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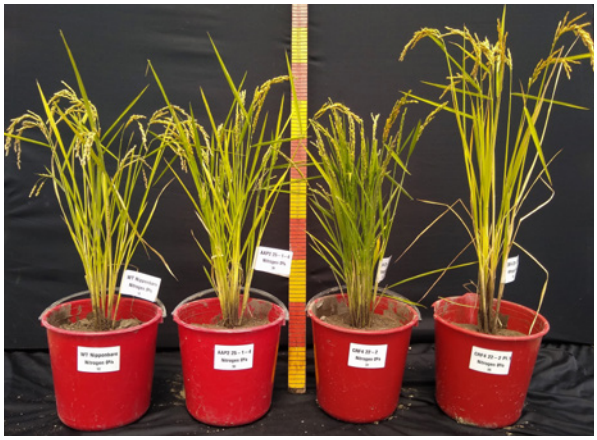
Selection and evaluation of gene-edited knockout mutants of *AtAAP2* and *AtCRF4* homologs of rice for agronomic nitrogen use efficiency (ANUE)

Selección y evaluación de mutantes knockout genéticamente editados de homólogos de *AtAAP2* y *AtCRF4* de arroz para la eficiencia agronómica del uso del nitrógeno (ANUE)



Gene-edited rice lines with different phenotypic features.

Photo: K. Wakatabi

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ABSTRACT

Nitrogen (N) is essential for amino acid synthesis in rice production, but its excessive use poses an environmental concern. This research aimed to improve rice agronomic nitrogen use efficiency (ANUE) by knockout (KO) of rice homologs of the two selected genes from *Arabidopsis thaliana*: *AtAAP2*, an amino acid permease involved in N transportation in shoots, and *AtCRF4*, a transcription factor participating in N uptake in roots. The homologs of these genes in rice were identified based on amino acid sequence similarity and knocked out using CRISPR/Cas9 mediated gene editing (GE). The *AAP2*-KO and *CRF4*-KO lines were subjected to agronomic evaluations with three N doses: 100% (180 kg ha^{-1}), 50% (90 kg ha^{-1}), and 0% (0 kg ha^{-1}) and showed a 130-175% increase in dry biomass weight and a 183-313% increase in panicle number compared to wild type (WT) in the first experiment. These lines also had slower leaf senescence, the so-called "stay-green" trait, indicating the KO effect of target genes in N metabolism. However, neither *AAP2*-KO nor *CRF4*-KO showed better yield or ANUE than WT. This study demonstrated the usefulness of GE technology in gene evaluation and highlighted the effects of *AtAAP2* and *AtCRF4* genes in the plant N cycle.

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Additional key words: amino acid permease 2 (*AAP2*); cytokinin response factor 4 (*CRF4*); *Oryza sativa* L.; remote sensing.

RESUMEN

El nitrógeno (N) es esencial para la síntesis de aminoácidos en la producción de arroz, pero su uso excesivo plantea una preocupación ambiental. Esta investigación tuvo como objetivo mejorar la eficiencia agronómica del uso del nitrógeno (ANUE) en el arroz mediante "knockout (KO)" de homólogos en arroz de los dos genes seleccionados de *Arabidopsis thaliana*: *AtAAP2*, una permeasa de aminoácidos involucrada en el transporte de N en los brotes, y *AtCRF4*, un factor de transcripción que participa en la absorción de N en las raíces. Los homólogos de estos genes en el arroz se identificaron por la similitud de la secuencia de aminoácidos y se desactivaron mediante la edición genética (GE) mediada por CRISPR/Cas9. Las líneas *AAP2*-KO y *CRF4*-KO fueron sometidas a evaluaciones agronómicas con tres dosis de N: 100% (180 kg ha^{-1}), 50% (90 kg ha^{-1}) y 0% (0 kg ha^{-1}) y mostraron un aumento de 130-175% en peso de biomasa seca y un aumento de 183-313% en número de panículas en comparación con el control (WT) en el primer experimento. Estas líneas también tenían una senescencia foliar más lenta, el denominado rasgo de "permanecer verde", lo que indica el efecto de desactivación de genes del objetivo en el metabolismo del N. Sin embargo, ni *AAP2*-KO ni *CRF4*-KO mostraron mejor rendimiento o ANUE que WT. Este estudio demostró la utilidad de la tecnología de edición genética en la evaluación de genes y destacó los efectos de los genes *AtAAP2* y *AtCRF4* en el ciclo del N de la planta.

Palabras clave adicionales: aminoácido permeasa 2 (*AAP2*); factor de respuesta de citoquinina 4 (*CRF4*); *Oryza sativa* L.; sensores remotos.

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INTRODUCTION

Rice is a vital crop, providing over 21% of global caloric needs (Fitzgerald *et al*., 2009). The "Green Revolution" in the 20th century significantly increased rice productivity through the breeding of new cultivars and the application of nitrogen (N) fertilizer (Pingali, 2012). However, excessive N use has negative environmental impacts, including greenhouse gas (GHG) emissions (Martínez-Dalmau *et al*., 2021). Improving rice nitrogen use efficiency (NUE) is crucial to reduce N fertilizer application and mitigating environmental consequences.

Strategies for enhancing NUE include agronomic approaches like site-specific nutrient management (SSNM) and alternate wetting and drying (AWD) (Mofijul *et al*., 2016; Chivenge *et al*., 2021), as well as genetic approaches targeting N-related genes (Sonoda *et al*., 2003; Hu *et al*., 2015). Advances in sequencing and genomic tools facilitated the introduction of molecular breeding techniques such as masker-assisted selection (MAS), genetically modified organisms (GMOs), and gene editing (GE) (Collard *et al*., 2008; Wang and Han, 2022). Currently, clustered regularly interspaced short palindromic repeats and Cas9 nuclease (CRISPR/Cas9) system has become a widely used GE technology for its simple structure and flexible applicability (Zhang *et al*., 2019).

Previous studies in *Arabidopsis thaliana* have shown that knockout (KO) mutation of *AtAAP2* function decreased seed protein levels (Zhang *et al*., 2010), but enhanced N allocation and improved the amino acid level in leaves, leading to higher photosynthesis efficiency, carbon fixation ability, and seed yields (Perchlik and Tegeder, 2018). *AtCRF4* is a transcription factor (TF) involved in N uptake and assimilation in *A. thaliana*, engaged in N signaling in shoots and roots (Brooks *et al*., 2019). Over-expression mutants of *AtCRF4* showed decreased N uptake, root biomass, primary root length, and lateral root number, due to inhibition of *SNZ* and *CDF1*, two TFs that are involved in N uptake and their expression is controlled by *CRF4* (Varala *et al*., 2018). Therefore, inhibiting *AtCRF4* expression could enhance N uptake efficiency by increasing *SNZ* and *CDF1* expressions, which may increase *NRT2.1* expression - another key nitrate transporter.

This research aimed to improve rice agronomic NUE (ANUE) by knocking out rice homologs of *AtAAP2* and *AtCRF4* using CRISPR/Cas9 and evaluating agronomic performance in the field.

MATERIALS AND METHODS

Creation of the KO rice lines by gene editing

The *AAP2*-KO and *CRF4*-KO rice lines were created in the National Agriculture and Food Research Organization (NARO) in Japan by identifying their homologs through bioinformatic research. Their homologs in rice were identified by BLAST search on RAP-DB (https://rapdb.dna.affrc.go.jp/) and SALAD Database (https://salad.dna.affrc.go.jp/salad/) based on the amino acid sequences of the candidate genes on TAIR (https://www.arabidopsis.org/) for *A. thaliana*. 4 *AtAAP2* homologs (LOC4324999, LOC4327987, LOC4336246, LOC4338844) and 1 *AtCRF4* homolog (LOC4326729) with certain expression level were identified in rice genome. The target sequences for the gRNA of target genes were selected based on the exon regions and PAM sequence locations, and CRISPR/Cas9 binary vectors with guide RNA (gRNA) were designed for each target gene (Fig. 1).

Agrobacterium tumefasiens competent cell EHA105 was transformed with the plasmids (binary vectors) through electroporation. The transformed *Agrobacterium* was cultured and selected on solid LB medium with hygromycin. Surviving colonies were stored as glycerol stock. Rice callus induction was achieved on solid N6D medium, followed by infection with transformed *Agrobacterium* on solid AB medium and co-cultivation on solid 2N6AS medium. After infection, rice calli were sterilized, transferred to N6D medium with hygromycin and meropenem for selection.

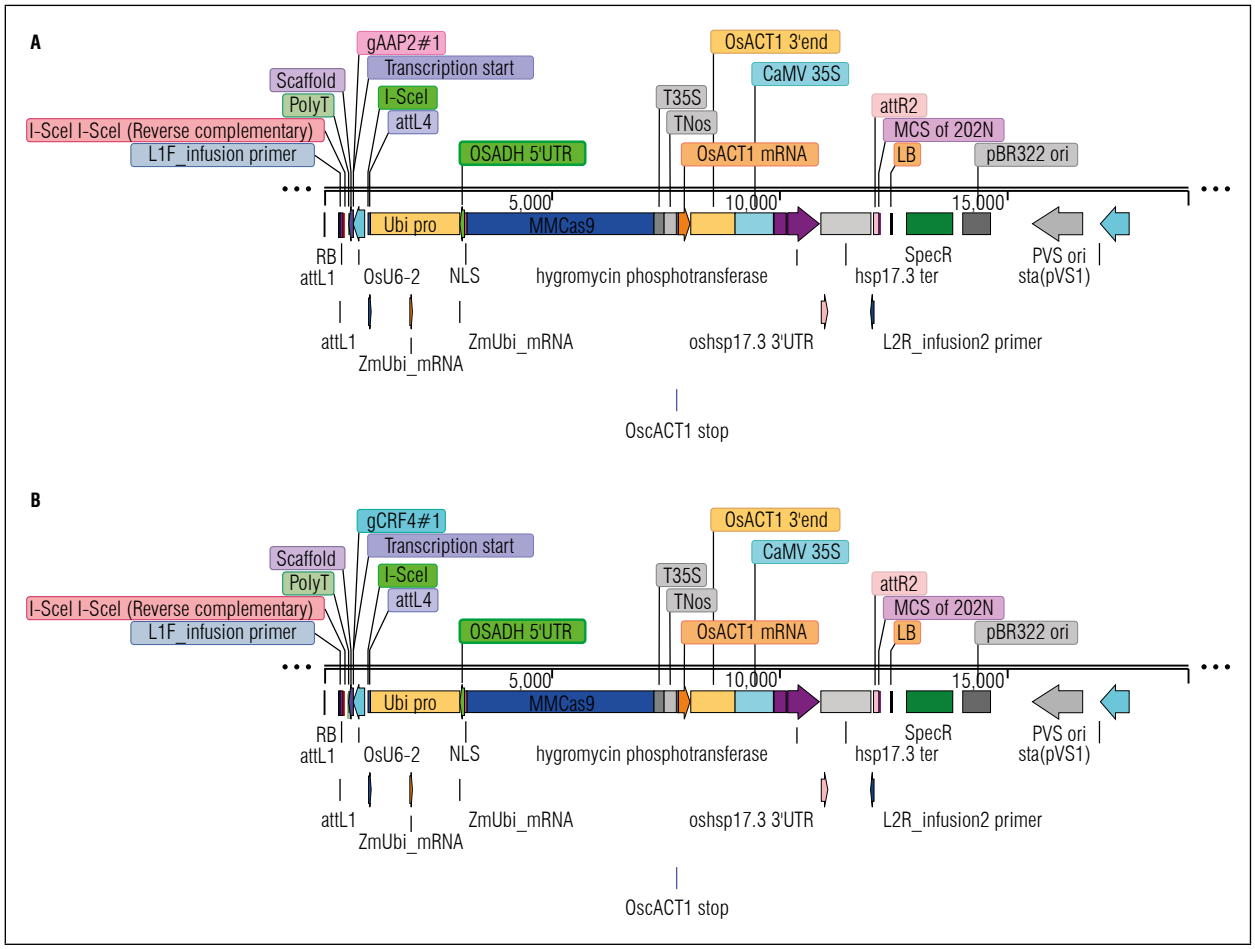

Figure 1. Vector designs for *AtAAP2* homolog (A), and *AtCRF4* homolog (B) in rice.

From those survived calli, DNA samples were extracted to detect candidate calli for regeneration. The selected calli were transferred onto ReIII regeneration medium with meropenem. Regenerated plants were transplanted onto a hormone-free medium, and their DNA was analyzed via PCR and capillary electrophoresis for KO confirmation. Both *AAP2*-KO and *CRF4*-KO lines were successfully obtained, and T_1 and T_2 seeds were sent to the International Center for Tropical Agriculture (CIAT) in Palmira, Valle del Cauca, Colombia for evaluation of agronomic parameters and ANUE. The experimental lines were put in genotyping before the seed shipment (Suppl. Tab. 1).

