nature portfolio

Peer Review File

Variation in a single allele drives divergent yield responses to elevated CO2 between rice subspecies

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript reports that elevated CO2 concentration (eCO2) leads to divergent yield response between indica than japonica, two of the main Asian cultivated subspecies. The yield divergence between the two subspecies is tightly related to the variation of DNR1 allele, with higher yield accumulation in indica variety or dnr1 mutant possessing weak- or null-DNR1 allele than that of japonica varieties with relatively intact DNR1 function. Further physiological assays reveal that i) eCO2 causes higher photosynthesis capacity and results in larger root system architecture and higher nitrogen use (uptake and assimilation) ability in indica variety, which is attributed to impaired DNR1-caused increment of IAA content in roots. Mechanistically, the increased IAA content activates AUXIN RESPONSE FACTORs (OsARF6/17) and by which several target genes of OsARF6/17, including NRTs, NPF2.4, and NIA2, are upregulated, thus facilitating the uptake, transportation, and assimilation of nitrate in rice roots. Additionally, eCO2 also induces plenty of photosynthetic system genes that is dependent on DNR1 while independent of OsARF6/17. Overall, this is an impressive work that uncovers the molecular link of eCO2 and nitrate use in rice, and also offers elite alleles to cope with low nitrogen use efficiency currently and eCO2 in the near future in agriculture.

The revised manuscript is much improved with more data logically presented throughout the text. Nonetheless, I still have some concerns and suggestions to the current manuscript.

Major concerns:
1. To prove the genetic regulation of “DNR1-OsARF6/17” module to nitrogen-use genes, the authors detected the expression level of these genes both in DNR1-overexpressing line and DNR1 OsARF6/17 double overexpression lines. And the generated data robustly support the hypothesis. Based on this, how about the transcriptional abundance of these nitrogen-use genes in dnr1 osarf6/17 double mutants under eCO2 condition?
2. Apart from the evidence from transgenic plants, the authors also performed transient transactivation assays to prove the direct regulation of OsARF6/17 to nitrogen-use genes. Nonetheless, more assay to confirm this regulatory effect is convincing, such as ChIP-qPCR assay between CO2 and eCO2 conditions, or just EMSA to further confirm the binding ability.
3. One of the novel points of this work is “the variation allele of DNR1 contributes to the divergent yield responses to eCO2 between rice subspecies”. However, my major concern, similar to Referee #2 mentioned in Q23 and Referee #3 mentioned in Q1, is how to expand the conclusion from few japonica and indica varieties (ZH11, YD6, HJX74) to the two subspecies. It is a pleasure to see the authors add more description in Discussion to explain the limitation of FACE system for testing more varieties (line 279-286) and prove the allelic divergence of DNR1 between indica and japonica in a larger rice population (line 302-309). If possible, I recommend the authors to confirm the DNR1 variation in more CO2 fertilization effect (CEF)-confirmed varieties from previous articles via bioinformatic analysis in rice database, and then display the allele frequency of DNR1-indica and DNR1-japonica among these varieties to further support their opinion. Alternatively, the authors may also perform a correlation analysis between the allele frequency of DNR1-indica and the yield response to eCO2 among these varieties.
4. Another attracting part of this work, as I see, might be the crosstalk/communication of eCO2 in shoots and nitrate use in roots. How does the “DNR1-auxin-OsARFs” module connect the two factors in different organs is fascinating. Indeed,

emerging evidence in the revised manuscript may suggest a possible role of mobile auxin in this process: i) eCO2 facilitates the accumulation of IAA in rice roots (Fig 2h), ii) it is shown that NPA treatment leads to remarkably reduced nitrate concentration in xylem sap, suggesting the impairment of nitrate transport from root to shoot (Referee #2, Q15). Besides, even though the authors conclude that "under eCO2, we observed that the aboveground and underground dry weights between ZH11 and dnr1 was similar to that under aCO2" in Qii, dnr1 mutant still appears to exhibit higher yield response to eCO2 than ZH11, especially for the shoot biomass with NPA treatment in Fig a. Based on these clues, may the authors further detect the possible role of auxin transport in the molecular link between eCO2 and nitrate use? Specifically, does NPA treatment affect IAA content and the expression level of OsARF6/17 as well as their regulatory effect to nitrogen-use genes in rice roots between ZH11 and dnr1?

Minor concerns:
1. Line2-3: "one of the two main rice subspecies" should be "one of the two main Asian cultivated rice subspecies"
2. I totally agree with the conclusion drawn from Fig 1 and Extended figures that weak or null allele of DNR1 facilitate yield response to eCO2. However, I still feel confusing to Fig 1a and its conclusion "eCO2 reduced DNR1 transcripts more strongly in the dnr1 mutants". As I see, both ZH11 and dnr1 possess the intact DNR1 promoter region, thereby their response to eCO2 in transcriptional level should be identical, if not (just as shown in Fig 1a), it's probably more related to posttranscriptional regulation such as nonsense-mediated mRNA decay (NMD) which is common in mutant lines. In comparison, the transcriptional divergence between HJX74 and NIL (Extended Data Fig 7) is more meaningful due to the different promoter sequence and functional coding sequence. Therefore, I suggest the authors just present the expression level of DNR1 in ZH11 in Fig 1a.
3. Although the antibody specificity of photosynthesis-related proteins is rarely confirmed due to the limitation of related mutants in Arabidopsis or rice, the unconfirmed or non-specific antibodies would be misleading to the future research. In fact, other assays such as quantitative proteomics in shoots/leaves could offer extra evidence for the western blot results. However, this might be beyond the scope of this study to request such assays unless it is available to the authors.

Reviewer #4

(Remarks to the Author)

Referee #1 (Remarks to the Author):

i) To my knowledge, how CO2 is sensed in leaves and thus triggers the downstream regulatory module has been elucidated in plants (Takahashi et al., 2022), which might be helpful for the current research.

Author's response Thanks for your suggestion. We analyzed the transcript levels of OsHT1 under aCO2 and eCO2 conditions, using the ZH11 and dnr1, and HJX74 and NIL. Under eCO2, the expression level of OsHT1 decreased in ZH11 but increased in dnr1. Yet, OsHT1 expression decreased in HJX74, while in NIL, it increased. The inconsistent results suggest OsHT1 may not contribute to DNR1-mediating differences in response to eCO2 between indica and japonica. Furthermore, to the best of our knowledge Takahashi et al. (2022) did not report on the difference in the response to eCO2 between indica and japonica. Thus, we believe this study largely falls outside the scope of our own investigation.

****MPK4/12 and HT1 together form the long-sought primary stomatal CO2/bicarbonate sensor upstream of the CBC1 kinase in plants (Takahashi et al., 2022). This MPK4/12/HT1 sensor may also act upstream of DNR1. Therefore, a more effective approach would be to verify whether the effect of eCO2 repressing DNR1 is absent in the ht1 mutant.

iii) Finally, the result that DNR1 is involved in regulating photosynthesis rate under elevated CO2 concentration is interesting (Fig 5), still the molecular regulation remains undiscovered.

Author's response Thank you for this comment. To elucidate how DNR1 influences photosynthesis, we initially analyzed the expression levels of photosynthesis-related genes (PsbA, PsaB, Rubisco (Rbcs, RbcL) and SBPase) in both the ZH11 and dnr1 mutant (as suggested by Reviewer #2). We found that the expression levels of OsPsaB, OsRbcs and OsSBPase were upregulated in dnr1 under both aCO2 and eCO2 conditions (new Fig 5 and Extended Data Fig. 15).

