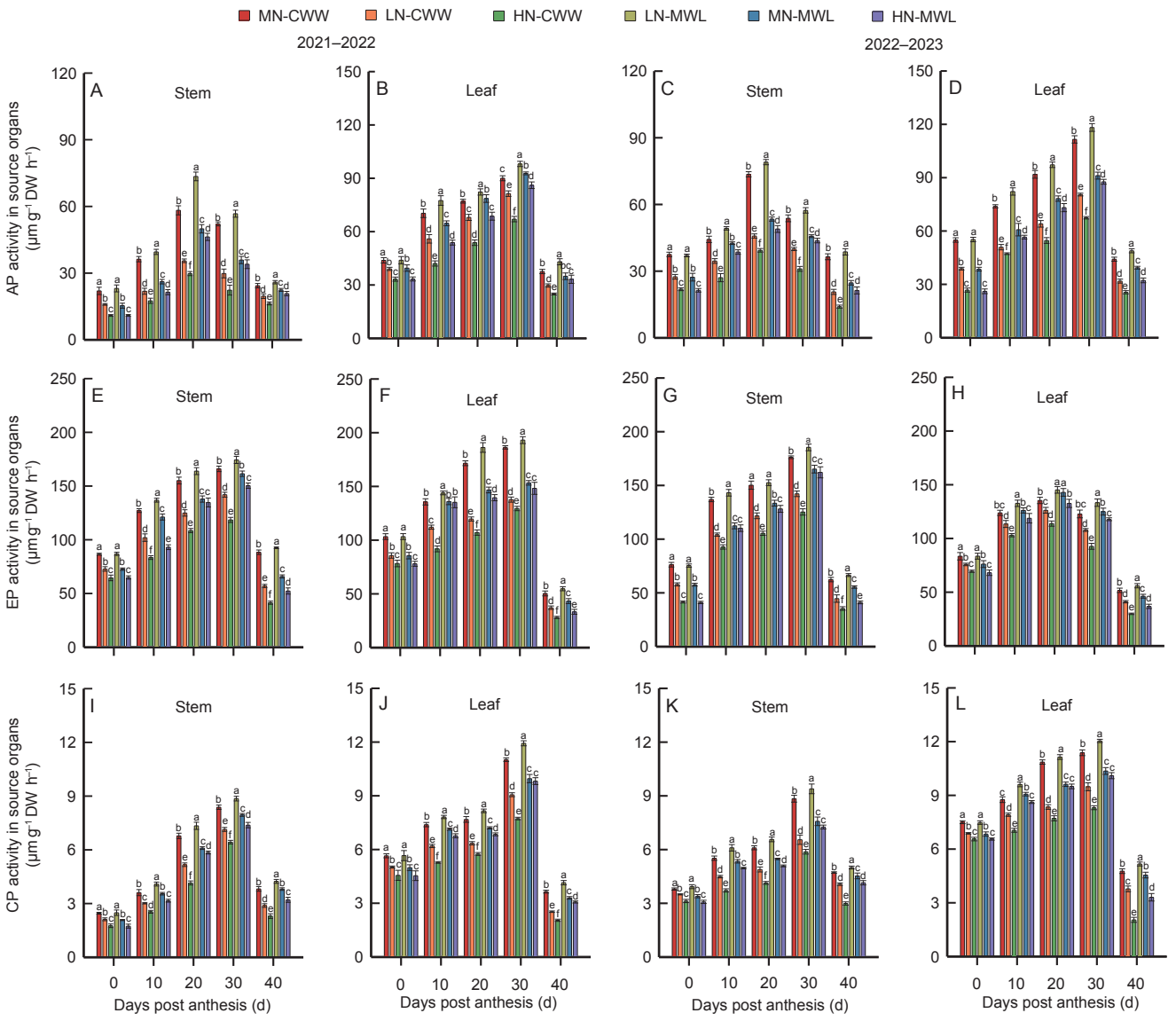


Fig. 3 Aminopeptidase (AP) (A–D), endopeptidase (EP) (E–H), and carboxypeptidase (CP) (I–L) activities in stems and leaves during grain filling under multiple soil moisture and nitrogen treatments in wheat. LN, MN, and HN stand for low, medium, and high nitrogen levels, respectively. CWW refers to conventional well-watered conditions, while MWL indicates a moderate water limitation during the grain filling phase. Bars are mean±SD (n=3). Differences indicated by lowercase letters represent the least significant difference at P=0.05 for the same measurement date.