Design of confined paddy field experiments

A paddy field experiment was conducted at CIAT, following the field design and management from (Selvaraj *et al*., 2017). To induce soil N depletion, three cycles of maize cultivation without fertilizer application were performed in previous seasons and soil samples were taken from each pool for analysis before transplanting. The experimental field had a split-plot design with three replicates. 14 lines including control were selected for the field experiment based on the mutagenesis location and types (Tab. 1). Seeds were sown on June 24, 2022, and transplanted on July 15 (21 days after sowing, DAS) with 20 cm gaps between plants and 25 cm gaps between rows. Statistical analysis was carried out on seven randomly selected plants per plot after the harvest on October 18 (118 DAS).

Nitrogen fertilizer was applied in the following treatments: (i) N 0% pool ($N_{0\%}$) received 0 kg ha^{-1}, (ii) N 50% pool ($N_{50\%}$) received 90 kg ha^{-1}, and (iii) N 100% pool ($N_{100\%}$) received 180 kg ha^{-1}. The rate of 180 kg ha^{-1} for $N_{100\%}$ was based on the farmers' practice in Colombia (Berrío *et al*., 2002). Actual N fertilizer weights were adjusted accordingly. In $N_{100\%}$, urea was applied in three splits, while $N_{50\%}$ received half the amount following the same application regime as $N_{100\%}$. Other nutrients were applied as basal fertilizer at the standard rate in Colombia.

For statistical analysis and ANUE calculation, seven randomly selected plants from each plot were harvested. Eight agronomic parameters were examined: plant height (PH), stem number (SN), panicle number (PN), panicle length (PL), filled grain weight (FG), empty grain weight (EG), dry biomass weight (DB), and 1,000-grain weight (1000GW).

Table 1. Rice lines for the field experiment.

No.	Line name	Description
1	AAP2-25-1-4	*AAP2*-KO
2	AAP2-30-8-1	*AAP2*-KO
3	AAP2-30-8-4	*AAP2*-KO
4	AAP2-30-8-10	*AAP2*-KO
5	CRF4-14-2	*CRF4*-KO
6	CRF4-22-2	*CRF4*-KO
7	CRF4-25-1	*CRF4*-KO
8	AAP2-25-1-3(2)-PI-2	*AAP2*-KO segregant
9	AAP2-30-8-1-PI-1	*AAP2*-KO segregant
10	AAP2-30-8-4-PI-1	*AAP2*-KO segregant
11	CRF4-14-2-PI-1	*CRF4*-KO segregant
12	CRF4-22-2-PI-1	*CRF4*-KO segregant
13	CRF4-25-1-PI-1	*CRF4*-KO segregant
14	WT Nipponbare	Control

ANUE calculation

ANUE is an NUE index calculated using yields (kg ha^{-1}) from the N-applied and non-fertilized control fields. It assesses the yield contribution of the N application by comparing it to the control. ANUE range is typically 10-30 kg kg^{-1}, with a value of 25 or higher indicating efficient management or low soil N supply (Congreves *et al*., 2021). The ANUE formula (Eq. 1) is derived from (Craswell and Godwin, 1984)

$$Agronomic\ NUE = \frac{Grain\ yield_F - Grain\ yield_C}{Applied\ N\ fertilizer}\ kg\ kg^{-1} \quad (1)$$

where, F = fertilized crop, C = unfertilized control.

NDVI and NDRE analysis

Plant monitoring by vegetation indices (VIs) was carried out during the paddy field experiment based on the protocols by Selvaraj *et al*. (2020). Normalized difference vegetation index (NDVI) and normalized difference red-edge (NDRE) were the VIs used to measure plant health and density. NDVI uses the near-infrared (NIR) and red (RED) spectral bands to measure greenness, while NDRE employs the red edge (REDGE) spectral band provided by MicaSense (Boiarskii and Hasegawa, 2019). A drone DJI MATRICE 600 and a multispectral camera MicaSense Altum (MicaSense, Seattle, Washington, USA) were

used to take aerial pictures. Ground control points (GCPs) were installed for georeferencing, and QGIS (https://www.qgis.org) was used to designate the regions of interest (ROIs) in the aerial pictures. The vegetation indices were extracted on CIAT Pheno-i (http://pheno-i.ciat.cgiar.org). Statistical analysis on NDVI and NDRE was conducted at 60 DAS (heading), 70 DAS (flowering), 81 DAS (milky-grain), and 88 DAS (grain-filling).

The formulas for NDVI (Eq. 2) and NDRE (Eq. 3) are as follows (Tucker, 1979; Barnes *et al*., 2000).

$$NDVI = \frac{NIR - RED}{NIR + RED} \quad (2)$$

$$NDRE = \frac{NIR - REDGE}{NIR + REDGE} \quad (3)$$

Statistical analysis

The statistical analyses were carried out on Statistical Analysis System SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The mixed model was employed for the analysis of the field experiments, assuming N treatment, genotype, and interactions of N treatment and genotype as fixed effects, and the blocks as random effects. Duncan's multiple range test was used to detect significant effects in the variation on the agronomic parameters and ANUE between all experimental lines. Meanwhile, Dunnett's test was used for NDVI and NDRE analysis to compare the extracted VI values between WT and KO lines at $P = 0.05$ in both tests.

Inserted marker *hpt* cassette analysis

Leaf samples were collected during the field experiment at CIAT for genomic DNA extraction to detect the remaining CRISPR/Cas9 cassette by PCR for *hpt* gene, the selection marker used in this study. Seven plants were randomly selected from each line in block 1 of $N_{100\%}$ on September 1st, 2022 and their flag leaves were collected. Genomic DNA from the samples was extracted based on the protocol by Risterucci *et al*. (2000) and modified by M. Lorieux in 2002. The dissolved genomic DNA of each sample was stored at -20°C until genetic analysis by PCR. To detect *hpt* gene, primers HPT-F3 (5´-AGT-TCG-ACA-GCG-TCT-CCG-ACC-TGA-3´) and HPT-R1 (5´-TGC-CGT-CAA-CCA-AGC-TCT-GAT-AGA-GT-3´) were used, which amplify a 792 bp fragment. The PCR mix contained 2X GoTaq Master MIX (Promega, Madison, WI, USA), 0.5 μL of genomic DNA, and 0.25 μM each HPT-F3 / HPT-R1 primer. The conditions of the thermocycler were: 98°C for 3 min, 35 cycles of 98 °C for 15 s and 68°C for 1 min were repeated. PCR products were checked on 1.2% agarose gel with SYBR Safe DNA Stain (Thermo Fisher Scientific Inc., Waltham, MA, USA).

RESULTS AND DISCUSSION

Field experiment of the KO lines in a paddy field for agronomic traits and ANUE

The soil analysis before the transplanting showed the average soil pH was 8.48 and the average POXC content was 11.81 g kg^{-1} which is low. The average NH_4^+ and NO_3^- contents were 7.711 and 5.944 mg kg^{-1}, respectively.

In the field experiment, visually noticeable phenotypic differences were observed between WT and KO lines. The KO lines, whether *AAP2*-KO or *CRF4*-KO, exhibited shorter plant height, a greater stem number, and greener color during the grain-filling stage (Fig. 2).

Significant differences were observed between genotypes (G), N levels, and G x N interactions in FG, EG, PH, SN, PN, and DB. PL did not show any G x N interaction (Suppl. Tab. 2). KO lines generally displayed increased EG, SN, PN, and DB, along with reduced FG, 1000GW, PH, and PL (Fig. 3). The ranges of SN, PN, and DB in *AAP2*-KO and *CRF4*-KO lines were 118-228%, 183-313%, and 130-175% compared to WT, respectively (Suppl. Tab. 2). The *AAP2*-KO segregant and *CRF4*-KO segregant lines exhibited different tendencies, with generally higher 1000GW, PH, and DB, and lower SN compared to WT (Suppl. Tab. 2). Most lines responded well to N applications in terms of DB, SN, PN, and FG, however, WT and the segregant lines were more sensitive to N application compared to the original *AAP2*-KO and *CRF4*-KO lines (Suppl. Fig. 1).

ANUE of the KO lines in the field experiment

ANUaE was calculated after collecting agronomic parameter data. Significant differences were observed in genotypes, N application rates, and G x N interactions in ANUE (Tab. 2). All the original *AAP2*-KO and *CRF4*-KO lines exhibited significantly

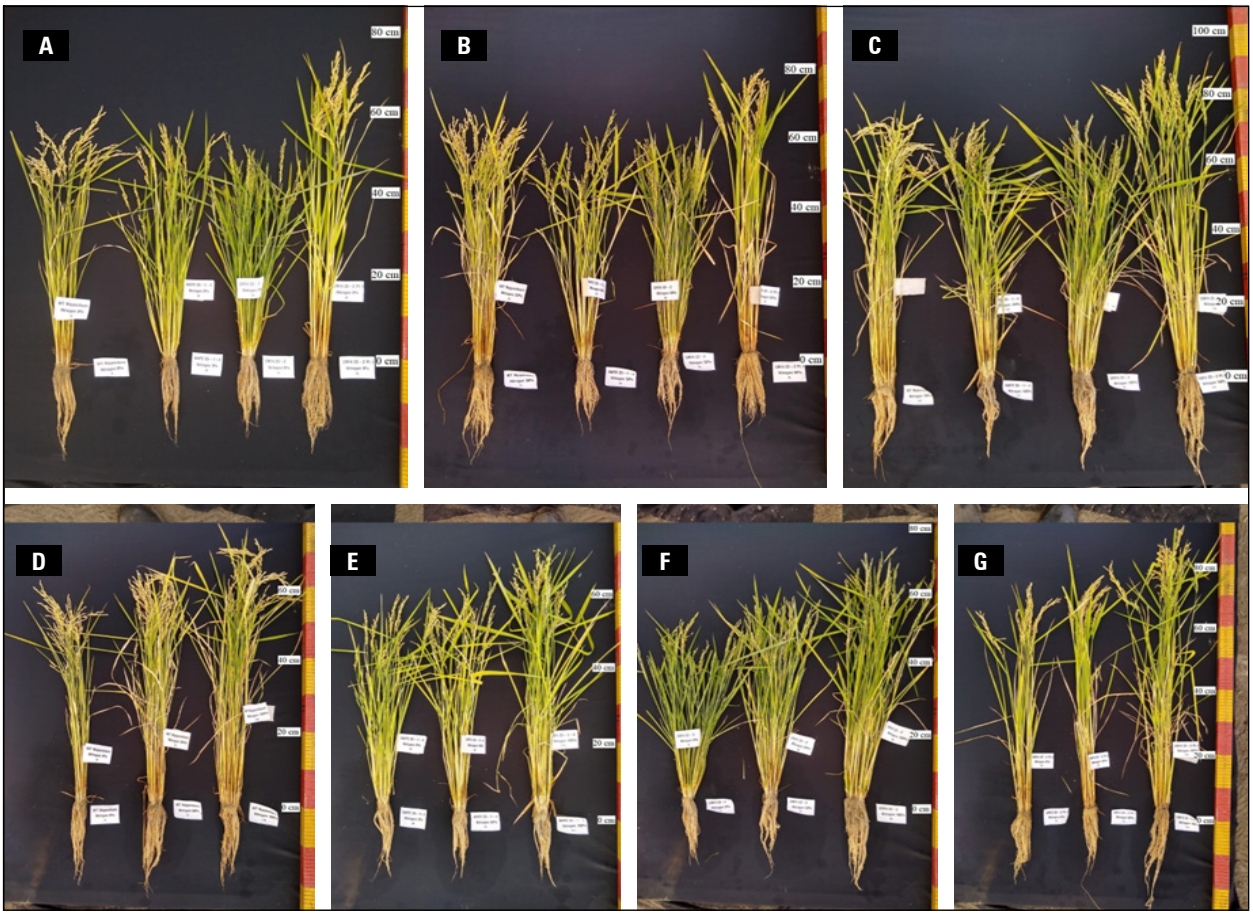

Figure 2. **Rice plants from the second paddy field experiment on October 1st (99 DAS) by N treatment; from left to right: WT, AAP2-25-1-4, CRF4-22-2 (Original GE line), and CRF4-22-2-PI-1 (segregant line) in 0% N pool ($N_{0\%}$) (A), 50% N pool ($N_{50\%}$) (B), and 100% N pool ($N_{100\%}$) (C). By genotypes, WT (D), AAP2-25-1-4 (E), CRF4-22-2 (F), and CRF4-22-2-PI-1 (G), from left to right: $N_{0\%}$, $N_{50\%}$, and $N_{100\%}$ in each picture.**