Subsequently, we examined the promoter sequences of these genes for ARF transcription factor binding elements, specifically TGTCTC/GAGACA. We found that OsARF6 and OsARF17 do not appear to possess transcriptional activation abilities for these photosynthesis-related genes under both aCO2 and eCO2 condition (Extended Data Fig. 17a).

Furthermore, we examined the expression levels of these six genes in plants overexpressing OsARF6 and OsARF17 within the ZH11/pACT::DNR1-Flag background. The results indicated that, compared to ZH11/pACT::DNR1-Flag, overexpression of OsARF6 and OsARF17 did not alter the expression levels of these genes with either aCO2 or eCO2. This collectively suggested that DNR1 does not regulate photosynthetic efficiency through OsARFs binding to the promoters of these photosynthesis-related genes and activating their expression (see below; Extended Data Fig. 17b-g). However, it is

noteworthy that eCO2 enhances photosynthetic efficiency by improving the carbon and nitrogen cycles through various mechanisms.

We have included this information in the Results and Discussion sections in the revised manuscript.

******Although through some effort, the questions remain unanswered, and the molecular regulation remains undiscovered. An effective approach to discover new transcription factors involved in this regulatory mechanism is to conduct RNA-seq analysis in both ZH11 and the dnr1 mutant under ambient (aCO2) and elevated (eCO2) CO2 conditions.

Other comments are listed below:
Q2: CO2 represses while nitrogen increases DNR1 in the DNR1-auxin-OsARFs model, which means that CO2 and nitrogen may act antagonistically in N use and finally plant growth. However, both of the two factors act positively in plant growth and eCO2-caused yield improvement deeply depends on nitrogen (Lv et al., 2020; Hu et al., 2021). Why?

Author's response Thank you for your question. We believe there may be a misunderstanding regarding the effects of eCO2 and nitrogen on the DNR1-auxin-OsARFs model. eCO2 likely influences DNR1 indirectly through changes in nitrogen status. eCO2 can increase photosynthesis and plant growth, thereby increasing the demand for nitrogen. This, in turn, could lead to a decrease in DNR1 abundance, stimulating nitrogen uptake. As a result, eCO2 significantly increased yield in the dnr1 mutant, primarily due to enhanced nitrogen uptake, which aligns with findings from previous studies (Lv et al., 2020; Hu et al., 2021).

*******To avoid misunderstandings regarding the effects of elevated CO2 (eCO2) and nitrogen on the DNR1-auxin-OsARFs model, your above statement "eCO2 likely influences DNR1 indirectly through changes in nitrogen status. eCO2 can increase photosynthesis and plant growth, thereby raising the demand for nitrogen. This, in turn, could lead to a decrease in DNR1 abundance, stimulating nitrogen uptake'" probably need to be included in the Discussion when presenting your model (Fig. 6).

Q4: It is confusing why the authors show the data that DNR1 is repressed by elevated CO2 in transcription level (Fig 1a) in dnr1 mutant, considering it's a loss-of-function mutant.

Author's response This is an important point, and we thank you for raising it. The dnr1 mutant harbors a 2-bp deletion within its second exon, resulting in premature termination and the disruption of its normal gene function. However, its promoter region remains intact and undamaged. Despite these mutations, we cautiously infer the mutant retains the ability to respond to signals from the carbon and nitrogen cycle.

********The dnr1 mutation was generated by CRISPR/Cas9 approach, which resulted in a 2-bp deletion in the second exon of DNR1 and therefore likely disrupts its normal gene functon(Zhang et al., 2021). The only difference between ZH11 and the dnr1 mutation is a 2-bp deletion in the second exon of DNR1; however, compared to ZH11, dnr1 exhibits a significantly enhanced response to elevated CO2 levels. (Fig 1a). Can the author discuss or explain this situation? Alternatively, did the CRISPR/Cas9 approach also edit other parts of the genome that the authors did not identify?

Referee #2 (Remarks to the Author):

Major comments

Q1: First, it does not fully examine whether differences in adaptation to eCO2 are caused solely by changes in nitrate use. The authors stated in the introduction that "in rice, nitrate (NO3-) and ammonium (NH4+) are the major sources of inorganic nitrogen17; up to 40% of the total nitrogen absorbed and utilized by rice by nitrification in the rhizosphere is NO3. It is noteworthy that, in general, indica has a higher capacity to absorb and assimilate NO3- than japonica, while the two subspecies have similar NH4+ uptake rates. However, it is unclear how Indica and japonica utilize the two nitrogen sources under high CO2 conditions. They need to explain the reason why they can focus on the changes in the use of nitrate differences in adaptation to high carbon dioxide by showing some experimental evidences.

Author's response We thank you for this and all following comments and insightful suggestions. Following your advice, we investigated the ammonium and nitrate absorption rates in 9 indica and 9 japonica rice varieties under aCO2 and eCO2 conditions. As shown below, our findings revealed significant differences in nitrate uptake between indica and japonica subspecies after eCO2 treatment: japonica varieties exhibited lower nitrate uptake rates that were less sensitive to changes in external CO2 status compared with indica varieties. However, we observed irregularities in the ammonium absorption patterns across both subspecies. This is why our focus on eCO2 highlights differing effects on nitrate, rather than ammonium, transport and metabolism in indica and japonica subspecies.

To address your concern, we added these results into the main text: "Consistent with these results, we found that japonica varieties exhibited significantly lower NO3- uptake rates that were less sensitive to changes in external CO2 status

compared with indica varieties (Extended Data Fig. 1)."

********This result has not been statistically analyzed. If the authors compare the fold change of NO3- and NH4+ influx between aCO2 and eCO2 in all varieties, it will more clearly explained that why they focus on eCO2 highlights differing effects on nitrate, rather than ammonium, transport and metabolism in indica and japonica subspecies.

Q4: In the FACE experiment, nitrogen was supplied in the form of urea, which was to be broken down into ammonium and then nitrified and absorbed by the plants. The authors then measured photosynthesis, aboveground weight, nitrogen uptake and yield. In hydroponic cultivation, it was given as 1.25 mM ammonium nitrate and root length, root area, number of root tips and nitrate uptake were measured. The growing conditions and measurements of the two are different and it is difficult to discuss the results of both directly, but they should show as clearly as possible a similar trend in both. For example, it should be specifically shown that trends in aboveground weight and nitrogen use efficiency are common between FACE and hydroponics.

Author's response We appreciate your feedback and acknowledge the importance of this point. Following your suggestion, we have included the weight of the aboveground part under hydroponic conditions (Figure 2c and Extended Data Fig. 9c) in the revised manuscript. We now also discussed its consistency and relevance in relation to our findings.

********When the two-way analysis of variance indicated a significant interaction between CO2 and variety (Fig. 1 and Fig. 2), it would be clearer if the authors use post-hoc tests to further explore those categories in CO2 or variety that are significantly different. This would help readers easily identify any differences or similar trends in FACE experiment and hydroponic systems.

Q6: I would also suggest that they show that the DNR1-dependent adaptation to carbon dioxide disappears when nitrification inhibitors are added.

Author's response Thank you for your suggestion. In hindsight, including nitrification inhibitors would have made our investigation into the impact of varying CO2 concentrations on nitrate metabolism more comprehensive. Unfortunately, the FACE experiments did not include treatments with these inhibitors, limiting our ability to observe phenotypic differences related to their use. Additionally, nitrification inhibitors slow down the microbial conversion of ammonium to nitrate in soil, which was not applicable in our hydroponic setup due to the absence of microbes. However, our findings revealed significant differences in nitrate uptake, rather than ammonium uptake, between indica and japonica subspecies after eCO2 treatment. Moreover, when nitrate was the sole nitrogen source, plants exhibited a higher responsiveness to variable CO2 levels compared to ammonium as the sole nitrogen source. Thus, although we lack data on nitrate inhibitors, our results strongly suggest that DNR1-dependent adaptation to eCO2 primarily involves nitrate metabolism.