mid-day leaf water potentials remaining above –1.5 MPa (Fig. 1; Appendices C and E). These findings suggest that a mid-day leaf water potential ranging from –1.00 to –1.45 MPa, or a soil water potential of –60 to –70 kPa at a depth of 15 to 20 cm, as alternatives to the CWW, can serve as universal indicators unaffected by soil type differences. Such a MWL regime can accurately monitor and reflect plant and soil water status and significantly enhance grain yield and NUE in wheat. This discovery holds substantial significance for improving grain yield and NUE in wheat, particularly in the face of global warming and water scarcity challenges.

4.2. Mechanism underlying the increase in grain yield and NUE by post-anthesis MWL under various N rates

The specific mechanism by which post-anthesis MWL enhances N remobilization from source to sink in wheat remains elusive. Studies show that the metabolism and signaling pathways of cytokinins are intimately linked to the modulation of source–sink interactions and nutrient partitioning within the plant (Tegeder and Masclaux-Daubresse 2018; Zhao *et al.* 2024). Present results showed that the Z+ZR levels were significantly lower in source organs (stems and leaves) but significantly higher in sink organs (spikes) in MWL compared to in CWW. Additionally, there was a significant and positive correlation between the change in the Z+ZR levels in sink organ and N accumulation in sink organ in CWW, relative to those in MWL at the same N rate (Figs. 1, 5, and 7). Additionally, applying KT (synthetic cytokinin) to the source organs significantly increased N accumulation in these organs while decreasing N accumulation in the sink organ, resulting in a lower HI_N. Conversely, when KT was applied to the sink organ, the