Table 2. **Agronomic nitrogen use efficiency (ANUE) calculated from the field experiment.**

Genotype	Treatment	Agronomic nitrogen use efficiency (ANUE)
WT Nipponbare	50%	13.85±2.78
WT Nipponbare	100%	19.57±1.25
AAP2-25-1-4	50%	3.10±0.11†
AAP2-25-1-4	100%	4.99±1.02†
AAP2-30-8-1	50%	5.68±2.91†
AAP2-30-8-1	100%	5.27±0.61†
AAP2-30-8-4	50%	2.60±2.47†
AAP2-30-8-4	100%	5.92±1.23†
AAP2-30-8-10	50%	4.81±0.14†
AAP2-30-8-10	100%	7.16±0.12†
CRF4-14-2	50%	-0.33±1.49†
CRF4-14-2	100%	3.63±0.32†

Continued

Table 2, continuation. **Agronomic nitrogen use efficiency (ANUE) calculated from the field experiment.**

Genotype	Treatment	Agronomic nitrogen use efficiency (ANUE)
CRF4-22-2	50%	0.90±0.64†
CRF4-22-2	100%	1.91±2.54†
CRF4-25-1	50%	-3.17±1.38†
CRF4-25-1	100%	1.26±0.39†
AAP2-25-1-3(2)-PI-2	50%	3.76±0.77†
AAP2-25-1-3(2)-PI-2	100%	4.42±0.39†
AAP2-30-8-1-PI-1	50%	9.20±2.08
AAP2-30-8-1-PI-1	100%	16.86±3.92
AAP2-30-8-4-PI-1	50%	11.85±2.13
AAP2-30-8-4-PI-1	100%	17.96±3.47
CRF4-14-2-PI-1	50%	-0.53±2.83†
CRF4-14-2-PI-1	100%	19.02±3.91

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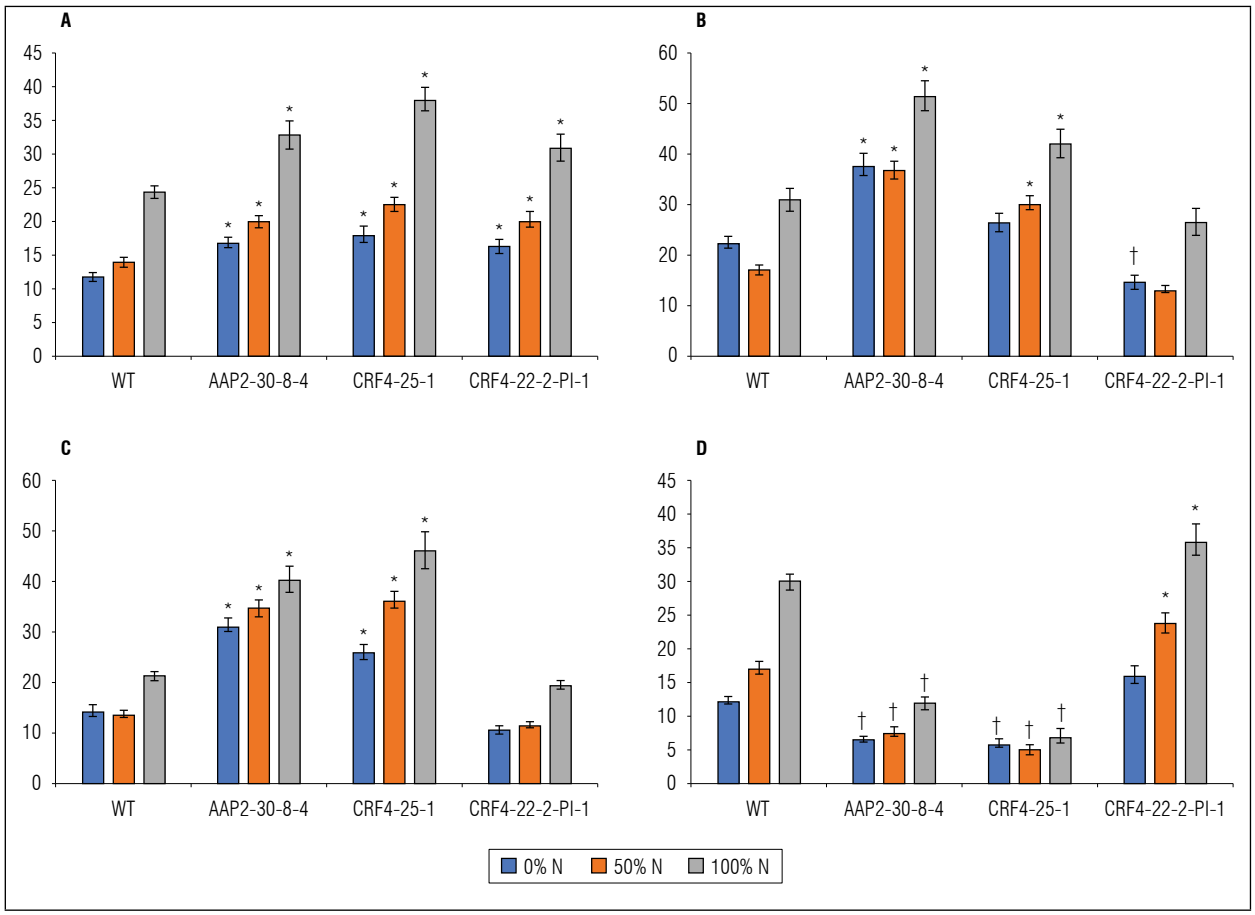

Figure 3. Means of WT, AAP2-30-8-4, CRF4-25-1, and CRF4-22-2-PI-1 on dry biomass weight, g/plant (A), stem number (B), panicle number (C), and filled grain weight, g/plant (D) under different N application regimes in the second field experiment. Values are means±SE of 21 plants. * indicates a significantly higher value and † indicates a significantly lower value at $P<0.05$.

Table 2, continuation. Agronomic nitrogen use efficiency (ANUE) calculated from the field experiment.

Genotype	Treatment	Agronomic nitrogen use efficiency (ANUE)
CRF4-22-2-PI-1	50%	16.96±3.13
CRF4-22-2-PI-1	100%	22.23±3.42
CRF4-25-1-PI-1	50%	20.21±12.12
CRF4-25-1-PI-1	100%	19.51±0.21
Genotype (G)		**
N level (N)		**
G x N		**
CV %		63.57
Mean		5.57

Values are means ± standard error (SE) from 21 plants. * significantly higher than wild type (WT) ($P<0.05$), ** significantly higher than WT ($P<0.01$), † significantly lower than WT ($P<0.05$).

lower ANUE than WT, regardless of N application rates. The segregant lines, such as CRF4-22-2-PI-1 at $N_{50\%}$ and $N_{100\%}$ and CRF4-25-1-PI-1 at $N_{50\%}$, demonstrated higher ANUE than WT, although these were not significant. Overall, the field experiment showed a superior increase of ANUE in WT and the segregant lines with increasing N application, meanwhile, the reaction to N application was weaker in the KO lines (Suppl. Fig. 2).

NDVI and NDRE analysis in the field experiments

There were no significant differences in NDVI and NDRE values between genotypes at 60 DAS, 70 DAS, and 81 DAS, except for some plots at 60 DAS and 81 DAS (Suppl. Tab. 3; Suppl. Tab. 4). However, NDRE detected more differences at 88 DAS, with 11 lines of KO and KO-segregated lines exhibiting higher NDRE

values than WT under different N conditions (Fig. 4). The dynamics of NDVI were similar between genotypes and N levels, while in NDRE, the dynamics between N levels were more distinct (Suppl. Fig. 3), and differences between genotypes were evident at certain time points. These findings align with the visual observations in the field, indicating that *AAP2*-KO and *CRF4*-KO lines retained their greenness for a longer duration compared to WT, particularly after flowering and grain filling.

Genetic analysis of CRISPR/Cas9 cassette

The existence of the *hpt* between original *AAP2*-KO lines varied, and all the original *CRF4*-KO lines possessed it as confirmed in the previous genotyping before the seed shipment (Fig. 5). Regarding the *CRF4*-KO segregant lines, the *hpt* gene was detected from six of the seven samples in CRF4-14-2-Pl-1 and CRF4-25-1-Pl-1, and all seven samples in CRF4-22-2-Pl-1. However, among the *AAP2*-KO segregant lines,

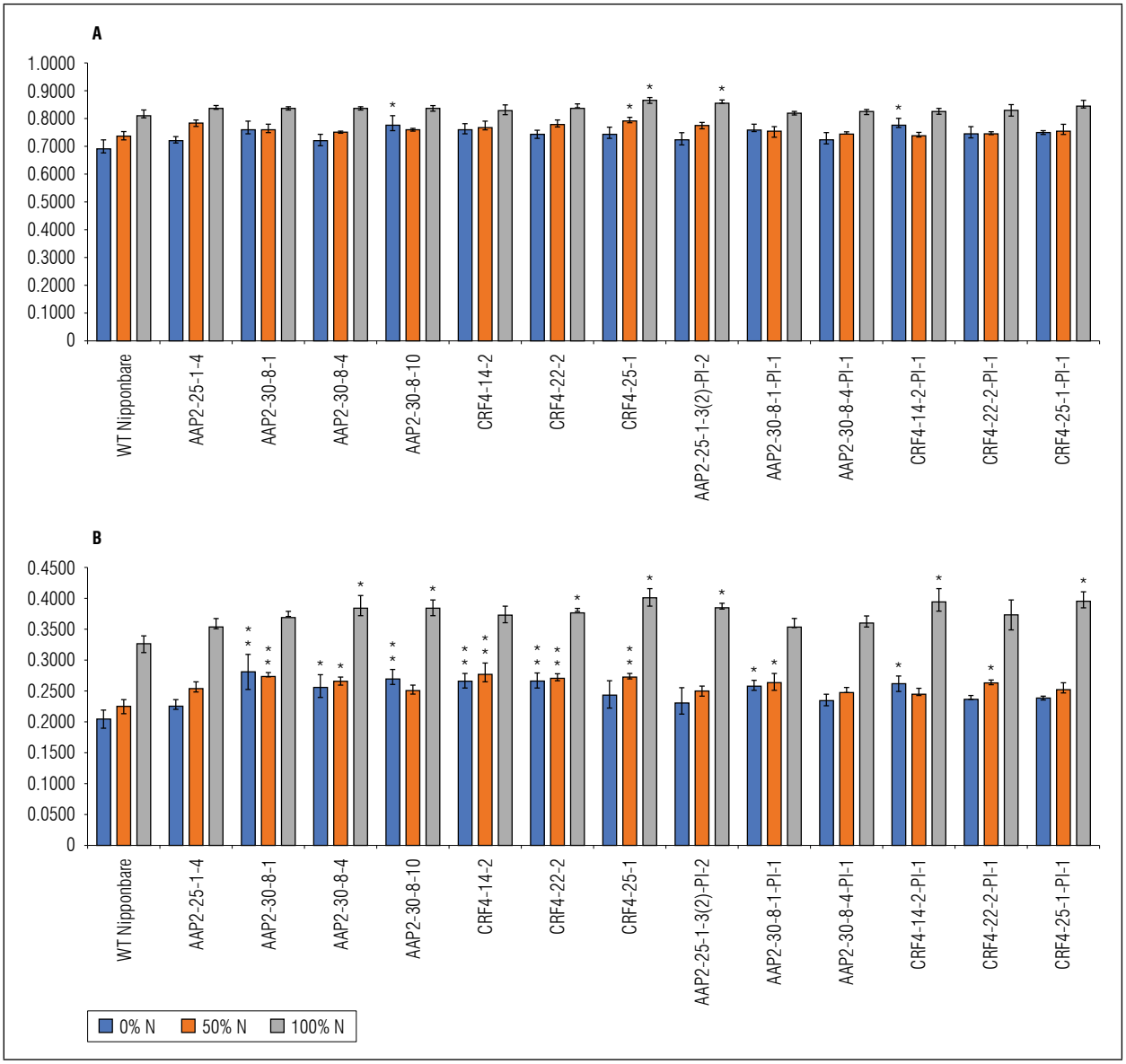

Figure 4. Normalized Difference Vegetation Index (NDVI) (A) and Normalized Difference Red Edge (NDRE) (B) values at 88 DAS. Values are means ±SE from three blocks. * and ** indicate a significantly higher value at $P<0.05$ and $P<0.01$, respectively. Genetic analysis of CRISPR/Cas9 cassette

AAP2-30-8-1-Pl-1, AAP2-30-8-4-Pl-1, and AAP2-25-1-3(2)-Pl-2 had the *hpt* gene in four, two, and three of the collected seven samples, respectively.