*****In our experience, the presence of microorganisms in hydroponic systems can vary. Therefore, when we treat with ammonium, we often add nitrification inhibitors to suppress the nitrification reaction. The authors could consider measuring nitrate levels just before collecting samples to check for any contamination.

Q10: It needs to be explained whether the substrate affinity of NRT1.1B, its response to nitrogen nutrient conditions, and the transport rate of nitric acid are consistent.

Author's response Thanks for your suggestion. Yes, these results are consistent. To address your concern, we now provide more detail and make this point explicitly. In our study, we used 2.5 mM K15NO3 to conduct rice root low-affinity 15NO3- influx measurements. To elucidate the influence of aCO2 and eCO2 on the affinity of OsNRT1.1B for nitrate, we conducted 15N absorption measurements with concentration gradients. This study involved HJX74 and a single segment substitution line (SSSL-064), generated by crossing IRAT261 (donor parent) with HJX74 (recurrent parent), which incorporates a chromosome segment containing OsNRT1.1B from japonica IRAT261 into the HJX74 genetic background. The results indicated that eCO2 enhances nitrate absorption capacity under both high and low 15N concentrations, although the extent of enhancement varies with substrate concentration.

*****This result has not been statistically analyzed. Compared to SSSL-064, HJX74 exhibits a significantly enhanced response to elevated CO2 levels under both nitrate concentrations. Does this suggest that OsNRT1.1B, independent of DNR1, can also sense elevated CO2 levels to regulate the CO2 fertilization effect (CEF) in rice?

Referee #3 (Remarks to the Author):
Q2: There are also some inconsistencies in the current study and the authors' previous publication. In Zhang et al. (2021), the dnr1 mutants in the ZH11 background had greater N uptake and yield in the hydroponic conditions, presumably at ambient CO2 concentrations. Why did these lines only show a benefit at elevated CO2 in the current experiments? That isn't clear. There was reduced DNR1 transcript and protein abundance at both ambient and elevated CO2, so if the hypothesis is correct, then there should have been a benefit under both conditions.

Author's response Thanks for your comments. We believe there may be a misunderstanding regarding the effects of DNR1 on rice yield and N uptake, likely due to some unclear figure formatting on our part. In our manuscript, dnr1 mutants in the ZH11 background showed higher nitrogen uptake and yields under both aCO2 and eCO2 concentrations. To clarify this, we have adjusted the scale of Y-axis in Figure 1.

******When the two-way analysis of variance indicated a significant interaction between CO2 and variety, it would be clearer if the authors use post-hoc tests to further explore those categories in CO2 or variety that are significantly different. This would help readers easily identify any differences under both aCO2 and eCO2 concentrations in Fig 1.

Reviewer #5

(Remarks to the Author)
I've reviewed NCOMMS-24-52534-T by Liu et al. The manuscript is an extension of a prior study that identified the role of the auxin homeostasis gene DNRI (dull nitrogen ren response1) on auxin generation and subsequent signalling cascades influencing primary drivers of nitrogen transport and assimilation in rice. The prior manuscript (Plant Cell, Siyu Shang et al 2021) revealed the allelic difference of DNR1 between two rice species Oryza indica and Oriya japonica, two of the primary rice varieties grown across Asia. The discovery of the DNR1 genetic module and its management of a positive nitrogen use efficiency response (improved nitrogen uptake, N assimilation etc) was an astounnding discovery for the respective research community. The initial highlighted a number of areas for further study and ways to enhance the discovery further to increase rice productivity on a minimised N budget.

The second manuscript being discussed is a solid new contribution to an extended understanding of DNR1 in both japonica and indica varieties. The data provided shows a strong and convincing relationship between DNR1 activity (expression and protein abundance) with changes in N response systems. The novel new focus on response to elevated CO2 using FACE delivery systems introduces a new level of regulatory control and a picture of how these two important plant species will respond to an elevated CO2 environment. Apparent allelic differences in DNR1 between indica (generally low DNR1 expression) and japonica (generally high DNR1 expression) provides a potential link between C and N metabolism and how a rich CO2 environment can influence N use in one crop (indica) better than japonica. I think this is very exciting and does make this manuscript an interesting read that delivers new ideas on how auxin mediated signalling cascades tie together C & N metabolic responses in plants. Experimentally, the authors have endeavoured (through it would appear multiple reviews) clear and reliable data that has been now adequately explained and documented. Re-reviewing the prior reviews and the authors responses, the newly compiled manuscript is rich with information (probably too much) to justify the author's claims.

I disagree this is a manuscript destined to a discipline specific journal, there is plenty of new information not previously covered in the previous Plant Cell Manuscript. Though after an exhaustive read, I think the presentation and explanation of the allelic variation between DNR1 alleles could use some further investigation or documentation. I'm perplexed why the polymorphisms are less abundant in japonica DNR1 relative to indica and why tillering and N/CO2 responsiveness is a trait not being selected in japonica relative to indica. Is there an unknown NUE penalty with the cultivation of japonica relative to indica to allow the former to still be produced. If a revised manuscript is asked, it would be nice to include some level of discussion on the allelic differences and their persistence in the breeding pools of indica and japonica varieties. This will complement the vast amounts of convincing data on why this is an important study and the necessity to be communicated through this chosen journal.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript has addressed my primary concerns regarding the correlation between different DNR1 alleles and the CO2 fertilization effect, as well as the implications of elevated CO2 (eCO2) on the enrichment of ARFs in N-use genes. Additionally, it is great to see that the authors have delved into the potential role of DNR1 in an auxin transport-dependent communication mechanism between CO2 and nitrate in rice, which involves shoot-to-root signaling.

Interestingly, the results suggest that although the activation of N-use genes in roots by eCO2 is independent of DNR1, it partially depends on auxin transport from the shoot, as indicated by the outcomes of NPA treatment in response to Q4. This finding implies the existence of other unknown mechanisms that facilitate the crosstalk between CO2 and nitrate from the shoot to the root. I recommend that the authors incorporate these results into the manuscript and provide a thorough discussion. This addition may capture significant interest within the plant science community.

Reviewer #4

(Remarks to the Author)

All the concerns have been properly addressed.

Reviewer #5

(Remarks to the Author)
I was satisfied with my last review of this manuscript. The authors had responded to all of my questions and collectively presented a much improved version of the manuscript. I'm satisfied with the current responses made by other reviewers and support the new included data as beneficial to the paper's story and clarity.

The following are our responses to the reviewers' comments and an indication of additional data and other changes we have made to the manuscript in response to these comments. For the sake of clarity, the reviewer comments are written in italics.