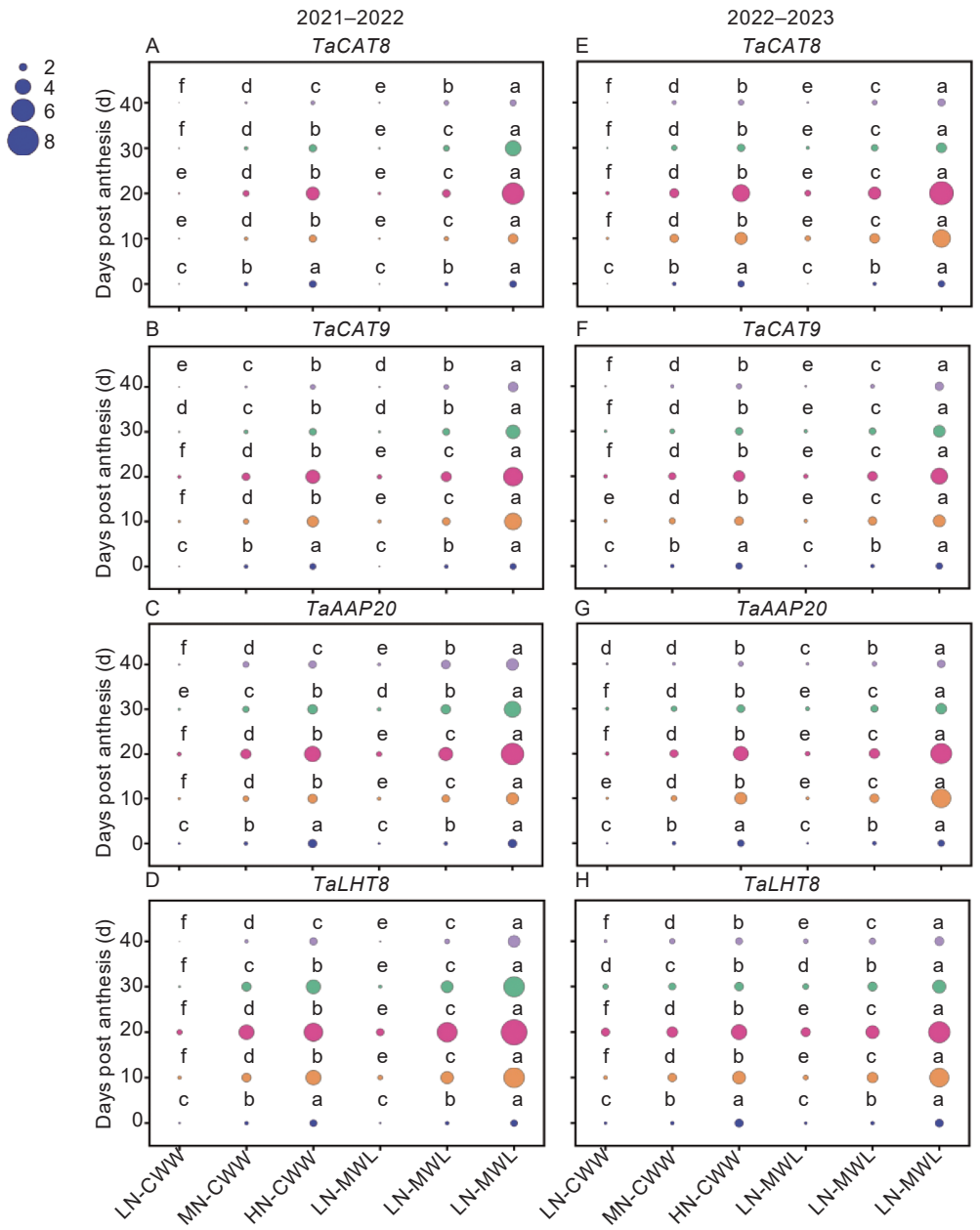


Fig. 4 Relative expression levels of *TaCAT8* (A and E), *TaCAT9* (B and F), *TaAAP20* (C and G), *TaLHT8* (D and H) in spikes during grain filling under multiple soil moisture and nitrogen treatments in wheat. LN, MN, and HN stand for low, medium, and high nitrogen levels, respectively. CWW refers to conventional well-watered conditions, while MWL indicates a moderate water limitation during the grain filling phase. Bars are mean±SD (n=3). Differences indicated by lowercase letters represent the least significant difference at P=0.05 for the same measurement date.

opposite effects were observed (Fig. 6-B; Appendix G). Upon application of KT to either source or sink organ, no notable disparities were discerned in the overall N uptake by the aboveground plant (Fig. 6-A). These findings lend further credence to the notion that Z+ZR play a minor role in N uptake processes. Furthermore, the heightened distribution rate of Z+ZR to the sink organ under the MWL regime is postulated to contribute favorably to the remobilization of pre-stored N from source organs to sink organs in wheat. Notably, this positive effect of enhanced N translocation from source to sink is particularly accentuated under HN conditions.

Information on how cytokinins regulate N remobilization from source organs to sink organs in wheat is very limited. A key factor in N redistribution from source to sink is the transport of FAA into the phloem and grains. The development of sinks and grain yield largely depends on the amount of FAA translocated, which mainly comes from the breakdown of proteins stored in vegetative tissues and is facilitated by the xylem (Lu *et al.* 2018; Gao *et al.* 2020; Guo *et al.* 2020; Dani *et al.* 2022). Our study revealed that the Z+ZR content in source organs (stems and leaves) was significantly lower in MWL than in CWW across all the N treatments, which was related to the higher proteolytic

