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Summary

- The elevation of CO_2 in the atmosphere increases plant biomass but decreases their mineral content. The genetic and molecular bases of these effects remain mostly unknown, in particular in the root system, which is responsible for plant nutrient uptake.
- To gain knowledge about the effect of elevated CO_2 on plant growth and physiology, and to identify its regulatory in the roots, we analysed genome expression in Arabidopsis roots through a combinatorial design with contrasted levels of CO_2, nitrate and iron.
- We demonstrated that elevated CO_2 has a modest effect on root genome expression under nutrient sufficiency, but in contrast leads to massive expression changes under nitrate or iron deficiencies. We demonstrated that elevated CO_2 negatively targets nitrate and iron starvation modules at the transcriptional level, associated with a reduction of high-affinity nitrate uptake. Finally, we inferred a gene regulatory network governing the root response to elevated CO_2. This network allowed us to identify candidate transcription factors including MYB15, WOX11 and EDF3 which we experimentally validated for their role in the stimulation of growth by elevated CO_2.
- Our approach identified key features and regulators of the plant response to elevated CO_2, with the objective of developing crops resilient to climate change.

Key words: Gene regulatory network — Elevated CO_2 — Growth stimulation — Mineral nutrition — Arabidopsis.

Introduction

The atmospheric concentration of carbon dioxide (CO_2) is expected to reach between 750 ppm and more than 1000 ppm at the end of the century (IPCC, 2021). Elevated atmospheric CO_2 (eCO_2) will profoundly modify plant physiology, as CO_2 is the primary substrate of photosynthesis. This is illustrated by the eCO_2 fertilization effect, which leads in C3 plants to an enhanced photosynthesis, a stimulation of growth and an accumulation of biomass for plants grown under eCO_2 condition (Ainsworth & Long, 2021). The stimulation of plant growth by eCO_2 has significant implications, as an augmentation of green biomass and yield is required for satisfying the increasing demand for food, and for mitigating the rise of the CO_2 concentration in the atmosphere. Nevertheless, a large number of CO_2-enrichment experiments, conducted both in fields and in laboratories, have returned that the gain of biomass for plants grown under eCO_2 is much lower than theoretically expected due to plant acclimation to eCO2 (Tausz-Posch *et al.*, 2020). Acclimation of plants to eCO_2 is usually associated to a negative feedback of photosynthesis due to the accumulation of sugars, and to a decrease in leaf Rubisco content (Thompson *et al.*, 2017; Tausz-Posch *et al.*, 2020; Ainsworth & Long, 2021), but the genetic basis remains poorly understood. In addition to this, growing C3 plants under eCO_2 leads to an unexpected decline of their mineral composition (Loladze, 2014; Myers *et al.*, 2014; Gojon *et al.*, 2022). Indeed, plants grown under eCO_2 show a decrease in the tissue concentrations of most mineral nutrients compared to those grown under ambient CO_2 (aCO_2), especially concerning nitrogen (N) and essential micronutrients like iron (Fe). The acclimation of plants to eCO_2 and the negative effect of eCO_2 on plant mineral content are concerning for food security, and present a serious threat of increasing starvations in the coming decades, especially for populations already at risk (Smith & Myers, 2018) Several physiological hypotheses have been proposed to explain this negative effect of eCO_2 on plant mineral composition. Among them, general hypotheses have been highlighted, such as a dilution of

nutrients in higher biomass or a reduced root-to-shoot translocation of nutrients due to a lowered transpiration rate and lower stomatal conductance under eCO_2 (Tausz-Posch *et al.*, 2020). For instance, it was shown recently that increasing transpiration in the *aca7* mutant partially restores the content of Fe in seeds under eCO_2 (Sun *et al.*, 2022). In Arabidopsis and wheat, it has been demonstrated that eCO_2 negatively affects the uptake and reduction of nitrate, highlighting a close link between eCO_2 and N nutrition (Bloom *et al.*, 2010; Bloom *et al.*, 2014). Strikingly, not much is known about the regulatory mechanisms that are associated to the plant acclimation to eCO_2 and to the negative effect of eCO_2 on plant mineral composition. Only a handful of transcriptomic experiments analyzing plants under eCO_2 have been performed. These experiments strongly suggested that eCO_2 has a minor effect on genome expression (Miyazaki *et al.*, 2004; Taylor *et al.*, 2005; Ainsworth *et al.*, 2006; Li *et al.*, 2006; Li *et al.*, 2008; Tallis *et al.*, 2010; Vicente *et al.*, 2019). In Arabidopsis leaves, transcriptomic markers similar to those found under N deficiency have been found under eCO_2, for example, the expression of several genes usually down-regulated by N limitation, was also lower under eCO_2 condition (Li *et al.*, 2006). Several genes coding for major actors of Fe acquisition, such as the transporter *IRT1*, have been also identified as down-regulated by eCO_2 in rice leaves (Yang *et al.*, 2020). On another hand, despite the crucial importance of roots for the homeostasis of nutrients and for growth, the effect of eCO_2 on root genome expression has been very poorly investigated. Several studies, nevertheless, suggested that eCO_2 leads to a misregulation of some genes associated with nitrate or Fe homeostasis, including nitrate transporter genes from the *NRT1* and *NRT2* families (Jauregui *et al.*, 2015; Vicente *et al.*, 2015; Vicente *et al.*, 2016; Bencke-Malato *et al.*, 2019). Collectively, these data suggest that eCO_2 has a significant impact on the regulation of signaling pathways associated to growth and mineral nutrition. However, there is no clear view concerning the effect of eCO_2 on these signaling pathways in the roots yet, and so far, no regulators of the response to eCO_2 in the roots have been identified. The objective of