Reviewer #1 (Remarks to the Author):

This manuscript reports that elevated CO_2 concentration (eCO_2) leads to divergent yield response between indica than japonica, two of the main Asian cultivated subspecies. The yield divergence between the two subspecies is tightly related to the variation of DNR1 allele, with higher yield accumulation in indica variety or dnr1 mutant possessing weak- or null-DNR1 allele than that of japonica varieties with relatively intact DNR1 function. Further physiological assays reveal that i) eCO_2 causes higher photosynthesis capacity and results in larger root system architecture and higher nitrogen use (uptake and assimilation) ability in indica variety, which is attributed to impaired DNR1-caused increment of IAA content in roots. Mechanistically, the increased IAA content activates AUXIN RESPONSE FACTORs (OsARF6/17) and by which several target genes of OsARF6/17, including NRTs, NPF2.4, and NIA2, are upregulated, thus facilitating the uptake, transportation, and assimilation of nitrate in rice roots. Additionally, eCO_2 also induces plenty of photosynthetic system genes that is dependent on DNR1 while independent of OsARF6/17. Overall, this is an impressive work that uncovers the molecular link of eCO_2 and nitrate use in rice, and also offers elite alleles to cope with low nitrogen use efficiency currently and eCO_2 in the near future in agriculture.

The revised manuscript is much improved with more data logically presented throughout the text. Nonetheless, I still have some concerns and suggestions to the current manuscript.

Author's response Thank you for acknowledging our revised manuscript and for your thoughtful suggestions.

Major concerns:

Q1: *To prove the genetic regulation of "DNR1-OsARF6/17" module to nitrogen-use genes, the authors detected the expression level of these genes both in DNR1-overexpressing line and DNR1 OsARF6/17 double overexpression lines. And the generated data robustly support the hypothesis. Based on this, how about the transcriptional abundance of these nitrogen-use genes in dnr1 osarf6/17 double mutants under* eCO_2 *condition?*

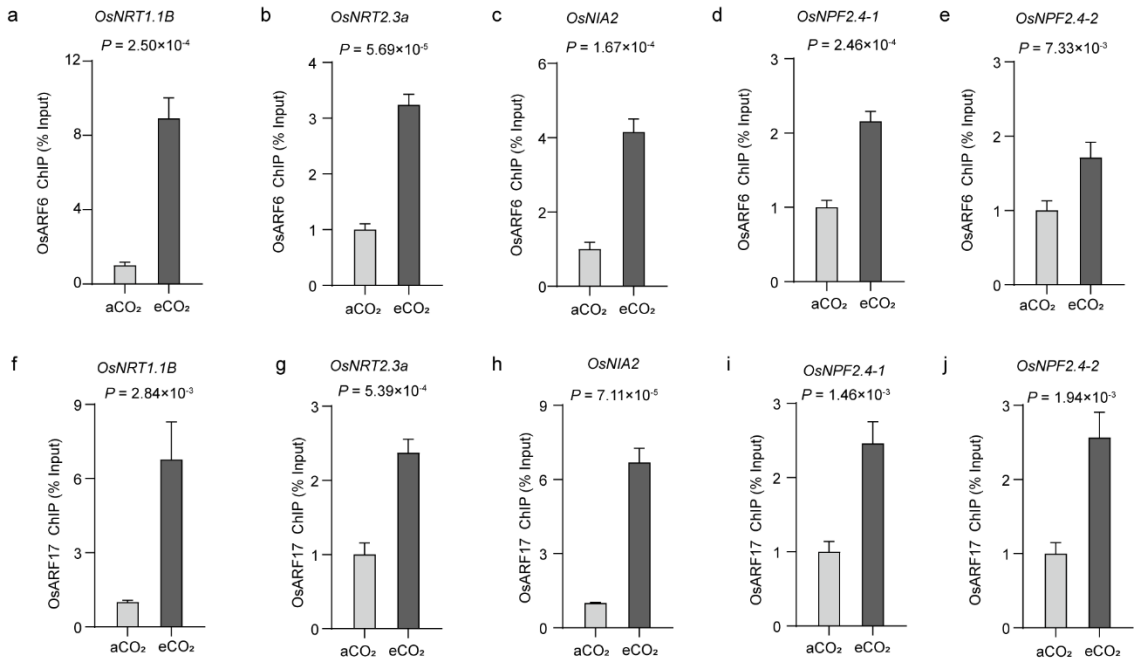
Author's response Thanks for your insightful comment. We acknowledge that detecting the transcriptional abundance of these nitrogen-use genes in the *dnr1 osarf6/17* double mutants would enhance the robustness of our results. However, due to time constraints, we are unable to generate these double mutants. Nonetheless, the presence or absence of *dnr1 arf6/17* double mutants does not affect the central focus of this article: the variations in *DNR1* that mediate differences in how rice subspecies respond to eCO_2.

Q2: *Apart from the evidence from transgenic plants, the authors also performed transient transactivation assays to prove the direct regulation of OsARF6/17 to nitrogen-use genes. Nonetheless, more assay to confirm this regulatory effect is convincing, such as ChIP-qPCR assay between* CO_2 *and* eCO_2 *conditions, or just EMSA to further confirm the binding ability.*

Author's response Thank you for your suggestion. In our previous *Plant Cell* article, ChIP-qPCR assays and EMSA in **Figures 3I** and **3J**, along with **Supplementary Figures S10** and **S11**, demonstrated that both OsARF6 and OsARF17 bind directly to the TGTCTC/GAGACA-containing segments within the promoter regions of four genes (*OsNRT1.1B*, *OsNRT2.3a*, *OsNPF2.4*, and *OsNIA2*) involved in nitrate metabolism. To further illustrate the regulatory effects of OsARF6 and OsARF17 under aCO_2 and eCO_2 conditions, we here performed ChIP-qPCR assays, which indicated that eCO_2 promoted the enrichment of TGTCTC/GAGACA motif-containing fragments from the promoters of these four genes, as shown in **Figures 3I**, **3J** and **Supplementary**

Figures S10（Zhang, et al., 2021). Collectively, the findings from ChIP-qPCR and transient reactivation assays in original manuscript strongly support the direct regulatory effects of OsARF6 and OsARF17 on nitrogen-related genes under both aCO_2 and eCO_2 conditions.

We have included these results in new **Fig.4** and **Extended Data Fig. 16** in the revised manuscript.



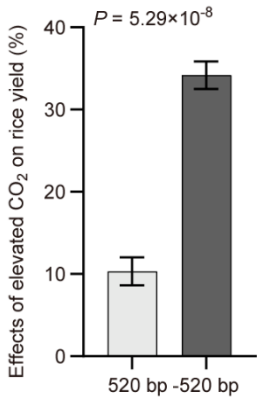
Q3: *One of the novel points of this work is "the variation allele of DNR1 contributes to the divergent yield responses to eCO_2 between rice subspecies". However, my major concern, similar to Referee #2 mentioned in Q23 and Referee #3 mentioned in Q1, is how to expand the conclusion from few japonica and indica varieties (ZH11, YD6, HJX74) to the two subspecies. It is a pleasure to see the authors add more description in Discussion to explain the limitation of FACE system for testing more varieties (line 279-286) and prove the allelic divergence of DNR1 between indica and japonica in a larger rice population (line 302-309). If possible, I recommend the authors to confirm the DNR1 variation in more CO_2 fertilization effect (CEF)-confirmed varieties from previous articles via bioinformatic analysis in rice database, and then display the allele*

frequency of DNR1-indica and DNR1-japonica among these varieties to further support their opinion. Alternatively, the authors may also perform a correlation analysis between the allele frequency of DNR1-indica and the yield response to eCO_2 among these varieties.

Author's response Thanks for your suggestion. We have collected eight additional rice varieties (highlighted in yellow) with confirmed CO_2 fertilization effects (CFE) and validated the *DNR1* variation. These varieties have been added to **Supplementary Table 3**. Additionally, we included a new **Extended Data Fig. 3** in the revised manuscript to show the differences in CFE between rice varieties with *indica DNR1* allele and with the *japonica DNR1* allele, based on previous FACE experiments.