Knockout of *AtAAP2* and *AtCRF4* homologs delayed senescence of rice plant

In our study, we used CRISPR/Cas9 to knock out the homologs of two selected *A. thaliana* genes in rice. The *AAP2*-KO and *CRF4*-KO lines exhibited prolonged greenness during the grain-filling stage, as indicated by NDVI and NDRE analysis (Fig. 4). This "stay-green" phenotype may contribute to increased biomass or yield (Borrell *et al*., 2001). However, the impact of "stay-green" is still debated in previous studies, with both positive (Borrell *et al*., 2000; Fu *et al*., 2009) and negative (Cha *et al*., 2002; Zang *et al*., 2022) effects reported. The *AAP2*-KO and *CRF4*-KO lines may possess a type B or C stay-green phenotype (Thomas and Howarth, 2000), as they showed similar VI dynamics to the wild type during vegetative growth but higher NDVI or NDRE values later on (Suppl. Tab. 3; Suppl. Tab. 4). The knockout of *AtAAP2* and *AtCRF4* homologs likely affected the N pathways in the rice plants, as senescence involves chlorophyll degradation and N remobilization (Zang *et al*., 2022).

One possible explanation for the observed features in the KO lines is that the knockout of the target gene(s) disrupted the translocation of N and photosynthetic products from the source to sink tissues. Another hypothesis suggests that the phenotypic features in *CRF4*-KO lines may be due to interactions between NRT2.1 and other nitrate transporters. *AtCRF4* regulates the expression of the high-affinity nitrate transporter NRT2.1 (Varala *et al*., 2018), and previous studies have shown that OsNRT2.1 requires the partner protein OsNAR2.1 to enhance its N uptake

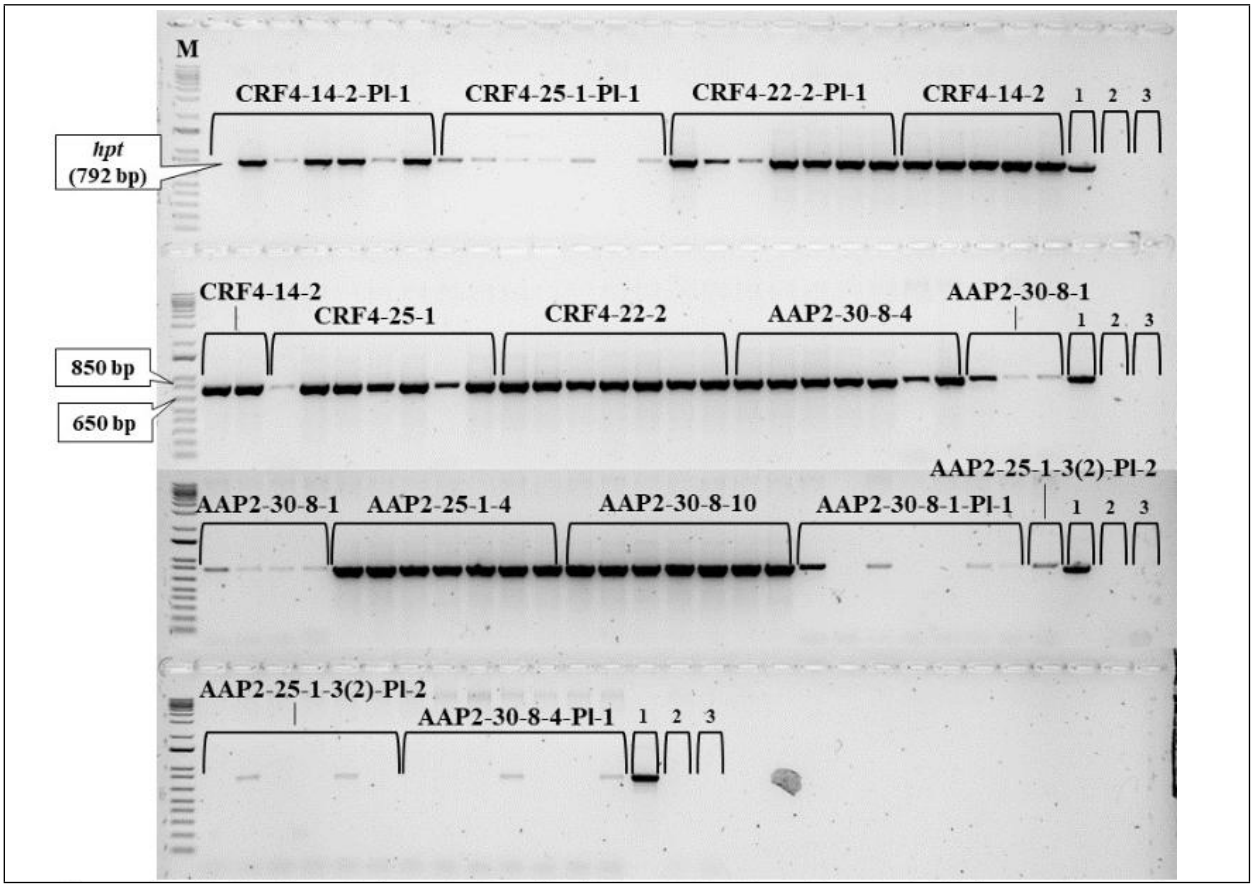

Figure 5. ***hpt*** **detection by PCR between the rice lines in the field experiment. 1.2 % agarose gel with SYBR Safe DNA Stain (Thermo Fisher Scientific). The numbers in the figure indicate: 1, plasmid with** ***hpt*** **(positive control); 2, WT Nipponbare (negative control); 3, distilled water (control negative). M: 1Kb PLUS DNA Ladder (Invitrogen), 0.5 μg μL.**

activity (Feng *et al*., 2011). Altered N uptake was not observed when *OsNRTs* were overexpressed under the constitutive S35 promoter (Katayama *et al*., 2009). Transgenic lines of *OsNRT2.1* with different promoters exhibited varying levels of agronomic nitrogen use efficiency (ANUE) and dry matter translocation to grains (Chen *et al*., 2016). The complete knockout of the *AtCRF4* homolog in rice likely led to an abundant expression of nitrate transporters throughout the plant body due to the lack of the TF that represses their expression and might induce an overexpression-like condition. In a similar experiment, transgenic lines with *OsNRT2.1* overexpression under a constitutive promoter displayed shorter plant height and lower yield (Chen *et al*., 2016), although not as extreme as observed in our *CRF4*-KO lines. These findings align with our observations.

The *AAP2*-KO and *CRF4*-KO lines did not exhibit improved ANUE compared to WT, likely due to lower yield and limited response to N application (Fig. 3; Tab. 2). The cause of this reduced yield is still uncertain. One possible explanation is that delayed senescence hindered N remobilization, leading to lower yield (Zang *et al*., 2022). Alternatively, lower seed setting rates could have inhibited shoot senescence by lacking the destination for N remobilization, as rice leaf senescence primarily occurs before plant death to facilitate nutrient transfer to seeds (Lee and Masclaux-Daubresse, 2021). Moreover, genetic variations among *AAP2*-KO genotypes might contribute to yield fluctuations and lower ANUE (Suppl. Tab. 1). The increased stem and panicle numbers in the KO lines could be related to the "stay-green" trait, which has been shown to positively correlate with fertile stem numbers (Luche *et al*., 2015). There is a study that observed similar trends with higher panicle numbers and shorter plant heights in stay-green lines (Sakuraba *et al*., 2015). Overexpressing the low-affinity nitrate transporter *OsNPF7.2* also enhanced the tiller number and grain yield (Wang *et al*., 2018), suggesting interactions between stay-green trait, tiller number, and nitrate transporters in rice.

The observed phenotypic difference in the segregant lines might be due to the recovery of fertility as most of them showed normal or higher yield per plant than WT (Suppl. Tab. 2). However, detecting the genetic differences between the original KO and segregant lines can be challenging since they may be due to somaclonal or off-target mutations, although our gRNAs were designed to reduce the possibility of this unfavorable event (Miyao *et al*., 2012; Zhang *et al*., 2015). Detecting genetic mutations in non-target regions is usually more laborious than sequencing the target regions (Shillito *et al*., 2021).

CONCLUSION

Both *AAP2*-KO and *CRF4*-KO rice lines showed distinct phenotypic features from WT such as shorter plants, increased stem and panicle numbers, and dry biomass weight in the two field experiments. The KO lines also showed delayed leaf senescence; thus, it is evident that the KOs of the target genes affected the N cycles and pathways in rice plants. However, neither *AAP2*-KO nor *CRF4*-KO showed better yield or ANUE than WT. This study demonstrated the usefulness of GE technology in gene evaluation and highlighted the effects of *AtAAP2* and *AtCRF4* genes in the plant N cycle.

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BIBLIOGRAPHIC REFERENCES

Barnes, E.M., T.R. Clarke, S.E. Richards, P.D. Colaizzi, J. Haberland, P. Waller, C. Choi, E. Riley, T. Thompson, R.J. Lascano, H. Li, and M.S. Moran. 2000. Coincident detection of crop water stress, nitrogen status, and canopy density using ground based multispectral data. In: Proc. 5th Int. Conf. Precision Agric. ASA-CSSA-SSSA, Madison WI.

Berrío, L.E., L.R. Sanint, F. Correa, and E. Tulande. 2002. Respuesta al uso de nitrógeno en variedades de arroz sembradas en Colombia, 1950-1999. Foro Arrocero Latinoam. 8(2), 22-23. http://ciat-library.ciat.cgiar.org/Articulos_CIAT/flar/respuesta.pdf

Boiarskii, B. and H. Hasegawa. 2019. Comparison of NDVI and NDRE indices to detect differences in vegetation and chlorophyll content. J. Mech. Continua Math. Sci. (Suppl. 4). Doi: https://doi.org/10.26782/jmcms.spl.4/2019.11.00003

Borrell, A.K., G.L. Hammer, and A.C.L. Douglas. 2000. Does maintaining green leaf area in sorghum improve yield under drought? I. Leaf growth and senescence. Crop Sci. 40(4), 1026-1037. Doi: https://doi.org/10.2135/cropsci2000.4041026x

Borrell, A., G. Hammer, and E. Van Oosterom. 2001. Stay-green: a consequence of the balance between supply and demand for nitrogen during grain filling? Ann. Appl. Biol. 138(1), 91-95. Doi: https://doi.org/10.1111/j.1744-7348.2001.tb00088.x

Brooks, M.D., J. Cirrone, A.V. Pasquino, J.M. Alvarez, J. Swift, S. Mittal, C.-L. Juang, K. Varala, R.A. Gutiérrez, G. Krouk, D. Shasha, and G.M. Coruzzi. 2019. Network walking charts transcriptional dynamics of nitrogen signaling by integrating validated and predicted genome-wide interactions. Nat. Commun. 10, 1569. Doi: https://doi.org/10.1038/s41467-019-09522-1

Cha, K.-W., Y.-J. Lee, H.-J. Koh, B.-M. Lee, Y.-W. Nam, and N.-C. Paek. 2002. Isolation, characterization, and mapping of the stay green mutant in rice. Theor. Appl. Genet. 104, 526-532. Doi: https://doi.org/10.1007/s001220100750

Chen, J., Y. Zhang, Y. Tan, M. Zhang, L. Zhu, G. Xu, and X. Fan. 2016. Agronomic nitrogen-use efficiency of rice can be increased by driving *OsNRT2.1* expression with the *OsNAR2.1* promoter. Plant Biotechnol. J. 14(8), 1705-1715. Doi: https://doi.org/10.1111/pbi.12531

Chivenge, P., S. Sharma, M.A. Bunquin, and J. Hellin. 2021. Improving nitrogen use efficiency—a key for sustainable rice production systems. Front. Sustain. Food Syst. 5, 737412. Doi: https://doi.org/10.3389/fsufs.2021.737412

Collard, B.C.Y., C.M. Vera Cruz, K.L. McNally, P.S. Virk, and D.J. Mackill. 2008. Rice molecular breeding laboratories in the genomics era: current status and future considerations. Int. J. Plant Genomics 2008, 524847. Doi: https://doi.org/10.1155/2008/524847

Congreves, K.A., O. Otchere, D. Ferland, S. Farzadfar, S. Williams, and M.M. Arcand. 2021. Nitrogen use efficiency definitions of today and tomorrow. Front. Plant Sci. 12, 637108. Doi: https://doi.org/10.3389/fpls.2021.637108

Craswell, E.T. and D.C. Godwin. 1984. The efficiency of nitrogen fertilizers applied to cereals in different climates. Adv. Plant Nutr. 1, 1-55.