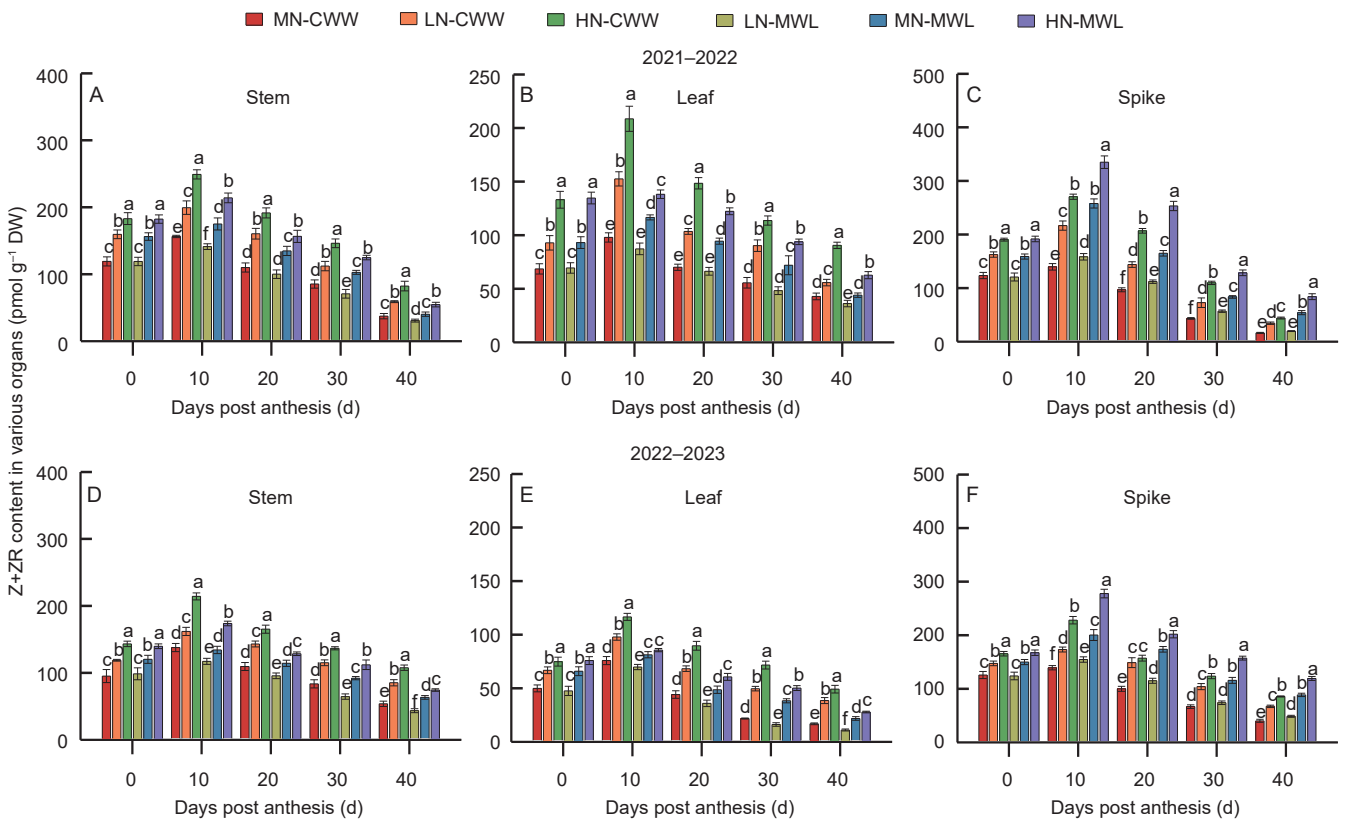


Fig. 5 Cytokinins contents in various organs during grain filling under multiple soil moisture and nitrogen treatments in wheat. LN, MN, and HN stand for low, medium, and high nitrogen levels, respectively. CWW refers to conventional well-watered conditions, while MWL indicates a moderate water limitation during the grain filling phase. Cytokinin contents of zeatin (Z)+zeatin riboside (ZR) were primarily measured. Bars are mean±SD (n=3). Differences indicated by lowercase letters represent the least significant difference at P=0.05 for the same measurement date.