Supplementary Table 3 | Rice varieties in FACE experiments exhibiting *DNR1* allelic variations.

Varieties	520 bp	*indica/japonica*
No8	+	*japonica*
Koshi	+	*japonica*
Aikoku	+	*japonica*
Akita	+	*japonica*
Nipponbare	+	*japonica*
Nanjing9108	+	*japonica*
Wuxiangjing14	+	*japonica*
Wuyunjing21	+	*japonica*
Wuyunjing23	+	*japonica*
YD6	-	*indica*
IR24	-	*indica*
IIyou084	-	*indica*
Shanyou63	-	*indica*
Liangyoupei9	-	*indica*
Huajingxian74	-	*indica*



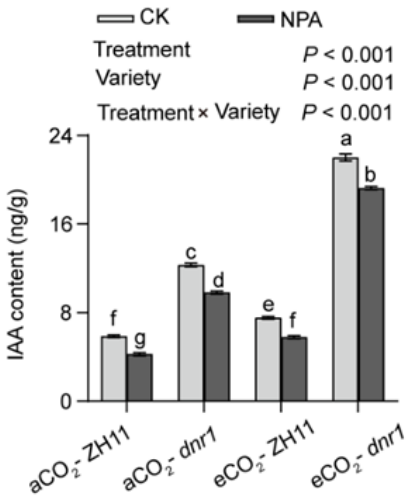
Extended Data Fig. 3 | Differences in effect of elevated CO_2 on rice yields between rice varieties with *indica DNR1* allele (-520 bp) and with the *japonica DNR1 allele* (520 bp) in FACE experiments. *P*-values were generated from two-sided Student's *t* tests.

Q4: *Another attracting part of this work, as I see, might be the crosstalk/communication of eCO_2 in shoots and nitrate use in roots. How does the "DNR1-auxin-OsARFs" module connect the two factors in different organs is fascinating. Indeed, emerging evidence in the revised manuscript may suggest a possible role of mobile auxin in this process: i) eCO_2 facilitates the accumulation of IAA in rice roots (Fig 2h), ii) it is shown that NPA treatment leads to remarkably reduced nitrate concentration in xylem sap, suggesting the impairment of nitrate transport from root to shoot (Referee #2, Q15). Besides, even though the authors conclude that "under eCO2, we observed that the aboveground and underground dry weights between ZH11 and dnr1 was similar to that under aCO2" in Qii, dnr1 mutant still appears to exhibit higher yield response to eCO2 than ZH11, especially for the shoot biomass with NPA treatment in Fig a. Based on these clues, may the authors further detect the possible role of auxin transport in the molecular link between eCO_2 and nitrate use? Specifically, does NPA treatment affect IAA content and the expression level of OsARF6/17 as well as their regulatory effect to nitrogen-use genes in rice roots between ZH11 and dnr1?*

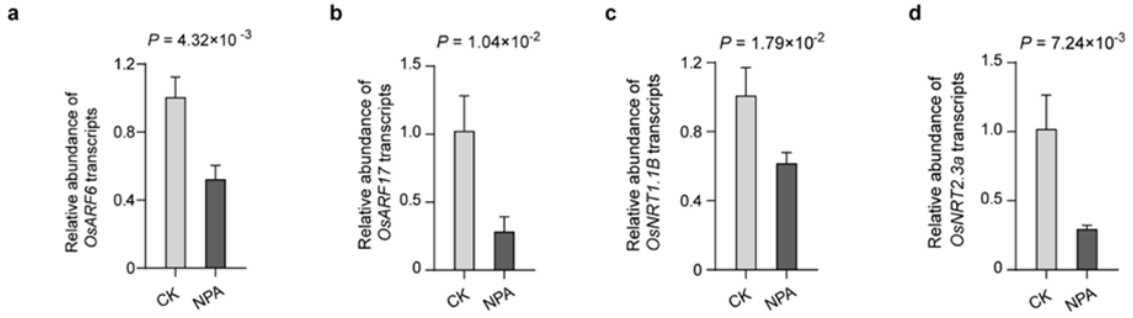
Author's response Thanks for your suggestion. In *Qii*, the statement "we observed that aboveground and underground dry weights between ZH11 and *dnr1* were similar under

aCO_2" lacks precision and may lead to misunderstandings. More accurately, regardless of the CO_2 condition (aCO_2 or eCO_2), NPA treatment resulted in a decrease in aboveground biomass of both ZH11 and *dnr1*, potentially attributed to reduced nitrate concentration in the xylem sap. However, the magnitude of this decrease is nearly identical for both lines. Therefore, we conclude that the growth repression induced by NPA treatment is unlikely to be significantly regulated by DNR1.

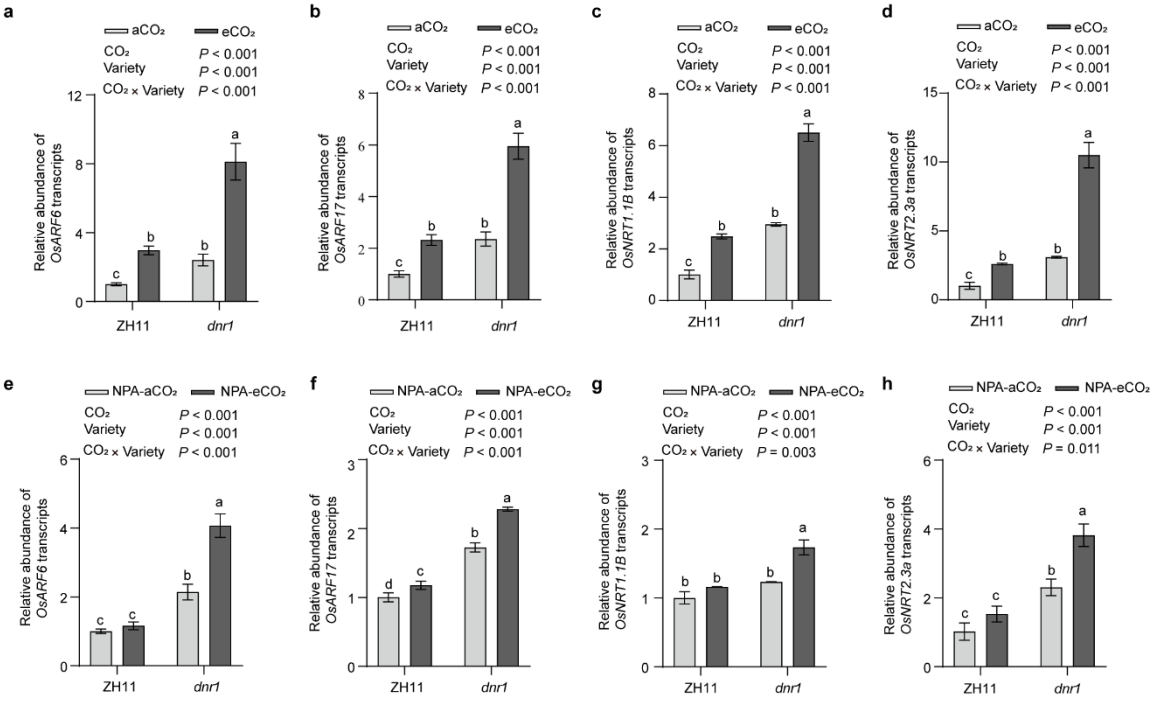
Following your suggestion, we first measured the auxin content in ZH11 and *dnr1* plants grown under both conditions after NPA treatment. The results (see below) revealed that, under both aCO_2 and eCO_2 conditions, NPA treatment led to a reduction in root auxin content in both ZH11 and *dnr1*. Notably, the magnitude of this decrease was similar in both lines, which is consistent with the previously observed changes in aboveground biomass before and after NPA treatment.