Feng, H., M. Yan, X. Fan, B. Li, Q. Shen, A.J. Miller, and G. Xu. 2011. Spatial expression and regulation of rice high-affinity nitrate transporters by nitrogen and carbon status. J. Exp. Bot. 62(7), 2319-2332. Doi: https://doi.org/10.1093/jxb/erq403

Fitzgerald, M.A., S.R. McCouch, and R.D. Hall. 2009. Not just a grain of rice: the quest for quality. Trends Plant Sci. 14(3), 133-139. Doi: https://doi.org/10.1016/j.tplants.2008.12.004

Fu, J.-D., Y.-F. Yan, and B.-W. Lee. 2009. Physiological characteristics of a functional stay-green rice "SNU-SG1" during grain-filling period. J. Crop Sci. Biotechnol. 12(1), 47-52. Doi: https://doi.org/10.1007/s12892-009-0078-8

Hu, B., W. Wang, S. Ou, J. Tang, H. Li, R. Che, Z. Zhang, X. Chai, H. Wang, Y. Wang, C. Liang, L. Liu, Z. Piao, Q. Deng, K. Deng, C. Xu, Y. Liang, L. Zhang, L. Li, and C. Chu. 2015. Variation in *NRT1.1B* contributes to nitrate-use divergence between rice subspecies. Nat. Genet. 47(7), 834-838. Doi: https://doi.org/10.1038/ng.3337

Katayama, H., M. Mori, Y. Kawamura, T. Tanaka, M. Mori, and H. Hasegawa. 2009. Production and characterization of transgenic rice plants carrying a high-affinity nitrate transporter gene (*OsNRT2.1*). Breed. Sci. 59(3), 237-243. Doi: https://doi.org/10.1270/jsbbs.59.237

Lee, S. and C. Masclaux-Daubresse. 2021. Current understanding of leaf senescence in rice. Int. J. Mol. Sci. 22(9), 4515. Doi: https://doi.org/10.3390/ijms22094515

Luche, H.S., J.A.G. Silva, L.C. Maia, and A.C. Oliveira. 2015. Stay-green: a potentiality in plant breeding. Cienc. Rural 45(10), 1755-1760. Doi: https://doi.org/10.1590/0103-8478cr20140662

Martínez-Dalmau, J., J. Berbel, and R. Ordóñez-Fernández. 2021. Nitrogen fertilization. A review of the risks associated with the inefficiency of its use and policy responses. Sustainability 13(10), 5625. Doi: https://doi.org/10.3390/su13105625

Miyao, A., M. Nakagome, T. Ohnuma, H. Yamagata, H. Kanamori, Y. Katayose, A. Takahashi, T. Matsumoto, and H. Hirochika. 2012. Molecular spectrum of somaclonal variation in regenerated rice revealed by whole-genome sequencing. Plant Cell Physiol. 53(1), 256-264. Doi: https://doi.org/10.1093/pcp/pcr172

Mofijul, S.M., Y.K. Gaihre, A.L. Shah, U. Singh, M.I.U. Sarkar, M.A. Satter, J. Sanabria, and J.C. Biswas. 2016. Rice yields and nitrogen use efficiency with different fertilizers and water management under intensive lowland rice cropping systems in Bangladesh. Nutr. Cycl. Agroecosyst. 106(2), 143-156. Doi: https://doi.org/10.1007/s10705-016-9795-9

Perchlik, M. and M. Tegeder. 2018. Leaf amino acid supply affects photosynthetic and plant nitrogen use efficiency under nitrogen stress. Plant Physiol. 178(1), 174-188. Doi: https://doi.org/10.1104/pp.18.00597

Pingali, P.L. 2012. Green revolution: impacts, limits, and the path ahead. Proc. Natl. Acad. Sci. U.S.A. 109(31), 12302-12308. Doi: https://doi.org/10.1073/pnas.0912953109

Risterucci, A.M., L. Grivet, J.A.K. N'Goran, I. Pieretti, M.H. Flament, and C. Lanaud. 2000. A high-density linkage map of *Theobroma cacao* L. Theor. Appl. Genet. 101, 948-955. Doi: https://doi.org/10.1007/s001220051566

Sakuraba, Y., W. Piao, J.H. Lim, S.-H. Han, Y.-S. Kim, G. An, and N.-C. Paek. 2015. Rice ONAC106 inhibits leaf senescence and increases salt tolerance and tiller angle. Plant Cell Physiol. 56(12), 2325-2339. Doi: https://doi.org/10.1093/pcp/pcv144

Selvaraj, M.G., M. Valderrama, D. Guzman, M. Valencia, H. Ruiz, and A. Acharjee. 2020. Machine learning for high-throughput field phenotyping and image processing provides insight into the association of above and below-ground traits in cassava (*Manihot esculenta* Crantz). Plant Methods 16, 87. Doi: https://doi.org/10.1186/s13007-020-00625-1

Selvaraj, M.G., M.O. Valencia, S. Ogawa, Y. Lu, L. Wu, C. Downs, W. Skinner, Z. Lu, J.C. Kridl, M. Ishitani, and J. van Boxtel. 2017. Development and field performance of nitrogen use efficient rice lines for Africa. Plant Biotechnol. J. 15(6), 775-787. Doi: https://doi.org/10.1111/pbi.12675

Shillito, R.D., S. Whitt, M. Ross, F. Ghavami, D. De Vleesschauwer, K. D'Halluin, A. Van Hoecke, and F. Meulewaeter. 2021. Detection of genome edits in plants—from editing to seed. In Vitro Cell. Dev. Biol.-Plant 57, 595-608. Doi: https://doi.org/10.1007/s11627-021-10214-z

Sonoda, Y., A. Ikeda, S. Saiki, N. Von Wirén, T. Yamaya, and J. Yamaguchi. 2003. Distinct expression and function of three ammonium transporter genes (*OsAMT1;1-1;3*) in rice. Plant Cell Physiol. 44(7), 726-734. Doi: https://doi.org/10.1093/pcp/pcg083

Thomas, H. and C.J. Howarth. 2000. Five ways to stay green. J. Exp. Bot. 51(Suppl. 1), 329-337. Doi: https://doi.org/10.1093/jexbot/51.suppl_1.329

Tucker, C.J. 1979. Red and photographic infrared linear combinations for monitoring vegetation. Remote Sens. Environ. 8(2), 127-150. Doi: https://doi.org/10.1016/0034-4257(79)90013-0

Varala, K., A. Marshall-Colón, J. Cirrone, M.D. Brooks, A.V. Pasquino, S. Léran, S. Mittal, T.M. Rock, M.B. Edwards, G.J. Kim, S. Ruffel, W.R. McCombie, D. Shasha, and G.M. Coruzzi. 2018. Temporal transcriptional logic of dynamic regulatory networks underlying nitrogen signaling and use in plants. Proc. Natl. Acad. Sci. U.S.A. 115(25), 6494-6499. Doi: https://doi.org/10.1073/pnas.1721487115

Wang, C. and B. Han. 2022. Twenty years of rice genomics research: from sequencing and functional genomics to quantitative genomics. Mol. Plant 15(4), 593-619. Doi: https://doi.org/10.1016/j.molp.2022.03.009

Wang, J., K. Lu, H. Nie, Q. Zeng, B. Wu, J. Qian, and Z. Fang. 2018. Rice nitrate transporter *OsNPF7.2* positively regulates tiller number and grain yield. Rice 11, 12. Doi: https://doi.org/10.1186/s12284-018-0205-6

Zang, Y., Y. Yao, Z. Xu, B. Wang, Y. Mao, W. Wang, W. Zhang, H. Zhang, L. Liu, Z. Wang, G. Liang, J. Yang, Y. Zhou, and J. Gu. 2022. The relationships among "STAY-GREEN" trait, post-anthesis assimilate remobilization, and grain yield in rice (*Oryza sativa* L.). Int. J. Mol. Sci. 23(22), 13668. Doi: https://doi.org/10.3390/ijms232213668

Zhang, L., Q. Tan, R. Lee, A. Trethewy, Y.-H. Lee, and M. Tegeder. 2010. Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in *Arabidopsis*. Plant Cell 22(11), 3603-3620. Doi: https://doi.org/10.1105/tpc.110.073833

Zhang, X.-H., L.Y. Tee, X.-G. Wang, Q.-S. Huang, and S.-H. Yang. 2015. Off-target effects in CRISPR/Cas9-mediated genome engineering. Mol. Ther. Nucleic Acids 4, e264. Doi: https://doi.org/10.1038/mtna.2015.37

Zhang, Y., K. Massel, I.D. Godwin, and C. Gao. 2019. Applications and potential of genome editing in crop improvement. Genome Biol. 20(1), 13. Doi: https://doi.org/10.1186/s13059-019-1622-6

Supplementary Table 1. Genotyping result of the KO lines.

Line	Plant No.	*hpt*	Chr.1	Chr.2	Chr.4	Chr.5
AAP2-25-1	1	+	mono-allelic or bi-allelic*	T insertion homo	WT	WT
	2				WT+T insertion	WT + T insertion
	4				T insertion homo	WT+T insertion
APP2-25-1-3(2)	1	+	mono-allelic or bi-allelic 12 bp after the cleavage site*	T insertion homo	T insertion homo	WT+T insertion
	2				T insertion homo	WT+T insertion
	3				WT+T insertion	WT+T insertion
	4				WT+T insertion	WT+T insertion
	5				T insertion homo	T insertion homo
APP2-25-1-4	1	+	T insertion homo	T insertion homo	WT	WT+T insertion
	2				WT	WT
	3				WT	10 bp deletion+WT
	4				WT	WT+T insertion
APP2-30-8	1	+	mono-allelic or bi-allelic*	T insertion homo	WT+T insertion	WT+T insertion
	2				T insertion homo	WT
	3				10 bp deletion + T insertion	WT+T insertion
	5				10 bp deletion + T insertion	WT+T insertion
APP2-30-8-1	2	-	T insertion homo	T insertion homo	T insertion homo	WT
	3				T insertion homo	WT
	4				10 bp deletion homo	WT
	5				T insertion homo	WT
APP2-30-8-2	1	+	T insertion homo	T insertion homo	10 bp deletion homo	WT+T insertion
	2				T insertion homo	WT+T insertion
	3				T insertion homo	WT+T insertion
	4				10 bp deletion homo	WT
	5				10 bp deletion+ T insertion	WT+T insertion
APP2-30-8-4	1	+	mono-allelic or bi-allelic*	T insertion homo	10 bp deletion homo	T insertion homo
	2				10 bp deletion homo	T insertion homo
	3				10 bp deletion homo	T insertion homo
	4				10 bp deletion homo	T insertion homo
	5				10 bp deletion homo	WT+T insertion
APP2-30-8-5	1	+	mono-allelic or bi-allelic*	T insertion homo	10 bp deletion+ T insertion	WT
	2				T insertion homo	WT
	3				10 bp deletion+ T insertion	WT
	4				T insertion homo	WT
	5				10 bp deletion+ T insertion	WT

Continued

Supplementary Table 1. Genotyping result of the KO lines.

Line	Plant No.	*hpt*	Chr.1	Chr.2	Chr.4	Chr.5
APP2-30-8-8	1	-	mono-allelic or bi-allelic*	T insertion homo	T insertion homo	WT
	2				T insertion homo	WT
	3				T insertion homo	WT
	4				T insertion homo	WT
	5				T insertion homo	WT
APP2-30-8-10	1	-	mono-allelic or bi-allelic*	T insertion homo	T insertion homo	WT+T insertion
	2				10 bp deletion + T insertion	WT+T insertion
	3				10 bp deletion homo	WT+T insertion
	4				10 bp deletion + T insertion	WT+T insertion
	5				T insertion homo	WT+T insertion
CRF4-14-2		+	A insertion homo			
CRF4-14-2-5		-	A insertion homo			
CRF4-14-2-6		-	A insertion homo			
CRF4-14-4		+	A insertion homo			
CRF4-14-4-2		-	A insertion homo			
CRF4-22-2		+	A insertion homo			
CRF4-25-1		+	A insertion homo			

* indicates mutation patterns are not determined. *AtAAP2* homologs were found on chromosomes 1, 2, 4 and 5, meanwhile *AtCRF4* homolog exists only on chromosome 1. Mutation types without specific location in this table means it occurred at the cleavage site of each target region.The genotyping of *AtAAP2* homologs chr.1, 2 and *AtCRF4* homolog chr.1 were carried out for one individual plant. Regarding the *AtAAP2* homologs on chr. 4 and 5, the genotyping was carried out for maximum five plants. All the lines in this list were shipped to CIAT, but the lines in grey color were removed from the experimental list due to their performances during the seed quarantine and multiplication.