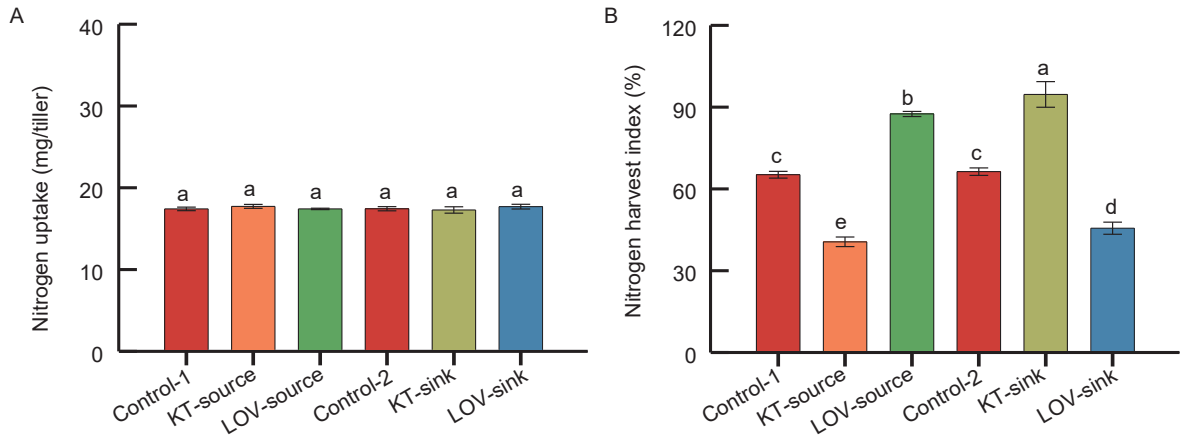


Fig. 6 Effects of applying chemical regulators on nitrogen uptake amount in aboveground plants and nitrogen harvest index in various organs of wheat. KT-source, apply 40 μmol L^{-1} kinetin on source; KT-sink, apply 40 μmol L^{-1} kinetin on sink; LOV-source, apply 40 μmol L^{-1} lovastatin on source; LOV-sink, apply 40 μmol L^{-1} lovastatin on source. Bars are mean±SD (n=3). Differences indicated by lowercase letters represent the least significant difference at P=0.05.

enzyme (AP, CP and EP) activities and the lower N accumulation, soluble protein content and total FAA levels in source organs in MWL (Figs. 1, 2, 3, 5, and 7; Appendix F). In contrast, the Z+ZR levels in the sink organ (spikes) were significantly higher in MWL than in CWW during grain filling. This increase was associated with the upregulated expression of key amino acid transporter genes (*TaCAT9*, *TaCAT8*, *TaAAP20*, and *TaLHT8*) in the sink organ by MWL during this period (Figs. 4, 5, and 7). Moreover, applying KT to the source organs notably reduced the proteolytic enzyme activities, resulting in a significant increase in the N accumulation in stems and leaves, while reducing the