Next, we examined the effect of NPA treatment on the expression levels of *OsARF6*, *OsARF17*, *OsNRT1.1B*, and *OsNRT2.3a* in the ZH11 roots. The results indicated that NPA treatment led to a reduction in the expression levels of these genes, which is likely a consequence of reduced auxin content in the roots following NPA treatment.



Finally, to further clarify the relationship between NPA treatment, varying CO_2 concentrations, and nitrate metabolism, we conducted qPCR analysis to assess the root transcript abundances of *OsARF6*, *OsARF17*, *OsNRT1.1B*, and *OsNRT2.3a* in ZH11 and *dnr1* plants grown under both aCO_2 and eCO_2 conditions, with or without NPA treatment. The results below revealed that NPA treatment resulted in a reduction in the root expression levels of *OsARF6*, *OsARF17*, *OsNRT1.1B*, and *OsNRT2.3a* in both ZH11 and *dnr1*, regardless of the CO_2 conditions (aCO_2 or eCO_2). Importantly, the degree of reduction for these genes was similar in both lines.



Collectively, these results suggest that NPA-induced changes in both root auxin content and the expression levels of *OsARF6*, *OsARF17*, *OsNRT1.1B*, and *OsNRT2.3a* are

independent of DNR1.

Minor concerns:

Q5: *Line2-3: "one of the two main rice subspecies" should be "one of the two main Asian cultivated rice subspecies"*

Author's response We appreciate this point and have made the suggested revisions in the revised manuscript.

Q6: *I totally agree with the conclusion drawn from Fig 1 and Extended figures that weak or null allele of DNR1 facilitate yield response to eCO_2. However, I still feel confusing to Fig 1a and its conclusion "eCO_2 reduced DNR1 transcripts more strongly in the dnr1 mutants". As I see, both ZH11 and dnr1 possess the intact DNR1 promoter region, thereby their response to eCO2 in transcriptional level should be identical, if not (just as shown in Fig 1a), it's probably more related to posttranscriptional regulation such as nonsense-mediated mRNA decay (NMD) which is common in mutant lines. In comparison, the transcriptional divergence between HJX74 and NIL (Extended Data Fig 7) is more meaningful due to the different promoter sequence and functional coding sequence. Therefore, I suggest the authors just present the expression level of DNR1 in ZH11 in Fig 1a.*

Author's response Thanks for your suggestion. We agree to display only the *DNR1* expression levels in ZH11 in the revised **Fig. 1a**.

Q7: *Although the antibody specificity of photosynthesis-related proteins is rarely confirmed due to the limitation of related mutants in Arabidopsis or rice, the unconfirmed or non-specific antibodies would be misleading to the future research. In fact, other assays such as quantitative proteomics in shoots/leaves could offer extra evidence for the western blot results. However, this might be beyond the scope of this study to request such assays unless it is available to the authors.*

Author's response We fully agree with your opinion and acknowledge that these antibodies need to be validated for use in the relevant mutants in *Arabidopsis* or rice. After reviewing the literature, we found that PsaB (AS10695) and PsbA (AS05084) have been confirmed to be effective in rice (Jiang et al., 2023), while RbcL (AS03037) and RbcS (AS07259) have been confirmed for use in *Arabidopsis* (DeTar et al., 2021). Additionally, the instruction manual indicates that SBPase (AS152873) has a confirmed reaction in both *Arabidopsis* and rice; however, it has not been reported as usable in either species.

Jiang D., et al. (2023). Toxic effects of lanthanum(III) on photosynthetic performance of rice seedlings: Combined chlorophyll fluorescence, chloroplast structure and thylakoid membrane protein assessment. Ecotoxicol Environ Saf. 267, 115627.
DeTar, R., et al. (2021). Loss of inner-envelope K^+/H^+ exchangers impairs plastid rRNA maturation and gene expression. Plant Cell, 33(7), 2479-2505.

Reviewer #4 (Remarks to the Author):

Q1: *MPK4/12 and HT1 together form the long-sought primary stomatal CO2/bicarbonate sensor upstream of the CBC1 kinase in plants (Takahashi et al., 2022). This MPK4/12/HT1 sensor may also act upstream of DNR1. Therefore, a more effective approach would be to verify whether the effect of eCO2 repressing DNR1 is absent in the ht1 mutant.*

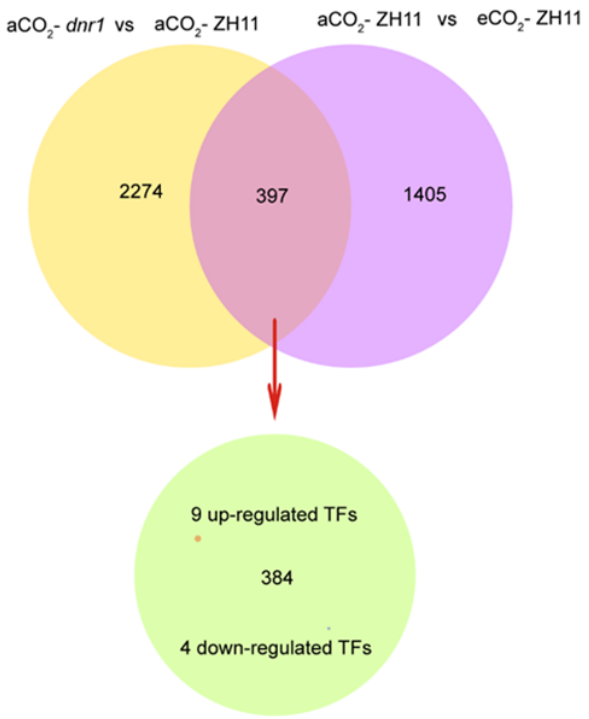
Author's response We greatly appreciate your suggestion and find it very insightful. However, it is important to note that Takahashi *et al*., (2022) utilized the *ht1* mutant in *Arabidopsis*. Thus, we are unable to determine whether the effect of elevated CO_2 on rice DNR1 is absent in the *Arabidopsis ht1* mutant.

Q2: *Although through some effort, the questions remain unanswered, and the molecular regulation remains undiscovered. An effective approach to discover new transcription*

factors involved in this regulatory mechanism is to conduct RNA-seq analysis in both ZH11 and the dnr1 mutant under ambient (aCO_2) and elevated (eCO_2) CO_2 conditions.