Supplementary Table 2. Summary of the statistical analysis of the agronomic parameters in the field experiment. The parameters are identical to Table 3.

Genotype	Treatment	FG	EG	1000 GW	PH	SN	PN	DB	PL
Nipponbare_WT	0%	12.12 ± 0.56	0.46 ± 0.09	25.20 ± 0.35	65.52 ± 1.36	22.24 ± 1.22	14.05 ± 1.21	11.65 ± 0.63	15.08 ± 0.37
	50%	17.02 ± 0.86	0.45 ± 0.06	26.19 ± 0.18	70.67 ± 0.86	16.81 ± 0.81	13.38 ± 0.64	13.79 ± 0.67	16.17 ± 0.31
	100%	29.73 ± 1.15	1.01 ± 0.07	25.73 ± 0.32	83.05 ± 1.34	30.76 ± 2.32	21.05 ± 0.83	24.11 ± 1.01	17.90 ± 0.78
AAP2-25-1-4	0%	5.80 ± 0.67 †	3.04 ± 0.32 *	20.93 ± 0.21 †	65.33 ± 1.04	34.33 ± 2.38 *	29.43 ± 1.98 *	17.05 ± 0.60 *	13.64 ± 0.34 †
	50%	8.99 ± 1.12 †	5.15 ± 0.81 *	21.54 ± 0.07 †	72.95 ± 1.86	37.86 ± 2.16 *	33.29 ± 2.07 *	22.52 ± 1.45 *	14.92 ± 0.35 †
	100%	10.30 ± 1.14 †	6.08 ± 0.50 *	21.78 ± 0.17 †	76.52 ± 1.06 †	45.81 ± 3.11 *	45.86 ± 3.34 *	29.41 ± 1.66 *	16.29 ± 0.33 †
AAP2-30-8-1	0%	5.59 ± 0.50 †	4.38 ± 0.30 *	22.87 ± 0.13 †	67.14 ± 1.81	29.52 ± 3.31 *	34.95 ± 2.55 *	17.89 ± 0.98 *	13.51 ± 0.23 †
	50%	8.15 ± 1.27 †	3.82 ± 0.43 *	23.22 ± 0.18 †	63.76 ± 0.63 †	37.71 ± 1.92 *	33.33 ± 2.44 *	20.83 ± 1.27 *	14.20 ± 0.31 †
	100%	10.33 ± 1.16 †	6.07 ± 0.44 *	22.35 ± 0.01 †	72.52 ± 1.45 †	50.14 ± 2.05 *	49.81 ± 2.75 *	33.74 ± 1.48 *	14.99 ± 0.23 †
AAP2-30-8-4	0%	6.34 ± 0.38 †	3.03 ± 0.25 *	22.07 ± 0.39 †	61.86 ± 0.71	37.76 ± 2.19 *	31.14 ± 1.46 *	16.62 ± 0.72 *	13.46 ± 0.38 †
	50%	7.51 ± 0.70 †	3.39 ± 0.23 *	22.01 ± 0.14 †	62.33 ± 0.83 †	36.57 ± 1.73 *	34.43 ± 1.72 *	19.88 ± 0.89 *	14.10 ± 0.23 †
	100%	11.67 ± 0.95 †	5.22 ± 0.31 *	22.84 ± 0.06 †	71.76 ± 1.08 †	51.33 ± 3.00 *	40.38 ± 2.66 *	32.72 ± 2.00 *	14.82 ± 0.28 †
AAP2-30-8-10	0%	7.54 ± 0.35 †	3.08 ± 0.27 *	22.46 ± 0.29 †	62.00 ± 0.97	35.57 ± 1.93 *	26.86 ± 1.57 *	17.77 ± 1.15 *	12.50 ± 0.31 †
	50%	8.79 ± 0.49 †	3.38 ± 0.33 *	22.87 ± 0.19 †	65.19 ± 1.64 †	33.19 ± 1.42 *	27.57 ± 1.58 *	17.94 ± 0.86	13.49 ± 0.24 †
	100%	13.99 ± 1.25 †	4.63 ± 0.32 *	22.74 ± 0.21 †	80.67 ± 2.61	40.62 ± 3.98 *	39.29 ± 1.69 *	30.51 ± 1.57 *	15.57 ± 0.27 †
CRF4-14-2	0%	3.25 ± 0.87 †	4.95 ± 0.39 *	23.53 ± 0.30 †	63.14 ± 0.97	35.05 ± 2.40 *	36.91 ± 2.94 *	19.30 ± 1.03 *	12.93 ± 0.37 †
	50%	3.10 ± 0.74 †	5.46 ± 0.38 *	22.69 ± 0.51 †	68.33 ± 1.00	32.57 ± 1.84 *	38.86 ± 2.51 *	22.36 ± 1.38 *	13.66 ± 0.30 †
	100%	4.34 ± 1.04 †	8.59 ± 0.44 *	22.37 ± 1.06 †	74.52 ± 0.82 †	48.91 ± 1.86 *	51.86 ± 2.38 *	36.31 ± 1.55 *	14.37 ± 0.25 †
CRF4-22-2	0%	3.80 ± 0.59 †	5.08 ± 0.34 *	21.43 ± 0.31 †	60.76 ± 0.94 †	35.48 ± 2.36 *	35.95 ± 1.50 *	19.63 ± 1.63 *	12.97 ± 0.24 †
	50%	4.21 ± 0.65 †	6.58 ± 0.47 *	22.20 ± 0.15 †	67.19 ± 0.91	38.33 ± 2.49 *	41.91 ± 2.75 *	24.13 ± 1.48 *	13.81 ± 0.23 †
	100%	5.52 ± 1.16 †	8.10 ± 0.66 *	21.67 ± 0.19 †	75.24 ± 1.19 †	46.19 ± 2.17 *	52.38 ± 3.62 *	32.32 ± 1.75 *	15.18 ± 0.38 †
CRF4-25-1	0%	5.85 ± 0.63 †	3.71 ± 0.35 *	21.66 ± 0.40 †	66.52 ± 1.52	26.24 ± 1.83	25.71 ± 1.52 *	17.80 ± 1.15 *	13.86 ± 0.38 †
	50%	4.90 ± 0.70 †	5.47 ± 0.33 *	21.57 ± 0.11 †	72.81 ± 1.16	30.14 ± 1.37 *	36.19 ± 1.73 *	22.30 ± 0.91 *	14.35 ± 0.24 †
	100%	6.98 ± 1.01 †	8.55 ± 0.52 *	21.72 ± 0.17 †	78.48 ± 1.09 †	41.95 ± 2.83 *	46.05 ± 3.67 *	37.87 ± 1.79 *	16.10 ± 0.44 †
AAP2-25-1-3(2)-PI-2	0%	5.48 ± 0.41 †	3.95 ± 0.45 *	21.55 ± 0.58 †	70.43 ± 0.93 *	26.48 ± 1.68	24.57 ± 1.36 *	17.54 ± 0.96 *	14.90 ± 0.35
	50%	6.60 ± 0.60 †	4.84 ± 0.36 *	21.74 ± 0.26 †	75.24 ± 1.07 *	29.48 ± 1.85 *	32.48 ± 1.72 *	23.47 ± 1.02 *	15.29 ± 0.22
	100%	9.46 ± 0.79	6.87 ± 0.36 *	21.08 ± 0.53 †	89.52 ± 2.08 *	30.48 ± 3.11	39.33 ± 2.38 *	39.23 ± 1.13 *	17.14 ± 0.43
AAP2-30-8-1-PI-1	0%	18.43 ± 1.69 *	1.02 ± 0.25	27.17 ± 1.12 *	79.29 ± 1.75 *	15.38 ± 2.16 †	10.95 ± 1.04	16.20 ± 1.04 *	16.31 ± 0.43
	50%	22.57 ± 1.94 *	1.08 ± 0.15	27.94 ± 0.16 *	85.91 ± 1.09 *	14.76 ± 1.04	12.43 ± 0.53	19.60 ± 0.97 *	16.93 ± 0.28
	100%	33.60 ± 2.97	1.48 ± 0.14	28.08 ± 0.96 *	92.05 ± 1.17 *	25.62 ± 2.51 †	17.33 ± 1.29	30.69 ± 1.90 *	17.70 ± 0.46
AAP2-30-8-4-PI-1	0%	15.87 ± 1.20	0.80 ± 0.13	26.94 ± 0.31 *	75.33 ± 1.11 *	17.95 ± 1.22	10.00 ± 0.33	15.88 ± 1.06 *	15.27 ± 0.37
	50%	19.79 ± 1.36	1.88 ± 0.27 *	27.56 ± 0.56	82.62 ± 1.08 *	17.14 ± 1.59	14.33 ± 1.04	21.23 ± 1.66 *	17.62 ± 0.47 *
	100%	32.04 ± 2.21	2.15 ± 0.31	27.40 ± 0.44 *	87.86 ± 2.04 *	18.71 ± 0.98 †	17.62 ± 0.96	28.89 ± 1.80 *	18.53 ± 0.32

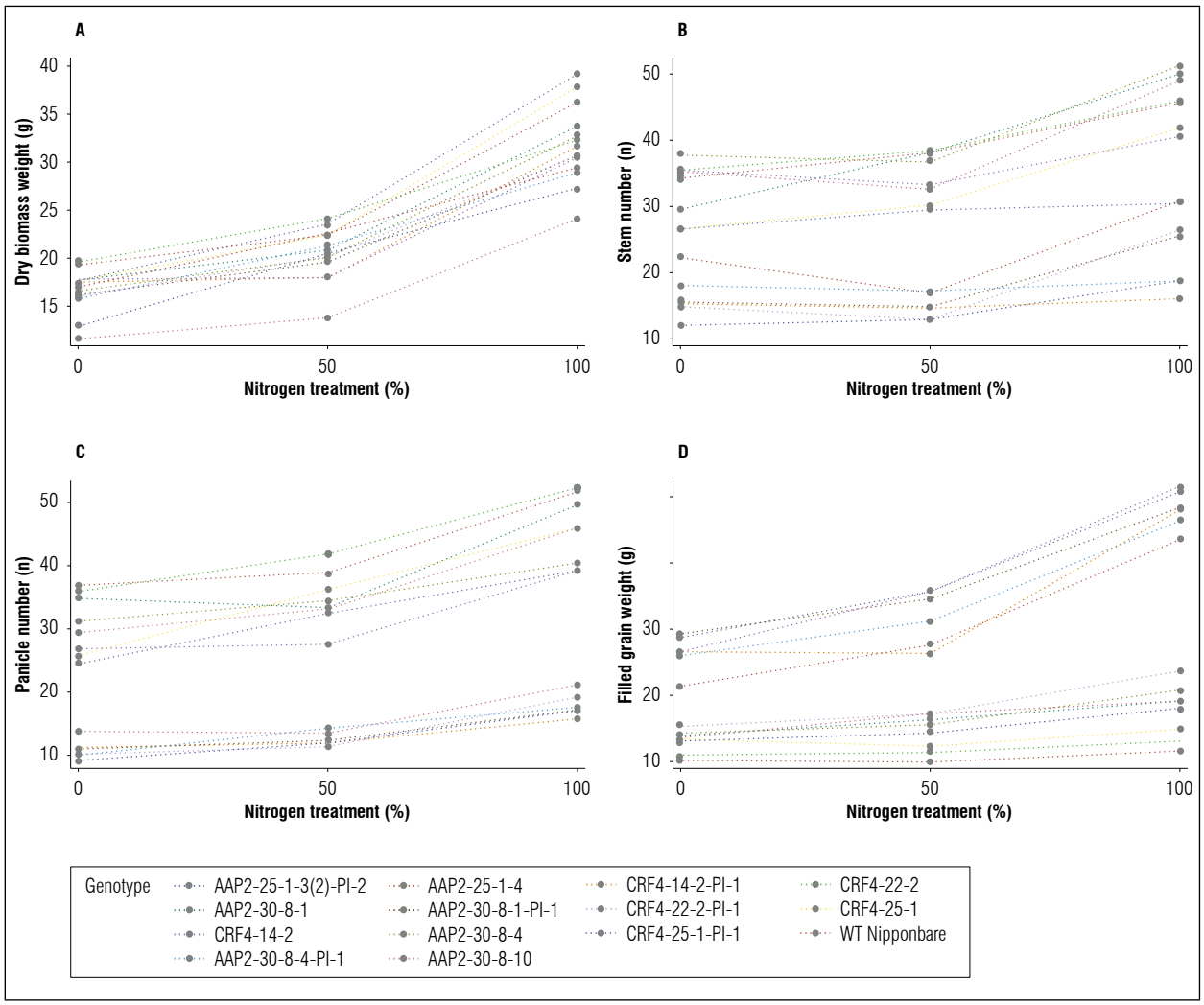
Continued

Supplementary Table 2, continuation. Summary of the statistical analysis of the agronomic parameters in the field experiment. The parameters are identical to Table 3.