this work was to contribute to the understanding of the effect of eCO_2 on plants through the study of regulatory mechanisms in the roots, and how they affect two main phenotypes induced by eCO_2: the stimulation of biomass production and the alteration of mineral content.

To do so, we performed a combinatorial analysis of the effect of eCO_2 on root genome expression under contrasting provision of nitrate and/or Fe, in order to reveal features by which eCO_2 disrupts the regulation of major root function, and to identify regulators of this response through the inference of gene regulatory networks. The targeted analysis of genome expression data demonstrated that eCO_2 severely disrupts the expression of regulatory modules associated to nutrient limitation, including the negative regulators of nitrate signaling and of Fe starvation. In addition, the inference of gene regulatory networks reveals several candidate genes for the regulation of the response to eCO_2 in the roots. The analysis of these candidate regulators notably demonstrated that *MYB15*, *WOX11* and *EDF3* transcription factors are required to reach the full potential of growth stimulation and biomass accumulation in a CO_2-rich atmosphere under nitrate limiting condition, without penalizing the mineral composition of plants.

Material and Methods

Biological material. Plant growth conditions and material *Arabidopsis thaliana* plants were grown in hydroponics using nutrient solution as described by (Gansel *et al.*, 2001). Nitrate concentration was either 10 mM (high nitrate) or 0.5 mM (low nitrate) KNO3 during all the experiment. Fe was supplied at a concentration of 50 μM during all the experiment for plants under Fe supply, or was removed from the medium during the last week of growth for plants undergoing Fe starvation. CO_2 conditions in the chambers were constantly maintained at air (around 420 ppm, aCO_2) or 900 ppm (eCO_2), under 200 μE light intensity, and 8-h/16-h light (22◦C) / dark (20◦C) photoperiod. Plant shoots and roots were sampled after 5 weeks of growth. Accessions and mutant alleles used in this study are Columbia (Col), Wassilewskija (WS), *rsl5*

(WiscDsLox384E5), *myb15* (SALK 151976), *myb85* (SALK 052089), *wrky59* (SALK 102984), *wox11-2* (SALK 004777), *edf3* (FLAG 606H09). Mutants were obtained from the Nottingham and the Versailles Arabidopsis Stock Centers.

RNA extraction, quantification of transcripts and RNA-sequencing. Five plant roots from identical conditions were pooled together into one biological replicate, flash frozen in liquid nitrogen, and stored at -80◦C. RNA were extracted from root tissues using TRIZOL (Invitrogen), and DNAse treated using RQ1 (Promega). Reverse transcription was achieved from 1 μg of total RNA with M-MLV reverse transcriptase (RNase H minus, Point Mutant, Promega) using an anchored oligo(dT)20 primer. Accumulation of transcripts was measured by qRT-PCR (LightCycler 480, Roche Diagnostics) using the SYBR Premix Ex TaqTM (TaKaRa). Gene expression was normalized using *UBQ10* and *ACT2* as internal standards. Results are presented as the expression relative to *UBQ10*. Sequences of primers used in RT-qPCR for gene expression analysis are listed in Table S1. RNA-sequencing libraries were done from root total RNA using standard RNA-Seq protocol method (Poly-A selection for mRNA species) by the Novogene company. RNA-sequencing was performed using Illumina technology on a NovaSeq6000 system providing PE150 reads.