Author's response Thank you for this comment. In our previous point-by-point response to Reviewer #1 (*ii* and *iii*), we indicated that DNR1 regulates the expression of photosynthesis-related genes independently of OsARFs. This suggests that eCO_2 enhances photosynthetic efficiency by improving the carbon and nitrogen cycles through various mechanisms. To discover new transcription factors involved in regulating photosynthesis, we conducted RNA-seq analysis as suggested and found that there are 397 target genes regulated by both CO_2 and DNR1, among which 9 transcription factors are upregulated by both eCO_2 and null-*DNR1* allele, and 4 transcription factors are downregulated by both eCO_2 and null-*DNR1* allele (see below). These 13 transcription factors may serve as potential candidates for regulating photosynthetic efficiency in response to eCO_2 and DNR1 interactions. Further investigation of these transcription factors could provide valuable insights into the molecular mechanisms underlying the photosynthetic adaptation to eCO_2 and DNR1.

a



b

Gene_id	Gene_name	aCO_2-ZH11	aCO_2-*dnr1*	eCO_2-ZH11	Gene_description	Family
Os01g0619900	OsTCL2	3.67854247	11.145769	8.4186984	B3H4X8|TCL2_ARATH MYB-like transcription factor TCL2	MYB_related
Os01g0603300	Os01g06033	2.34656956	5.7615265	4.8160231	Q9LVS0|KUA1_ARATH Transcription factor KUA1	MYB_related
Os06g0166400	OsERF#007	1.79827178	4.6616301	6.2959249	O22174|ERF08_ARATH Ethylene-responsive transcription factor ERF008	ERF
Os10g0562900	DERF12	1.02452559	3.0628627	2.7739188	O80341|EF102_ARATH Ethylene-responsive transcription factor 5	ERF
Os12g0116600	OsWRKY95	1.08848663	2.7483758	2.6939105	Q9SKD9|WRK46_ARATH Probable WRKY transcription factor 46	WRKY
Os12g0618600	OsHAP2F	0.25728782	1.3735229	1.5013162	Q9LVJ7|NFYA6_ARATH Nuclear transcription factor Y subunit A-6	NF-YA
Os11g0117600	OsWRKY50	0.53217407	1.5587078	1.4284482	Q0DFT7|WRK19_ORYSJ Transcription factor WRKY19	WRKY
Os07g0158500	OsHAP2J	0.46411483	1.3593662	1.3540927	Q8LFU0|NFYAA_ARATH Nuclear transcription factor Y subunit A-10	NF-YA
Os03g0366800	OsHsfB4d	0.04163242	0.5466231	0.5830366	Q10KX8|HFB4D_ORYSJ Heat stress transcription factor B-4d	HSF
Os04g0541100	GT-2	4.94324734	1.9248515	0.6950518	Q9C6K3|DF1_ARATH Trihelix transcription factor DF1	Trihelix
Os04g0381700	OsbHLH156	0.94869456	0.3048701	0	A0A0N7KIY3|BH156_ORYSJ Transcription factor BHLH156	bHLH
Os04g0686200	Os04g06862	0.58889244	0.2411229	0.111447	Q947D2|PRAF1_ARATH PH, RCC1 and FYVE domains-containing protein 1	HB-other
Os08g0483900	OsbHLH047	0.42634186	0	0.0497555	Q9SK91|BH094_ARATH Transcription factor bHLH94	bHLH

We have included this point in the revised manuscript. “We conducted RNA sequencing analysis on ZH11 and the *dnr1* mutant under both aCO_2 and eCO_2 conditions and identified 397 target genes regulated by both CO_2 and DNR1. Among these, 9 transcription factors are upregulated by both eCO_2 and null-*DNR1* allele, and 4 transcription factors are downregulated by both eCO_2 and null-*DNR1* allele (Supplementary Table 5). These 13 transcription factors may serve as potential candidates for regulating photosynthetic efficiency in response to eCO_2 and DNR1 interactions, offering promising avenues for future research. Overall, eCO_2 enhances photosynthetic efficiency by improving the C and N cycles through various mechanisms.”

However, it is important to note that our article primarily focuses on the molecular mechanism by which varying CO_2 concentrations regulate DNR1 abundance, thereby influencing leaf nitrogen content and ultimately enhancing photosynthetic capacity.

Other comments are listed below:

Q3: *To avoid misunderstandings regarding the effects of elevated CO_2 (eCO_2) and nitrogen on the DNR1-auxin-OsARFs model, your above statement "eCO_2 likely influences DNR1 indirectly through changes in nitrogen status. eCO_2 can increase photosynthesis and plant growth, thereby raising the demand for nitrogen. This, in turn, could lead to a decrease in DNR1 abundance, stimulating nitrogen uptake'" probably need to be included in the Discussion when presenting your model (Fig. 6).*

Author's response As suggested, we have included these sentences in the Discussion when we presenting our model in **Fig. 6**.

Q4: *The dnr1 mutation was generated by CRISPR/Cas9 approach, which resulted in a 2-bp deletion in the second exon of DNR1 and therefore likely disrupts its normal gene function (Zhang et al., 2021). The only difference between ZH11 and the dnr1 mutation is a 2-bp deletion in the second exon of DNR1; however, compared to ZH11, dnr1 exhibits a significantly enhanced response to elevated CO_2 levels. (Fig 1a). Can the author discuss or explain this situation? Alternatively, did the CRISPR/Cas9 approach also edit other parts of the genome that the authors did not identify?*

Author's response Thank you for your question. As Reviewer #1 noted, the regulation may be more closely related to post-transcriptional mechanisms, such as nonsense-mediated mRNA decay (NMD), which is common in mutant lines and can be difficult to explain clearly. To avoid any potential misunderstandings, we have followed Reviewer #1's suggestion and now display only the expression level of *DNR1* in ZH11 in the new **Fig. 1a**.

To be candid, the CRISPR/Cas9 approach may lead to off-target editing of other genes. However, we have demonstrated the transcriptional divergence between HJX74 and NIL in **Extended Data Fig. 8**, which is sufficient to illustrate the effect of eCO_2 on DNR1. This divergence is particularly evident in their distinct promoter sequences and functional coding sequences.

Referee #2 (Remarks to the Author):

Major comments

Q5: *This result has not been statistically analyzed. If the authors compare the fold change of* NO_3^- *and* NH_4^+ *influx between* aCO_2 *and* eCO_2 *in all varieties, it will more clearly explained that why they focus on* eCO_2 *highlights differing effects on nitrate, rather than ammonium, transport and metabolism in indica and japonica subspecies.*

Author's response Thanks for your comments. We performed the statistical analysis according to your comments and included this result into the main text.

"Furthermore, we observed an interactive effect on NO_3^- uptake ($P = 0.001$), but not on NH_4^+ absorption ($P = 0.373$), between eCO_2 and rice species. The NO_3^- uptake rates in *japonica* varieties were less responsive to eCO_2 (+40%) compared to *indica* varieties (+69%) (Extended Data Fig. 1)."

Q6: *When the two-way analysis of variance indicated a significant interaction between* CO_2 *and variety (Fig. 1 and Fig. 2), it would be clearer if the authors use post-hoc tests to further explore those categories in* CO_2 *or variety that are significantly different. This would help readers easily identify any differences or similar trends in FACE experiment and hydroponic systems.*

Author's response: Thanks for your suggestion. We conducted the post-hoc tests and incorporated the results into new **Fig. 1** and **2**. Additionally, we have included the results of post-hoc tests in all relevant figures and tables.

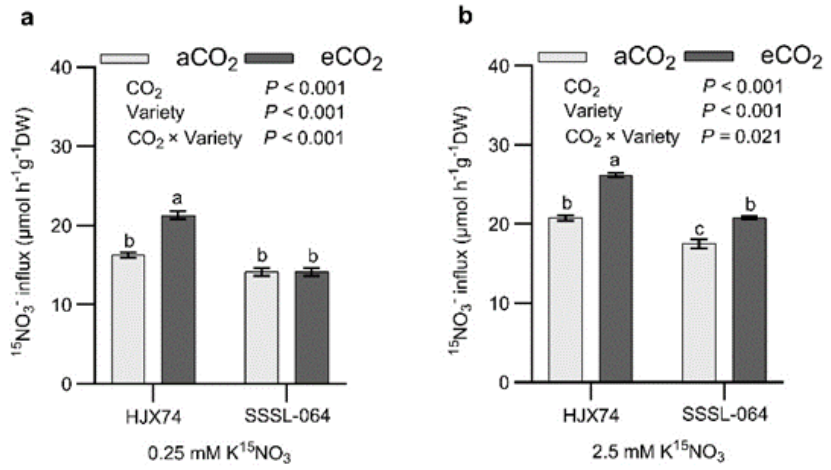
Q7: *In our experience, the presence of microorganisms in hydroponic systems can vary. Therefore, when we treat with ammonium, we often add nitrification inhibitors to suppress the nitrification reaction. The authors could consider measuring nitrate levels just before collecting samples to check for any contamination.*

Author's response Thanks for your suggestion. We agree that microorganisms can affect the nitrification process. Indeed, we collected the water samples to measure nitrate concentration before collecting plant samples. We found that NO_3^- concentrations were extremely low, making accurate measurements impossible.