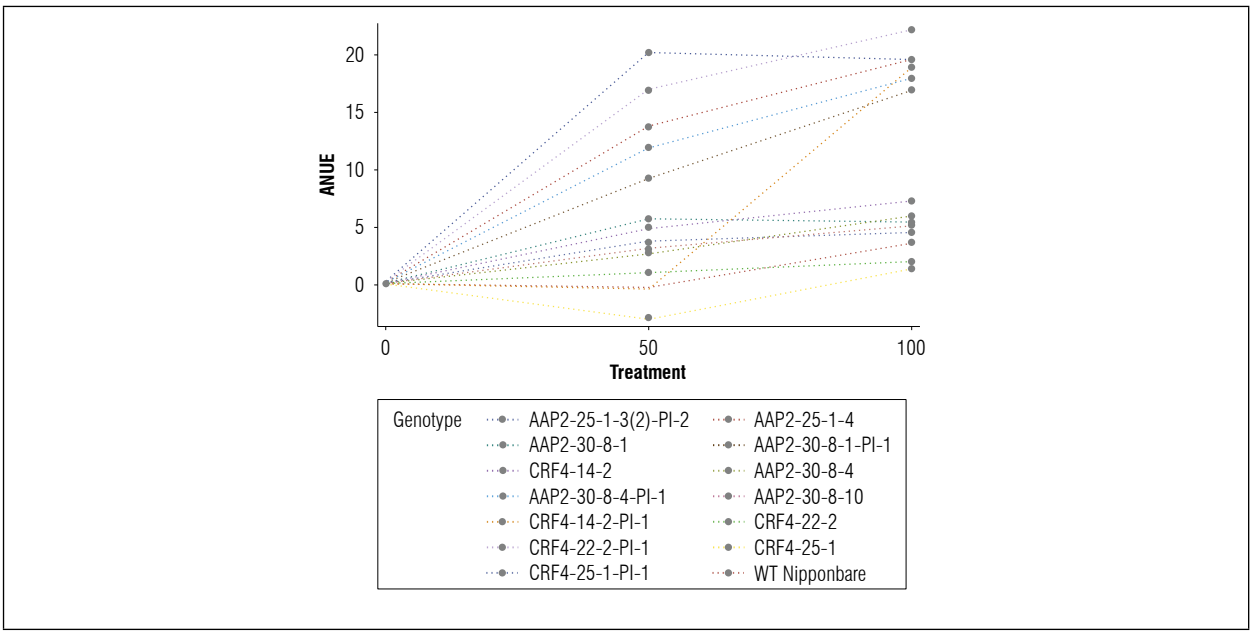
Genotype	Treatment	FG	EG	1000 GW	PH	SN	PN	DB	PL
CRF4-14-2-PI-1	0%	16.31 ± 1.18	1.20 ± 0.16	27.31 ± 0.40 *	76.76 ± 1.44 *	15.24 ± 1.28 †	11.19 ± 0.91	17.38 ± 1.39 *	15.63 ± 0.36
	50%	16.07 ± 1.25	1.46 ± 0.20	26.80 ± 0.28	80.67 ± 1.58 *	14.48 ± 1.07	11.99 ± 0.93	18.05 ± 0.94	16.32 ± 0.46
	100%	33.43 ± 3.10	2.19 ± 0.29	28.22 ± 0.08 *	98.29 ± 2.19 *	16.05 ± 1.02 †	15.76 ± 0.96	31.57 ± 1.71 *	18.34 ± 0.30
CRF4-22-2-PI-1	0%	15.96 ± 1.30	0.91 ± 0.13	27.35 ± 0.66 *	79.95 ± 1.02 *	14.76 ± 1.53 †	10.24 ± 0.78	16.07 ± 1.05 *	16.59 ± 0.44 *
	50%	23.60 ± 1.47 *	1.37 ± 0.26	26.56 ± 0.49	88.38 ± 1.68 *	12.86 ± 0.77	11.33 ± 0.51	20.12 ± 1.09 *	18.28 ± 0.51 *
	100%	35.97 ± 2.36 *	1.84 ± 0.26	27.96 ± 0.08 *	88.71 ± 2.38 *	26.43 ± 2.63	19.19 ± 0.84	30.73 ± 1.91 *	18.69 ± 0.34
CRF4-25-1-PI-1	0%	17.97 ± 1.93 *	0.66 ± 0.11	26.60 ± 0.70 *	79.91 ± 1.82 *	11.91 ± 1.10 †	9.19 ± 0.67	12.87 ± 1.07	15.90 ± 0.42
	50%	23.56 ± 1.66 *	1.71 ± 0.55	25.36 ± 0.40	83.86 ± 1.14 *	12.86 ± 0.81	11.95 ± 0.64	20.43 ± 1.62 *	17.33 ± 0.40
	100%	35.53 ± 2.14 *	1.85 ± 0.25	25.56 ± 0.44	96.71 ± 0.87 *	18.62 ± 0.90 †	17.10 ± 0.88	27.31 ± 1.22	18.30 ± 0.52

Genotype (G)	**	**	**	**	**	**	**	**
N Level (N)	**	**	ns	**	**	**	**	**
G x N	**	**	ns	**	**	**	**	ns
CV %	44.09 %	45.96 %	3.09 %	8.46 %	32.48 %	31.51 %	26.32 %	10.90 %
Mean	14.00 g	3.50 g	24.16 g	75.71 cm	28.91	27.09	22.99 g	15.55 cm

Values are mean±SE from 21 plants. * significantly higher than WT ($P<0.05$), ** significantly higher than WT ($P<0.01$), † significantly lower than WT ($P<0.05$), ns=not significant ($P>0.05$).



Supplementary Figure 1. Interactions between the lines and N treatments in the field experiment. A) dry biomass weight (DB), B) stem number (SN), C) panicle number (PN), and D) filled grain weight (FG).



Supplementary Figure 2. Interactions between genotypes and N levels on ANUE in the field experiment.

Supplementary Table 3. NDVI values in the field experiment.

Genotype	Treatment	60DAS	SE		70DAS	SE		81DAS	SE		88DAS	SE	
WT Nipponbare	0%	0.8440	± 0.0064		0.7295	± 0.0202		0.7160	± 0.0060		0.6924	± 0.0246	
	50%	0.8435	± 0.0022		0.7247	± 0.0112		0.7375	± 0.0091		0.7333	± 0.0125	
	100%	0.8949	± 0.0051		0.7466	± 0.0454		0.8082	± 0.0101		0.8090	± 0.0129	
AAP2-25-1-4	0%	0.8459	± 0.0033	ns	0.7441	± 0.0120	ns	0.7218	± 0.0056	ns	0.7173	± 0.0107	ns
	50%	0.8470	± 0.0065	ns	0.7274	± 0.0485	ns	0.7566	± 0.0078	ns	0.7767	± 0.0114	ns
	100%	0.8857	± 0.0101	ns	0.7556	± 0.0600	ns	0.8244	± 0.0130	ns	0.8355	± 0.0042	ns
AAP2-30-8-1	0%	0.8325	± 0.0025	ns	0.7390	± 0.0291	ns	0.7330	± 0.0072	ns	0.7610	± 0.0227	ns
	50%	0.8427	± 0.0038	ns	0.7427	± 0.0087	ns	0.7399	± 0.0080	ns	0.7570	± 0.0136	ns
	100%	0.8822	± 0.0072	ns	0.7680	± 0.0099	ns	0.8216	± 0.0097	ns	0.8290	± 0.0031	ns
AAP2-30-8-4	0%	0.8414	± 0.0036	ns	0.7180	± 0.0326	ns	0.7322	± 0.0089	ns	0.7166	± 0.0201	ns
	50%	0.8397	± 0.0014	ns	0.7217	± 0.0160	ns	0.7477	± 0.0039	ns	0.7478	± 0.0043	ns
	100%	0.8916	± 0.0080	ns	0.7534	± 0.0343	ns	0.8252	± 0.0068	ns	0.8337	± 0.0056	ns
AAP2-30-8-10	0%	0.8342	± 0.0040	ns	0.7302	± 0.0390	ns	0.7372	± 0.0028	*	0.7781	± 0.0250	*
	50%	0.8414	± 0.0024	ns	0.7092	± 0.0118	ns	0.7552	± 0.0031	ns	0.7555	± 0.0048	ns
	100%	0.8823	± 0.0059	ns	0.7871	± 0.0045	ns	0.8146	± 0.0050	ns	0.8317	± 0.0098	ns
CRF4-14-2	0%	0.8303	± 0.0017	*	0.7263	± 0.0223	ns	0.7351	± 0.0081	*	0.7576	± 0.0195	ns
	50%	0.8385	± 0.0016	ns	0.6943	± 0.0093	ns	0.7522	± 0.0081	ns	0.7685	± 0.0133	ns
	100%	0.8828	± 0.0045	ns	0.7443	± 0.0297	ns	0.8094	± 0.0033	ns	0.8258	± 0.0156	ns

Continued

Supplementary Table 3, continuation. NDVI values in the field experiment.