Biomass and nutrient-related measurements. 15 to 20 rosettes were dried in a 70◦C-oven for 72 hours, and plant shoot biomass was measured using a precision weighing scale. Nitrogen and Carbon composition of shoots was obtained using an Elementar Pyrocube analyzer. Fe content was measured using acidic digestion and a microwave plasma atomic emission spectrometer (MP-AES, Agilent). Nitrate uptake was measured by supplying $^{15}NO_3$- (1 atom% excess (15N) in hydroponic solution for 72 hours. Roots and shoots were then dried at 70◦C for 72 hours, and samples were analyzed for total N and atom% ^{15}N using a continuous flow isotope ratio mass spectrometer coupled with a C/N elemental analyzer (model Euroflash; Eurovector, Pavia, Italy).

Processing of raw RNASeq files. The quality control and adapter trimming of raw paired-end fastq files was done with *fastp* and its default parameters. Mapping to the TAIR10 reference genome was performed with *STAR*, and the options following :

--outSAMtype BAM SortedByCoordinate

--outFilterMismatchNmax 1

--outFilterMismatchNoverLmax 0.15

--alignIntronMin 30

--alignIntronMax 5000

Quantification of the bam files against the TAIR10 GFF3 annotation file was done using *htseq-count* with options:

-f bam --type gene -r pos

--idattr=Name --stranded=no

Statistical analyses of phenotypic data. We fit linear models to quantitative traits using categorical predictors via the *lm()* R function. We focused on the interpretation of interaction terms, of which we assessed the significance based on the t-test performed on each regression coefficients given by the *summary()* R function.

Transcriptomic analyses. Transcriptomes normalization, PCA and differential expression: the raw expression matrix was normalized using the TMM method. Lowly expressed genes with an average value across conditions under 10 were excluded from the analysis. PCA was carried out on normalized transcriptomes via the *ade4* R package. Differential expression was tested using the *EdgeR* R package as proposed in the *DIANE* R package (Cassan *et al.*, 2021), with no fold change constraint, and an adjusted p-value threshold (FDR) of 0.05.

Multivariate expression-based gene clustering. The *coseq* (Rau & Maugis-Rabusseau, 2018) package embedded in *DIANE* was used to partition genes based on their expression changes across conditions. The underlying framework is the framework of mixture models : Gaussian

mixtures were fit to each cluster after applying a prior arcsin transformation to the normalized counts. The model parameters, i.e the mixing proportions and the cluster-specific distributions parameters are estimated through an Expectation-Maximisation algorithm. A global quality score can be computed to evaluate a given clustering model : we used the Integrated Complete Likelihood. The final number of clusters (9) was chosen where the Integrated Complete Likelihood reached a plateau (elbow method).

Gene regulatory network inference. To reconstruct the transcriptional dependencies between genes, we relied on the network inference method GENIE3 (Huynh-Thu *et al.*, 2010), extended by a permutation-based approach to sparsify its output as implemented in *DIANE* (Cassan *et al.*, 2021). GENIE3 was shown to be among the best performers in benchmark studies such as the DREAM challenges (Marbach *et al.*, 2012), and allows to quantify the strength of regulatory influences between regulator genes and their targets. This influence is extracted from a regression framework : random forests are fit to predict the expression of target genes using the expression of regulator genes as predictors. In the process of fitting the regression trees, the importance of the predictors can be extracted, so that a ranking of all regulator-target pairs is obtained. To select the strongest regulatory interactions among that ranking, we first built a biologically relevant network with a connectivity density of 0.03, made of the regulatory pairs with the strongest importance values. Then, we used a permutation-based procedure to estimate null distributions of random forest importance values against which we tested the observed importance, and selected the interactions with an adjusted p-value (FDR method) below 5%. Network handling and the extraction of network-related metrics were allowed by the *igraph* library.

Gene regulatory Network validation. To validate the inferred network, we made use of the R package *AraNetBench*. In the network evaluation process, the inferred network is first transformed so that grouped regulators are ungrouped, duplicating their interactions with target

genes. Then, each one-to-one regulator-to-target link is compared to DAPSeq, CHIPSeq or TARGET databases. The validation rate of a network is computed as the number of links supported by at least one experiment, divided by the total number of links for which the regulator was experimentally studied. The statistical significance of this validation rate is then assessed by comparing it to the validation rates of a large population of networks with randomly swapped edges. To avoid confusing biases, those random networks and the inferred network are composed of the same nodes, and the regulator's degrees remain unchanged so that the overall connectivity distribution is preserved.

Community discovery. The Stochastic Block Model partitioning was determined via the *sbm* R package. The optimal number of communities was determined automatically, as the number of communities maximizing the inferred Block Model's quality criteria: the Integrated Complete Likelihood.