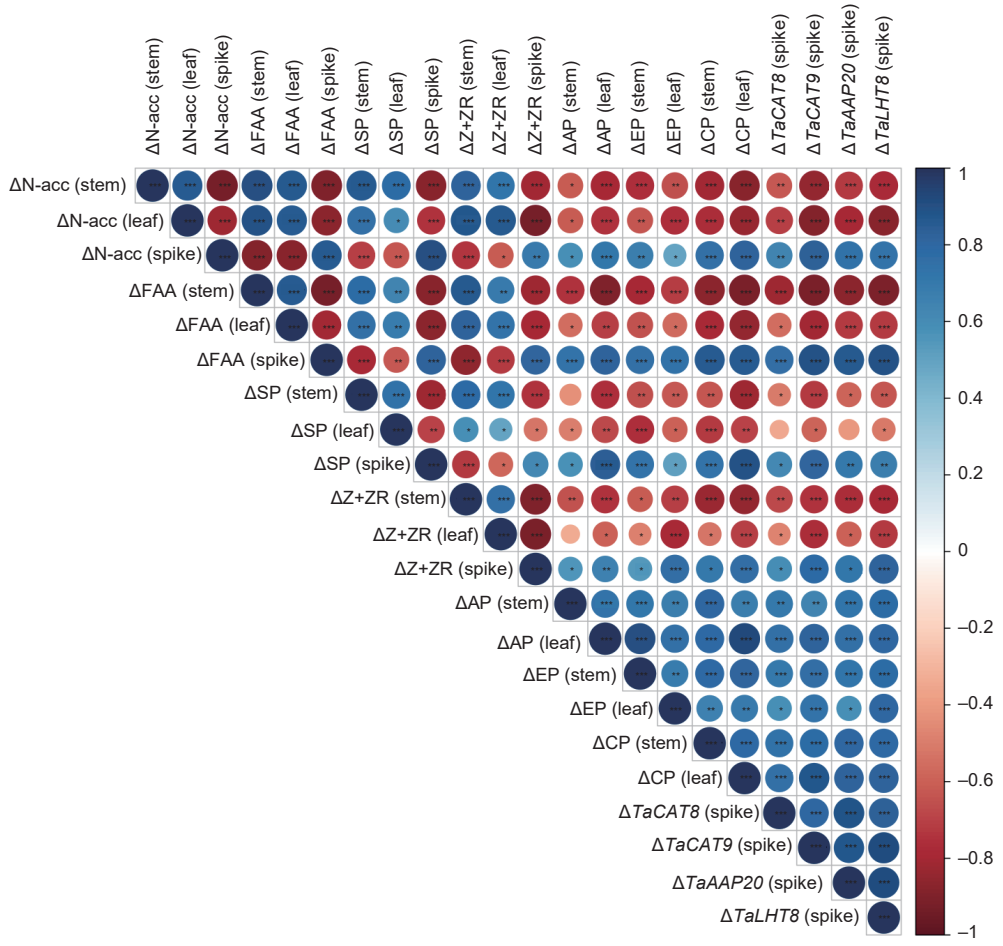


Fig. 7 Correlation analysis of the changes (Δvalues) in parameters of wheat in the moderate water limitation regime relative to the well-watered regime under the same nitrogen rates. Red and blue circles represent negative and positive correlations between parameters, respectively. Data for these calculations were drawn from Figs. 1–5 and Appendix F. N-acc, nitrogen accumulation; FAA, total free amino acids; SP, soluble protein; Z+ZR, *trans*-zeatin+*trans*-zeatin riboside; AP, aminopeptidase; EP, endopeptidase; CP, carboxypeptidase. Δ indicates the extent of change in the physiological parameters under low nitrogen level (LN)-moderate water limitation during grain filling (MWL), medium nitrogen level (MN)-MWL, and high nitrogen level (HN)-MWL relative to those under the LN-conventional well-watered (CWW), MN-CWW, and HN-CWW, respectively. ***, **, and * denote significance levels at $P<0.001$, $P<0.01$, and $P<0.05$, respectively (n=12, i.e., 1 wheat cultivar×2 relative differences×3 measurement times×2 study years).

N accumulation in grains and lowering HI_N. In contrast, boosting the expression of key amino acid transporter genes by applying KT to the sink organ significantly reduced the N accumulation in the source organs while markedly increasing the N content in the grains and HI_N (Fig. 6; Appendices G, H, and I). These results suggest that MWL during grain filling reduced Z+ZR levels in source organs, thereby enhancing the activities of proteolytic enzymes in the source organs, accelerating the degradation of proteins into amino acids, and promoting N output from the source organs in wheat. Simultaneously, the MWL increased cytokinin levels in sink organs, enhanced the expression levels of key amino acid transporter genes involved in the transport of amino acids in the sink organ, facilitating receptivity in sink organs for N, and ultimately leading to improvement in N accumulation in grains, HI_N, and grain yield in wheat.

Interestingly, we found that the inhibitory effect on the soluble protein content and total FAA levels in the source organs (stems and leaves) was more pronounced in MWL, along with a stronger enhancement of the proteolytic enzyme activities in the source organs and a greater upregulation of the key amino acid transporter gene expression in the sink organ (spikes) under HN conditions compared to those under LN or MN treatments (Figs. 2–4; Appendix F). Such an occurrence can be attributed to the enhancement of grain yield and NUE by the MWL regime, while simultaneously compensating for the potential loss in grain yield that may arise from excessive application of N fertilizer.

4.3. Post-anthesis MWL amplifies the positive impact of N application on grain yield and HI_N under HN

It is important to note that in the MWL regime, the increase in the N accumulation in sink organs (spikes), HI_N, and grain yield was more pronounced at HN levels compared to those at medium MN and LN levels. A plausible explanation is that, under HN application, the source organs (stems and

leaves) are richer in N, which may encourage the plant to more generously export N to the sink organ, driven by Z+ZR in the MWL regime, compared to those under MN and LN applications (Figs. 1 and 5; Table 1). Important evidence supporting this hypothesis is that in the MWL regime, the Z+ZR content, FAA content, as well as N content and accumulation in the source organs (stems and leaves) were lower, while these parameters were higher in the sink organ (spikes) compared to those in the CWW regime. This trend was consistent across LN, MN, and HN conditions, with the differences between CWW and MWL being particularly pronounced under HN conditions (Figs. 1, 2, and 5). Results suggest that source-sink coordination is influenced not only by the levels of Z+ZR but also by the distribution of Z+ZR between source and sink. Excessively high levels of Z+ZR in source organs during grain filling may negatively impact source-sink coordination, resulting in lower HI_N and reduced grain yield in wheat.