Q8: *This result has not been statistically analyzed. Compared to SSSL-064, HJX74 exhibits a significantly enhanced response to elevated CO_2 levels under both nitrate concentrations. Does this suggest that OsNRT1.1B, independent of DNR1, can also sense elevated CO_2 levels to regulate the CO_2 fertilization effect (CEF) in rice?*

Author's response Thanks for your comments. We analyzed this data through two-way *ANOVA* and post-hoc tests. Our results suggest the *OsNRT1.1B*, independent of *DNR1*, can also sense elevated CO_2 levels to regulate the CO_2 fertilization effect in rice. We included this information into the revised manuscript.

"These results indicate that *OsNRT1.1B* itself can influence $^{15}NO_3^-$ absorption and thereby affect growth to some extent in response to eCO_2."



Referee #3 (Remarks to the Author):

Q9: *When the two-way analysis of variance indicated a significant interaction between CO2 and variety, it would be clearer if the authors use post-hoc tests to further explore those categories in CO_2 or variety that are significantly different. This would help readers easily identify any differences under both aCO_2 and eCO_2 concentrations in Fig 1.*

Author's response: Thanks for your comments. We conducted the post-hoc tests and incorporated the results into new **Fig. 1**.

Reviewer #5 (Remarks to the Author):

I've reviewed NCOMMS-24-52534-T by Liu et al. The manuscript is an extension of a prior study that identified the role of the auxin homeostasis gene DNRI (dull nitrogen ren response1) on auxin generation and subsequent signalling cascades influencing primary drivers of nitrogen transport and assimilation in rice. The prior manuscript (Plant Cell, Siyu Shang et al 2021) revealed the allelic difference of DNR1 between two rice species Oryza indica and Oriya japonica, two of the primary rice varieties grown across Asia. The discovery of the DNR1 genetic module and its management of a positive nitrogen use efficiency response (improved nitrogen uptake, N assimilation etc) was an astounnding discovery for the respective research community. The initial

highlighted a number of areas for further study and ways to enhance the discovery further to increase rice productivity on a minimised N budget.

The second manuscript being discussed is a solid new contribution to an extended understanding of DNR1 in both japonica and indica varieties. The data provided shows a strong and convincing relationship between DNR1 activity (expression and protein abundance) with changes in N response systems. The novel new focus on response to elevated CO_2 using FACE delivery systems introduces a new level of regulatory control and a picture of how these two important plant species will respond to an elevated CO_2 environment. Apparent allelic differences in DNR1 between indica (generally low DNR1 expression) and japonica (generally high DNR1 expression) provides a potential link between C and N metabolism and how a rich CO_2 environment can influence N use in one crop (indica) better than japonica. I think this is very exciting and does make this manuscript an interesting read that delivers new ideas on how auxin mediated signalling cascades tie together C & N metabolic responses in plants. Experimentally, the authors have endeavoured (through it would appear multiple reviews) clear and reliable data that has been now adequately explained and documented. Re-reviewing the prior reviews and the authors responses, the newly compiled manuscript is rich with information (probably too much) to justify the author's claims.

I disagree this is a manuscript destined to a discipline specific journal, there is plenty of new information not previously covered in the previous Plant Cell Manuscript. Though after an exhaustive read, I think the presentation and explanation of the allelic variation between DNR1 alleles could use some further investigation or documentation. I'm perplexed why the polymorphisms are less abundant in japonica DNR1 relative to indica and why tillering and N/CO_2 responsiveness is a trait not being selected in japonica relative to indica. Is there an unknown NUE penalty with the cultivation of japonica relative to indica to allow the former to still be produced. If a revised manuscript is asked, it would be nice to include some level of discussion on the allelic differences and their persistence in the breeding pools of indica and japonica varieties. This will complement the vast amounts of convincing data on why this is an important study and the necessity to be communicated through this chosen journal.

Author's response Thanks for your positive feedback. As suggested, we have included the discussion on the allelic differences and their persistence in the breeding pools of *indica* and *japonica* varieties.

"Importantly, our phylogenetic analysis of ~3,000 rice accessions showed that *indica* and *japonica DNR1* alleles belong to two separate clades[23,24]. Haplotype analysis of the *DNR1* gene of these varieties revealed four distinct haplotypes (Hap. I-IV). Notably, 98.1% of the *indica* subpopulation belongs to Hap. I, while 75.7% and 22.2% of the *japonica* subpopulation belongs to Hap. II and Hap. III, respectively[24], demonstrating consistent differentiation across existing varieties. This divergence may be attributed to high-fertilizer breeding conditions that have led to the effective utilization of the indica-type *DNR1*, while it remains underutilized in *japonica* rice. Together, these results suggest that the CO_2 fertilization effect for the vast majority *japonica* varieties can be increased by manipulating DNR1."

The following are our responses to the reviewers' comments and an indication of additional data and other changes we have made to the manuscript in response to these comments. For the sake of clarity, the reviewer comments are written in italics.

Reviewer #1 (Remarks to the Author):

The revised manuscript has addressed my primary concerns regarding the correlation between different DNR1 alleles and the CO_2 fertilization effect, as well as the implications of elevated CO_2 (eCO_2) on the enrichment of ARFs in N-use genes. Additionally, it is great to see that the authors have delved into the potential role of DNR1 in an auxin transport-dependent communication mechanism between CO_2 and nitrate in rice, which involves shoot-to-root signaling.

Interestingly, the results suggest that although the activation of N-use genes in roots by eCO_2 is independent of DNR1, it partially depends on auxin transport from the shoot, as indicated by the outcomes of NPA treatment in response to Q4. This finding implies the existence of other unknown mechanisms that facilitate the crosstalk between CO_2 and nitrate from the shoot to the root. I recommend that the authors incorporate these results into the manuscript and provide a thorough discussion. This addition may capture significant interest within the plant science community.

Author's response Thank you for acknowledging our revised manuscript and for your thoughtful suggestions.

Based on the results from Q4, it is clear that NPA treatment influences auxin transport in a DNR1-independent manner, both under aCO_2 and eCO_2 conditions, and the relationship between CO_2 concentration changes and NO_3^- transport certainly warrants further investigation. However, we did not include these results in the previously revised manuscript, as they do not directly align with its primary focus. To ensure that

readers can easily grasp the key points of the manuscript, we would still like to highlight this point in our response.

Reviewer #4 (Remarks to the Author):

All the concerns have been properly addressed.

Author's response Thanks for your kind feedback.

Reviewer #5 (Remarks to the Author):

I was satisfied with my last review of this manuscript. The authors had responded to all of my questions and collectively presented a much improved version of the manuscript. I'm satisfied with the current responses made by other reviewers and support the new included data as beneficial to the paper's story and clarity.

Author's response We thank you for taking the time to review our manuscript, and really appreciate your positive feedback.