Gene Ontology enrichment analyses. DIANE's wrapper of *clusterProfiler* was used to detect significantly overrepresented ontologies, which relies on fisher's exact test with an adjusted p-value threshold of 0.05. The gene background used to assess enrichments was the list of all Arabidopsis genes.

Results

eCO_2 leads to profound reprogramming of genome expression under nutrient starvation conditions

In order to explore the effect of eCO_2 on the root regulatory responses under nutrient limitation, Arabidopsis plants were subjected to a combination of treatments including CO_2 (ambient, 420 ppm or elevated, 900 ppm), nitrate (high provision, 10 mM or low provision, 0.5 mM) and Fe (sufficient provision, 50 µM or starvation for 1 week) (Fig. 1a). First, we looked at the expression in the roots of known marker genes of nitrate or Fe nutrition. We observed that eCO_2

leads to a decrease in the expression of genes involved in nitrate uptake and assimilation such as the high-affinity root nitrate transporter genes *NRT2.1* and *NRT1.1*, and the nitrate reductase gene *NIA1*, especially under nitrate limitation (Fig. 1a). We also showed that eCO_2 inhibits the induction of the expression of major markers involved in the Fe starvation response such as the Fe transporter gene *IRT1* and the Fe chelate reductase gene *FRO2* (Fig. 1b). Therefore, eCO_2 seems to disrupt the expression of key actors involved in the response to nitrate or Fe deficiencies. In order to fully explore the genome expression changes in the roots induced by CO_2 elevation and how they affect responses to nutrient starvation, we performed root RNA-seq for the 8 combinations of CO_2 levels, nitrate supply and Fe supply.

Firstly, a principal component analysis (PCA) on the whole dataset revealed that the first principal component, which explains more than 50% of gene expression variation, mainly discriminated genes differentially expressed in response to Fe starvation (Fig. 2a, b). The second principal component, explaining 12.9% of gene expression variation, separated genes differentially expressed in response to nitrate provision, especially under Fe starvation. The effect of nitrate provision under Fe supply on the root transcriptome, mainly visible through the third principal component, explained less than 10% of gene expression variation in the dataset. Lastly, the effect of eCO_2 on the root transcriptome was explained by further principal components 4, 5 and 6, carrying together 11.2% of gene expression change. Therefore, we concluded that the effect of eCO_2 on the root transcriptome was modest in comparison to those of nutrient starvation.

However, a more striking observation was made when looking at the number of genes differentially expressed by eCO_2 depending on the provision of nitrate and Fe. Indeed, very few genes were found to be differentially expressed by eCO_2 when plants were grown under sufficient nitrate and Fe provision (124 genes up- or down-regulated; FDR ≤ 0.05) (Fig. 2c, Table S2-5). On the contrary, many more genes were found to be differentially expressed when

eCO_2 was combined to at least one nutrient limitation. Growth under eCO_2 condition leads to 1550 differentially expressed genes under nitrate limitation, to 3524 genes under Fe starvation, and to 2429 genes under the combination of nitrate limitation and Fe starvation (Fig. 2c, Table S2-5). Therefore, we concluded that eCO_2 has a limited effect on root transcriptome when plants grow under sufficient nutrient conditions, but leads to profound reprogramming of genome expression when plants grow under nutrient starvation conditions.

eCO_2 disrupts gene expression associated to nitrate and Fe starvation signaling pathways

First, we adopted a targeted approach by exploring the expression profile of marker genes involved in nitrate and Fe responses, and their regulation. In the case of nitrate response, we observed that an important number of genes involved in nitrate transport and assimilation were significantly down-regulated by eCO_2, especially under nitrate limitation (Fig. 3a). In line with the qRT-PCR data shown above (Fig. 1b), this was the case for nitrate transporter genes *NRT2.1*, *NAR2.1* and *NRT1.1*, nitrate and ammonium assimilation genes *NIR1* and *GLN1.2*, and nitrate-responsive genes like *G6PD3*. Other genes like *NRT2.2*, *NIA1* or *NIA2* were also down-regulated but to a lesser extent (Table S2-5). In parallel to this, we found that genes involved in the positive regulation of the nitrate starvation response, such as *NLP2*, *TGA4* or *CEP9* were also down-regulated by eCO_2. In opposition, we strikingly observed that numerous genes involved in the negative regulation of nitrate transport and assimilation were up-regulated by eCO_2, specifically under nitrate limitation. This was the case of *BT1* and *BT2*, known to down-regulate the expression of high-affinity nitrate transporters such as *NRT2.1*, but also for members of the *NIGT* transcription factor family that repress nitrate transporter genes under satiety conditions (Araus *et al.*, 2016; Kiba *et al.*, 2018) (Fig. 3a). In addition, we also found that the expression of *LBD41*, a close homolog of the *LBD* transcription factors sub-clade that repress nitrate transport and assimilation (Rubin *et al.*, 2009), was also induced by eCO_2. Altogether, these observations show that under nitrate limitation, eCO_2 markedly affects the