5. Conclusion

A MWL regime imposed during grain filling can facilitate N remobilization from source to sink by redistributing cytokinins (Z+ZR), thereby boosting HI_N, grain yield, and NUE in wheat. MWL not only mitigates grain yield losses caused by LN stress but also amplifies the positive impact of N application on grain yield and HI_N under HN in wheat. The MWL regime reduces in Z+ZR concentrations in source organs (stems and leaves) and stimulates proteolytic enzyme activity, accelerating protein degradation. Simultaneously, MWL increases cytokinin (Z+ZR) levels in sink organs, enhances the expression levels of key amino acid transporter genes involved in amino acid transport in sink organs, improves N receptivity in sink organs, and ultimately contributes to improved N accumulation in grains, HI_N, grain yield, and NUE in wheat (Fig. 8).

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used

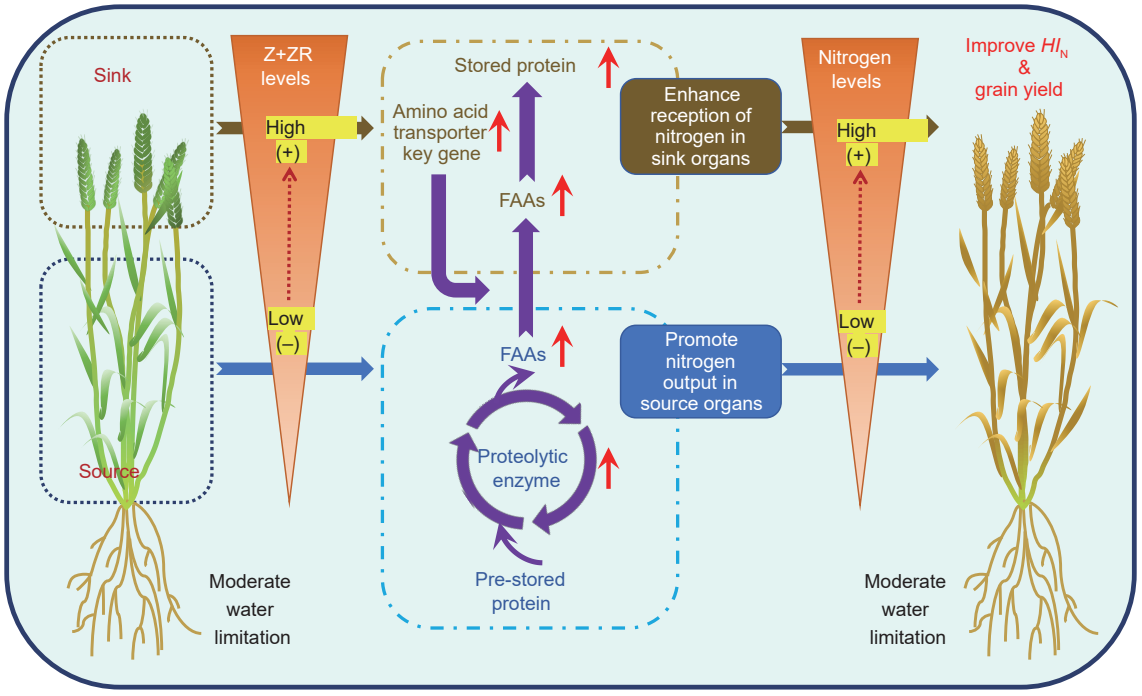


Fig. 8 A descriptive model of cytokinin mediates nitrogen remobilization from source to sink under moderate water limitation conditions in wheat. Moderate water limitation (MWL) reduced cytokinin (*trans*-zeatin (Z)+*trans*-zeatin riboside (ZR)) levels in source organs, thereby enhancing activities of proteolytic enzymes (endopeptidase, carboxypeptidase, and aminopeptidase) in the source organs, accelerating the degradation of proteins into amino acids, and promoting nitrogen output from the source organs in wheat. At the same time, the MWL increased Z+ZR levels in sink organs, enhanced expression levels of amino acid transporter key genes involved in the transport of amino acids in sink organs, facilitating receptivity in sink organs for nitrogen (amino acid), and ultimately leading to improved nitrogen harvest index (HI_N) and grain yield in wheat. FAA, free amino acids.

generative AI tools to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

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