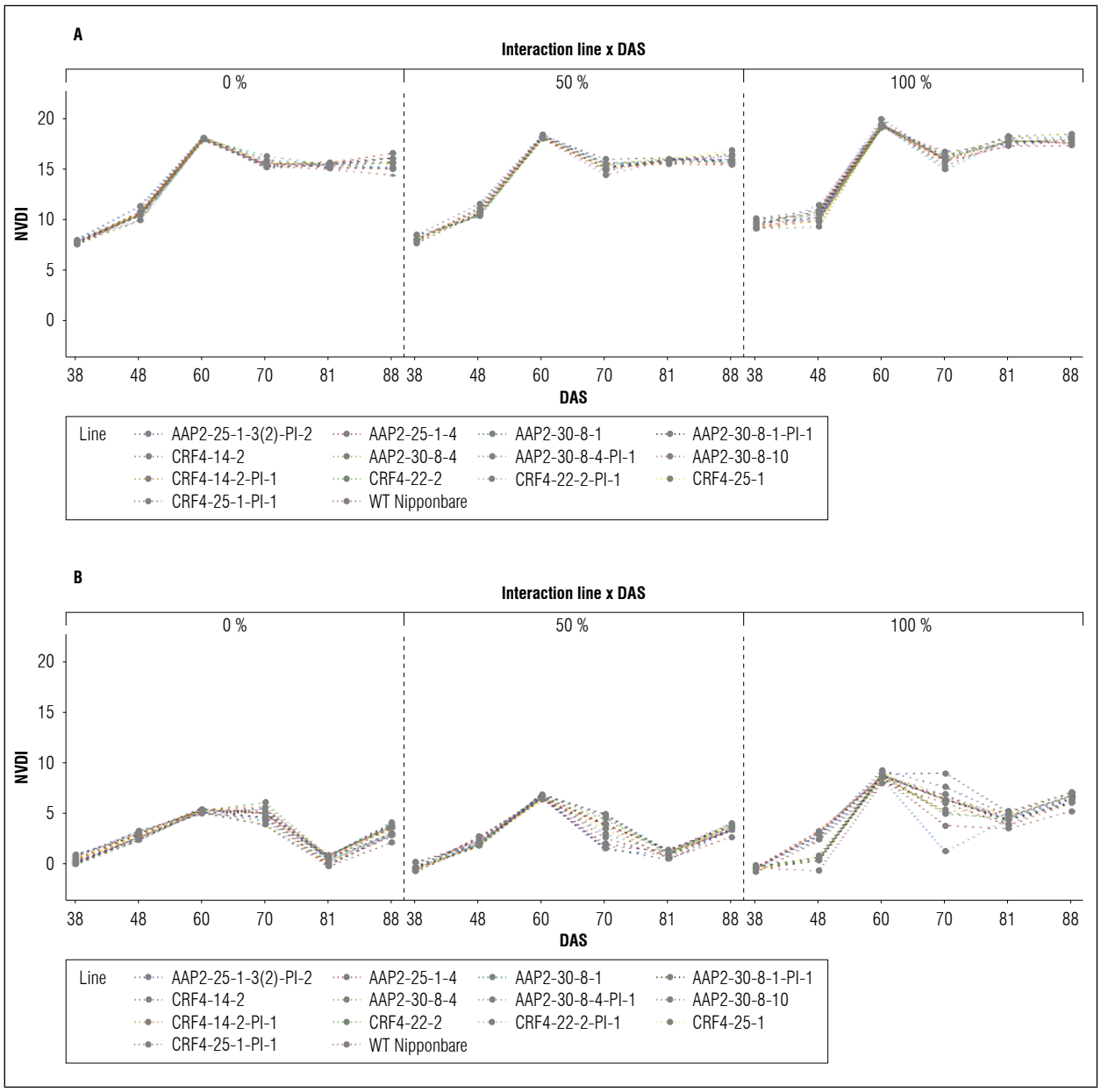
Genotype	Treatment	60DAS	SE		70DAS	SE		81DAS	SE		88DAS	SE	
CRF4-22-2	0%	0.8363 ±	0.0017	ns	0.7675 ±	0.0269	ns	0.7336 ±	0.0022	ns	0.7390 ±	0.0125	ns
	50%	0.8380 ±	0.0054	ns	0.7190 ±	0.0213	ns	0.7561 ±	0.0067	ns	0.7773 ±	0.0110	ns
	100%	0.8838 ±	0.0059	ns	0.7624 ±	0.0339	ns	0.8256 ±	0.0053	ns	0.8391 ±	0.0067	ns
CRF4-25-1	0%	0.8382 ±	0.0039	ns	0.7463 ±	0.0205	ns	0.7341 ±	0.0117	ns	0.7423 ±	0.0224	ns
	50%	0.8421 ±	0.0023	ns	0.7319 ±	0.0149	ns	0.7621 ±	0.0046	ns	0.7884 ±	0.0091	*
	100%	0.8935 ±	0.0064	ns	0.7706 ±	0.0351	ns	0.8409 ±	0.0052	*	0.8592 ±	0.0083	*
AAP2-25-1-3(2)-PI-2	0%	0.8356 ±	0.0034	ns	0.7532 ±	0.0339	ns	0.7239 ±	0.0079	ns	0.7218 ±	0.0217	ns
	50%	0.8492 ±	0.0023	ns	0.7216 ±	0.0452	ns	0.7511 ±	0.0053	ns	0.7694 ±	0.0096	ns
	100%	0.9149 ±	0.0101	ns	0.7523 ±	0.0145	ns	0.8490 ±	0.0069	**	0.8545 ±	0.0039	*
AAP2-30-8-1-PI-1	0%	0.8438 ±	0.0020	ns	0.7342 ±	0.0360	ns	0.7428 ±	0.0033	**	0.7611 ±	0.0107	ns
	50%	0.8496 ±	0.0016	ns	0.7556 ±	0.0154	ns	0.7618 ±	0.0071	ns	0.7450 ±	0.0163	ns
	100%	0.8963 ±	0.0072	ns	0.7274 ±	0.0325	ns	0.8311 ±	0.0072	ns	0.8166 ±	0.0054	ns
AAP2-30-8-4-PI-1	0%	0.8430 ±	0.0069	ns	0.7511 ±	0.0245	ns	0.7380 ±	0.0052	*	0.7234 ±	0.0195	ns
	50%	0.8484 ±	0.0041	ns	0.7363 ±	0.0045	ns	0.7558 ±	0.0014	ns	0.7422 ±	0.0046	ns
	100%	0.8920 ±	0.0025	ns	0.7140 ±	0.0122	ns	0.8318 ±	0.0031	ns	0.8194 ±	0.0062	ns
CRF4-14-2-PI-1	0%	0.8347 ±	0.0018	ns	0.7318 ±	0.0336	ns	0.7453 ±	0.0080	**	0.7787 ±	0.0163	*
	50%	0.8439 ±	0.0023	ns	0.7155 ±	0.0098	ns	0.7498 ±	0.0080	ns	0.7371 ±	0.0067	ns
	100%	0.8842 ±	0.0079	ns	0.7537 ±	0.0167	ns	0.8303 ±	0.0077	ns	0.8205 ±	0.0100	ns
CRF4-22-2-PI-1	0%	0.8397 ±	0.0011	ns	0.7287 ±	0.0309	ns	0.7270 ±	0.0027	ns	0.7448 ±	0.0191	ns
	50%	0.8580 ±	0.0023	ns	0.7410 ±	0.0145	ns	0.7572 ±	0.0035	ns	0.7432 ±	0.0052	ns
	100%	0.9003 ±	0.0101	ns	0.7528 ±	0.0468	ns	0.8314 ±	0.0161	ns	0.8248 ±	0.0202	ns
CRF4-25-1-PI-1	0%	0.8347 ±	0.0042	ns	0.7239 ±	0.0257	ns	0.7372 ±	0.0028	*	0.7454 ±	0.0055	ns
	50%	0.8529 ±	0.0056	ns	0.7221 ±	0.0353	ns	0.7550 ±	0.0102	ns	0.7539 ±	0.0167	ns
	100%	0.8917 ±	0.0054	ns	0.7657 ±	0.0508	ns	0.8392 ±	0.0108	*	0.8442 ±	0.0096	ns

Values are mean±SE from 21 plants. * significantly higher than WT ($P<0.05$), ** significantly higher than WT ($P<0.01$), † significantly lower than WT ($P<0.05$), ns=not significant ($P>0.05$).

Supplementary Table 4. NDRE values in the field experiment.

Genotype	Treatment	60DAS	SE		70DAS	SE		81DAS	SE		88DAS	SE	
WT Nipponbare	0%	0.3327	± 0.0076		0.3210	± 0.0208		0.1099	± 0.0197		0.2031	± 0.0147	
	50%	0.3705	± 0.0020		0.1802	± 0.0935		0.1399	± 0.0110		0.2253	± 0.0108	
	100%	0.4629	± 0.0131		0.3128	± 0.1148		0.2592	± 0.0187		0.3292	± 0.0145	
AAP2-25-1-4	0%	0.3195	± 0.0059	ns	0.3068	± 0.0309	ns	0.1176	± 0.0149	ns	0.2284	± 0.0081	ns
	50%	0.3922	± 0.0014	*	0.2313	± 0.0617	ns	0.1485	± 0.0119	ns	0.2587	± 0.0086	ns
	100%	0.4398	± 0.0171	ns	0.3607	± 0.0064	ns	0.2708	± 0.0083	ns	0.3619	± 0.0083	ns
AAP2-30-8-1	0%	0.3383	± 0.0023	ns	0.2982	± 0.0103	ns	0.1503	± 0.0230	ns	0.2826	± 0.0287	**
	50%	0.3954	± 0.0105	*	0.3163	± 0.0094	ns	0.1680	± 0.0022	ns	0.2780	± 0.0025	**
	100%	0.4695	± 0.0090	ns	0.3773	± 0.0295	ns	0.2945	± 0.0075	ns	0.3781	± 0.0032	ns
AAP2-30-8-4	0%	0.3393	± 0.0063	ns	0.3217	± 0.0311	ns	0.1408	± 0.0199	ns	0.2588	± 0.0192	*
	50%	0.3929	± 0.0067	*	0.3033	± 0.0123	ns	0.1637	± 0.0060	ns	0.2672	± 0.0064	*
	100%	0.4739	± 0.0280	ns	0.3454	± 0.1149	ns	0.3047	± 0.0221	ns	0.3919	± 0.0168	*
AAP2-30-8-10	0%	0.3294	± 0.0049	ns	0.3394	± 0.0793	ns	0.1513	± 0.0114	ns	0.2731	± 0.0116	**
	50%	0.3853	± 0.0009	ns	0.2181	± 0.0700	ns	0.1626	± 0.0066	ns	0.2528	± 0.0074	ns
	100%	0.4712	± 0.0199	ns	0.3737	± 0.0533	ns	0.3049	± 0.0187	ns	0.3895	± 0.0122	*
CRF4-14-2	0%	0.3241	± 0.0054	ns	0.3216	± 0.0482	ns	0.1481	± 0.0176	ns	0.2687	± 0.0117	**
	50%	0.3828	± 0.0053	ns	0.1916	± 0.0307	ns	0.1710	± 0.0098	ns	0.2818	± 0.0157	**
	100%	0.4406	± 0.0034	ns	0.3938	± 0.0343	ns	0.2745	± 0.0099	ns	0.3775	± 0.0134	ns
CRF4-22-2	0%	0.3311	± 0.0057	ns	0.3658	± 0.0612	ns	0.1522	± 0.0131	ns	0.2687	± 0.0128	**
	50%	0.3801	± 0.0047	ns	0.2960	± 0.0122	ns	0.1606	± 0.0059	ns	0.2721	± 0.0065	**
	100%	0.4585	± 0.0105	ns	0.3584	± 0.1355	ns	0.2930	± 0.0055	ns	0.3851	± 0.0023	*
CRF4-25-1	0%	0.3259	± 0.0082	ns	0.2773	± 0.0158	ns	0.1280	± 0.0188	ns	0.2452	± 0.0222	ns
	50%	0.3771	± 0.0039	ns	0.2579	± 0.0321	ns	0.1505	± 0.0072	ns	0.2735	± 0.0038	**
	100%	0.4790	± 0.0108	ns	0.3922	± 0.1071	ns	0.3137	± 0.0177	ns	0.4058	± 0.0145	**
AAP2-25-1-3(2)-PI-2	0%	0.3215	± 0.0092	ns	0.2742	± 0.0355	ns	0.1139	± 0.0153	ns	0.2343	± 0.0231	ns
	50%	0.3825	± 0.0055	ns	0.2000	± 0.0622	ns	0.1346	± 0.0010	ns	0.2515	± 0.0079	ns
	100%	0.4897	± 0.0131	ns	0.4243	± 0.0224	ns	0.3094	± 0.0016	ns	0.3909	± 0.0046	*
AAP2-30-8-1-PI-1	0%	0.3350	± 0.0063	ns	0.3209	± 0.0163	ns	0.1545	± 0.0055	ns	0.2602	± 0.0079	*
	50%	0.3926	± 0.0099	*	0.2748	± 0.0203	ns	0.1761	± 0.0119	*	0.2663	± 0.0149	*
	100%	0.4690	± 0.0075	ns	0.3753	± 0.0395	ns	0.2921	± 0.0039	ns	0.3629	± 0.0083	ns
AAP2-30-8-4-PI-1	0%	0.3244	± 0.0085	ns	0.2912	± 0.0325	ns	0.1311	± 0.0132	ns	0.2364	± 0.0084	ns
	50%	0.3887	± 0.0061	ns	0.2407	± 0.0335	ns	0.1591	± 0.0042	ns	0.2522	± 0.0046	ns
	100%	0.4572	± 0.0062	ns	0.2843	± 0.1355	ns	0.2985	± 0.0088	ns	0.3649	± 0.0090	ns
CRF4-14-2-PI-1	0%	0.3318	± 0.0081	ns	0.3488	± 0.0473	ns	0.1477	± 0.0198	ns	0.2634	± 0.0129	*
	50%	0.3797	± 0.0061	ns	0.2804	± 0.0226	ns	0.1660	± 0.0074	ns	0.2483	± 0.0069	ns
	100%	0.4653	± 0.0257	ns	0.3671	± 0.1355	ns	0.3299	± 0.0246	*	0.4013	± 0.0188	**
CRF4-22-2-PI-1	0%	0.3150	± 0.0088	ns	0.3055	± 0.0253	ns	0.1249	± 0.0092	ns	0.2399	± 0.0025	ns
	50%	0.3931	± 0.0041	*	0.3156	± 0.0150	ns	0.1644	± 0.0058	ns	0.2639	± 0.0038	*
	100%	0.4726	± 0.0114	ns	0.3928	± 0.0082	ns	0.3018	± 0.0221	ns	0.3757	± 0.0244	ns
CRF4-25-1-PI-1	0%	0.3203	± 0.0049	ns	0.3414	± 0.0458	ns	0.1399	± 0.0141	ns	0.2405	± 0.0083	ns
	50%	0.3821	± 0.0046	ns	0.2165	± 0.0953	ns	0.1553	± 0.0151	ns	0.2540	± 0.0119	ns
	100%	0.4754	± 0.0157	ns	0.2949	± 0.1874	ns	0.3236	± 0.0193	*	0.4013	± 0.0132	**

Values are mean±SE from 21 plants. * significantly higher than WT ($P<0.05$), ** significantly higher than WT ($P<0.01$), † significantly lower than WT ($P<0.05$), ns=not significant ($P>0.05$).



Supplementary Figure 3. Interactions between genotypes and N applications in NDVI (A) and NDRE (B) in the field experiment.