expression of nitrate signaling modules by up-regulating the expression of negative regulators of nitrate uptake and assimilation, and accordingly, by down-regulating the expression of nitrate uptake and assimilation genes. In the case of Fe-related gene expression, we observed a similar deregulation of signaling modules under eCO_2. Indeed, several genes that are induced by Fe starvation under ambient CO_2, such as *IRT1*, *FRO2* or the coumarin transporter *PDR9*, were much less induced or even not induced anymore under eCO_2 (Fig. 3b). In addition to this, we found that the regulators of Fe starvation response such as the transcription factors *FIT* or *BHLH39*, that are induced by Fe starvation under ambient CO_2, were much less induced or even not induced anymore under eCO_2 (Fig. 3b). These observations show that eCO_2 also disrupts the Fe starvation response, and then, more generally, that eCO_2 has a strong negative effect on the expression of signaling modules associated to deficiencies in major nutrients.

In order to see whether these observations coincide with the well-known effects of eCO_2 on physiological parameters in shoots, we measured biomass accumulation, N and Fe concentrations in shoots under each condition. We observed that eCO_2 significantly led to increased biomass, and this regardless of nutrients availability (Fig. 4a). While biomass is consistently increased by eCO_2, we observed contrasted effects on the mineral composition of plants. Growth under eCO_2 led to a strong and significant decline in shoot N concentration when plants were grown under low nitrate conditions, but not under high nitrate conditions (Fig. 4b). From aCO_2 to eCO_2, N concentration dropped by 30% under low nitrate and Fe supply, and by 15% under low nitrate and Fe starvation (Fig. 4b). Surprisingly, the growth under eCO_2 did not lead to a decrease in Fe content, regardless of the nutritional condition (Fig. 4c), suggesting that Fe provision or Fe accumulation in plants was too high to be affected by eCO_2, even after one week of Fe starvation. Finally, we investigated whether the alterations in the expression of nitrate signaling modules under eCO_2 translated into altered nitrate uptake capacities. Indeed, in accordance with the observations made at the level of gene expression

and total N concentration levels, we clearly observed that the nitrate uptake rate was significantly and negatively affected by eCO_2 under limiting nitrate condition, but not affected under high nitrate condition (Fig. 4c).

eCO_2 largely disrupts the response to nitrate limitation

Considering that the major effect of eCO_2 on shoot biomass and N concentration were observed under low nitrate conditions, we investigated in a broader way the impact of eCO2 on the reprogramming of genome expression by nitrate limitation. To do so, we performed a clustering approach, focusing our analysis on the interaction between eCO_2 and nitrate limitation response. The 1550 differentially expressed genes in response to high CO_2 under nitrate limitation (Fig. 2c) were partitioned based on their co-expression in the 4 (triplicated) experimental conditions with reference and perturbation levels of CO_2 and nitrate supply (in all cases with sufficient Fe). The mixture models-based approach we employed (Rau & Maugis-Rabusseau, 2018) led to an organization in 9 clusters (Fig. 5). In line with the profile of nitrate-responsive marker genes (Fig. 3a), we observed that the regulation of the expression of a large number of genes by nitrate is strongly modified under eCO_2 condition. In clusters 5 and 9, we observed more than 300 genes that are down-regulated by nitrate limitation under aCO_2 condition, but not under eCO_2 condition, as observed for *HRS1*, *BT1* and *BT2*. This suppression of regulation by eCO_2 was also observed for genes induced under nitrate limitation; in clusters 7 and 8, more than 200 genes have their expression induced by nitrate limitation under aCO_2 condition but not under eCO_2 condition, as observed for *CEP9*, *NLP2* and *NRT1.1*. In an even more pronounced way, we observed that growth under eCO_2 reversed the regulation by nitrate availability of several hundreds of genes, found in clusters 2, 3 and 6. Finally, cluster 4 contains almost 200 genes showing modest or no expression changes induced by nitrate limitation, but that are considerably repressed under the combination of nitrate limitation and eCO_2. Several nitrate transport or assimilation genes, like *NRT2.1*, *NAR2.1*, *NIR* or *GLN1.2*, belong to this

cluster. Based on this co-expression study, we show that the growth under eCO_2 either lessens, cancels, or even reverses nitrate-induced regulation of the expression of a large number of genes. This strengthens the hypothesis that eCO_2 severely affects regulatory pathways involved in the adaptation to nitrate limitation.

GRN inference yields insightful genes communities and candidate regulators of the response to eCO_2 under low nitrate

Secondly, we adopted a non-targeted approach to identify in the roots the regulators that drive the response mediated by eCO2, and that would be able to modulate the stimulation of growth or the alteration of N concentration under eCO_2. To do so, we inferred a gene regulatory network (GRN) using the 1550 genes found differentially expressed by eCO_2 under low nitrate supply (Table S5). Using as input the expression values obtained under aCO_2, eCO_2, limiting or abundant nitrate supply (12 samples in total), we employed random forest regressions combined with permutation-based statistics (Huynh-Thu *et al.*, 2010; Cassan *et al.*, 2021) to draw connections between regulator and target genes. To do so, we used a list of regulators composed of Arabidopsis transcription factors, enriched with indirect regulators of gene expression (i.e chromatin regulators) and previously known indirect regulators of nitrate-related gene expression such as *BT1*/*BT2* (Table S6). In this approach, a regulator was linked to a target if its expression was a robust predictor of the expression of the target in the input samples (see Methods section). The resulting GRN is made of 796 nodes (647 target genes, 149 regulators) and 1700 connections (Fig. S1).

In order to validate the inferred edges against known regulatory interactions, we leveraged the ConnecTF database (Brooks *et al.*, 2021), composed of in vitro (DAPSeq) and in vivo (CHIPSeq) binding experiments, as well as in planta regulation assays. Considering all the regulators for which validation information is available, 31.3% of the predicted interactions in the inferred network were supported by at least one experiment in ConnecTF (Fig. S2). To

determine the significance of this validation percentage, we compared it to the validation percentages of a population of 200 shuffled networks, in which the interactions between the same genes were randomly unmatched. No random network had a higher validation rate than our inferred network, leading us to conclude that our inference approach captured consistent biological information linked to the regulation of gene expression in the roots by eCO_2 under low nitrate conditions (Fig. S3).

We tested the presence of the known regulators of the nitrate response signaling pathway and their targets on this GRN, in order to appreciate their influence on the effect of eCO_2 on root genome expression under low nitrate conditions (Fig. 6A). Surprisingly, most of the important actors of nitrate uptake and assimilation like *NRT2.1*, *NRT1.1* or *NIA* genes were not present in the network, with the exception of *NAR2.1* and *NIR1*. In contrast, an important number of known regulators of the nitrate response signaling pathway were found in the GRN. Notably, *BT1*, *BT2* and *HRS1* were grouped together because of the high correlation value of their expression profiles in the 12 samples, which supports their similar function in the repression of nitrate limitation signaling pathways. To continue the general analysis of this GRN, we used the Stochastic Block Model (SBM) framework to analyze the topological structure of the GRN and identify gene communities. This revealed a modular and disassortative topology, typical of biological networks (Fig. 6B). In each of the 8 gene communities identified, we performed gene ontology (GO) enrichment. Notably, genes included in the communities 1 and 3 displayed significant enrichment for GO related to N like "response to N compound", "response to nutrient levels" or "response to nitrate" (Fig. S4). This supports again the hypothesis according to which eCO_2 severely affects the regulation of genome expression associated with N and with nitrate in particular.

MYB15, _EDF3_ and _WOX11_ regulate the stimulation of plant growth by eCO_2 under low nitrate

In order to identify candidate regulators of the response to eCO_2, we hypothesized that the regulators displaying the highest degree of connectivity to target genes are the most relevant to control a large part of the GRN, and thus of the response to eCO_2. Therefore, we ranked the regulators present in the GRN by their degree (Table S7). We analyzed the topological distribution of the 12 most connected regulators (Fig. 6C, D). Most of them belonged to the topological cluster 1, which is enriched for genes associated to the response to N compounds, nutrients or nitrate. We used knock-out mutant lines for several of these most connected regulators to test their involvement in the plant's response to eCO_2. Mutants for *MYB15*, *MYB85*, *WOX11*, *EDF3*, *WRKY59* and *RSL5* transcription factors were grown under eCO_2 and limiting nitrate conditions, and phenotyped for their shoot biomass and N concentration. We further analyzed whether biomass and N concentration changes caused by eCO_2 in each genotype were statistically different from the change observed in the WT. First, there was no evidence in our analysis that any of these transcription factors were involved in the control of N concentration in the shoot under eCO_2, as no single mutation in these regulators led to a significant change in the decrease in N concentration induced by high CO_2 compared to that of wild types (WT) (Fig. 7A, B). In contrast, we observed that the stimulation of growth induced by eCO_2 was significantly lower in myb15, wox11 and edf3 than in the WT (Fig. 7C, D). The stimulation of growth by eCO2 was reduced by 40%, 25% and 49% in myb15, wox11 and edf3, respectively. In *myb15* and *wox11*, the biomass appears already affected under aCO_2. However, the effect of *myb15* and *wox11* mutations was significantly stronger on the biomass stimulation by eCO_2 than on the biomass under aCO_2 (Table S8). This shows that these transcription factors have a general role in biomass production, which is significantly exacerbated by the elevation of CO_2. In *edf3*, the biomass under aCO_2 condition biomass remains unaffected in comparison to the WT (Table S8). Therefore, the strong reduction of biomass observed in *edf3* under eCO_2

was strictly associated to a defect of the stimulation of growth induced by high CO_2. This shows that EDF3 is a major regulator of the gain of biomass that is observed under eCO_2.

Discussion

In this study, we analyzed the effect of eCO_2 on plant growth and on nitrate and Fe nutrition in Arabidopsis. Several studies have explored the effect of eCO_2 on plant genome expression. Most of them concluded to a minor effect of eCO_2 on genome expression, with a relatively limited number of differentially expressed genes (Taylor *et al.*, 2005; Ainsworth *et al.*, 2006; Fukayama *et al.*, 2009; Tallis *et al.*, 2010; Jauregui *et al.*, 2015; Bencke-Malato *et al.*, 2019). Our results show that eCO_2 actually leads to modest changes in gene expression under sufficient nutrient conditions, but in opposition leads to a profound reprogramming of genome expression as soon as plants grow under limiting nitrate or under Fe starvation condition. More precisely, we demonstrated that eCO_2 has a strong effect on the expression of genes associated to nitrate or Fe signaling. A striking contrast was observed between the repressive effect of eCO_2 on the expression of nitrate transport and assimilation genes like *NRT2.1*, *NRT1.1* or *NIR1*, and the opposite inductive effect on the expression of genes like *BT1*/*BT2* or *NIGT* transcription factors, known to be among the main negative regulators of nitrate transport and assimilation (Araus *et al.*, 2016; Kiba *et al.*, 2018). The same observation made on Fe signaling genes led us to postulate that eCO_2 may broadly affect the response to nutrient limitation at the gene expression level, by altering the expression of major signaling modules.

Concerning nitrate, our results provide highly consistent lines of evidence supporting the original hypothesis that the repressive effect of eCO_2 on root nitrate uptake specifically targets the High-Affinity Transport System (HATS), and not the Low-Affinity Transport System (LATS). Indeed, the nitrate transport genes shown to be markedly down-regulated by eCO_2 (*NRT2.1*, *NAR2.1*, *NRT1.1*) all encode major contributors to the HATS activity (O'Brien *et al.*,

2016; Jacquot *et al.*, 2020). In line with this, root nitrate uptake rate is repressed by eCO_2 at 0.5 mM external nitrate (at which the HATS is largely predominant over the LATS), but not at 10 mM external nitrate (at which the HATS plays a negligible role as compared to the LATS). Accordingly, total N concentration in shoots is reduced by eCO_2 at 0.5 mM, but not at 10 mM nitrate. To our knowledge, such a differential response of the nitrate HATS and LATS to eCO_2 was not previously reported, and may provide some explanation to contradictory conclusions found in the literature. Indeed, the response of root nitrate acquisition to eCO_2 was found to be highly variable, ranging from strong inhibition to no effect or even stimulation, therefore preventing any clear conclusion on this point (BassiriRad *et al.*, 2001; Coskun *et al.*, 2016; Gojon *et al.*, 2022). However, most studies did not precisely monitor external nitrate availability, leaving the possibility that part of the above discrepancies may result from the fact that the plants in these studies either relied on the HATS or the LATS for their N nutrition. In opposition to root nitrate uptake, the response of shoot growth to eCO_2 was similar regardless of nutrient supply, with a large stimulation in all cases. Our observations show that the responses of the growth and mineral composition of shoots exposed to eCO_2 are not strictly correlated, which is evidence against the hypothesis of a growth dilution of plant N under eCO_2. This supports recent reports (Myers *et al.*, 2014; Feng *et al.*, 2015; Wujeska-Klause *et al.*, 2019), and thus suggests direct negative effects of eCO_2 on plant N acquisition.

We used a machine-learning approach to infer the GRN in the roots of Arabidopsis in response to eCO_2 under nitrate limitation, and to identify putative regulatory factors involved in the plant's response to eCO_2. This revealed that large communities of genes with a function associated to the response to nitrate or to N were affected by the growth under eCO_2, suggesting again that rising CO_2 in the atmosphere will markedly alter the physiological mechanisms of plant nutrition. In addition, ranking the regulators by their degree of connection to target genes in the GRN led to the identification of potential actors of the response to eCO_2 in plants. By

phenotyping loss-of-function mutants for a subset of these highly-connected regulators, we found that three of them, *MYB15*, *WOX11* and *EDF3*, were involved in the stimulation of growth by eCO_2. To date, only a handful of genes involved in the stimulation of growth by eCO_2 have been identified (Bouain *et al.*, 2022; Oguchi *et al.*, 2022). To our knowledge, our work provides the first identification of root related genes involved in the regulation of the stimulation of plant growth by eCO_2. We have demonstrated here that *MYB15*, *WOX11* and *EDF3* function are essential to fully reach the potential of eCO_2 fertilization under nitrate limitation, with a reduction of the growth stimulation by almost half in *myb15* or *edf3* mutants. *MYB15* has been previously identified as a regulator of the expression of the *PHO1;H3* phosphate transporter in the roots (Pal *et al.*, 2017). Interestingly, phosphate accumulation in the shoot has been recently proposed to regulate plant growth under eCO_2, through the expression of the *PHT4;3* phosphate transporter in shoots (Bouain *et al.*, 2022). In our data, the expression of these two phosphate transporters were not deregulated by eCO_2. Two other phosphate transporter genes were actually deregulated by eCO_2, but were not predicted as *MYB15* target genes in the GRN. Nevertheless, the link between the regulation of growth under eCO_2 by *MYB15* and phosphate accumulation will deserve further attention. Much less is known about the function of *EDF3*, apart from its role in the transcriptional network of N-associated growth inferred in Arabidopsis, were *EDF3* regulates essential N-associated genes like *NIR1*, *NAR2.1* or *GLN1.2* (Gaudinier *et al.*, 2018). Finally, the absence of variation in the decrease of N concentration led by the growth under eCO_2 in *myb15* or *edf3* mutants supports the physiological observations abovementioned, both strongly suggesting a decoupling between the growth stimulation and the decrease of N concentration established under eCO_2, at the physiological and genetic levels. Altogether, the identification of these genes as important components of the response to eCO_2 paves the way for the optimization of plant biomass

production under future CO_2 atmosphere without penalizing nutrient content, and nutritional value of crops.

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Author contributions

C.D., A.G. and A.M. designed the project. O.C, L-L.P., C.D., A.G., L.B., and A.M. planned the experiments. O.C. and L-L.P. performed most of the experiments. O.C., C.D., A.G., L.B., S.L. and A.M. analyzed and interpreted the data. O.C. and A.M. wrote the manuscript with help from every authors.

Competing interests

None declared.

Data availability

Data and R notebooks containing the analyses performed in this article can be found at https://github.com/OceaneCsn/CO2_root_networks_inference. RNA-seq data are available at

https://www.ebi.ac.uk/biostudies/arrayexpress/studies using the accession number: E-MTAB-12483.

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Supporting information

Fig. S1: Metrics of the GRN inferred from the effect of elevated CO_2 under low nitrate condition.

Fig. S2: Validation of edges in the GRN.

Fig. S3: Statistical significance of the comparison between validated edges in the GRN and in random networks made by permutation from DEGs included in the GRN.

Fig. S4: Gene Ontology Enrichment in the communities of the GRN.

Table S1: List and sequence of primers used in this study.

Table S2: Differentially Expressed Genes by ECO2 under high nitrate and iron starvation.

Table S3: Differentially Expressed Genes by ECO2 under high nitrate and iron supply.

Table S4: Differentially Expressed Genes by ECO2 under low nitrate and iron starvation.

Table S5: Differentially Expressed Genes by ECO2 under low nitrate and iron supply.

Table S6: List of regulators used in the inference of GRN.

Table S7: Full ranking of regulators from their degree in the GRN.

Table S8: Statistics of eCO2*genotype effects using a linear model.

Figure Legends

Figure 1. Combinatorial analysis of the effects of eCO_2, nitrate limitation and Fe starvation. (a) Design of the combinatorial experiment combining contrasting levels of CO_2, nitrate and Fe. Arabidopsis plants were grown in hydroponics for 5 weeks under contrasted levels of CO_2, nitrate and Fe. High nitrate (10 mm), Low nitrate (0,5 mM), +Fe (50 μM), -Fe (0 μM), Amb. (ambient CO_2, 420 ppm), Elev. (elevated CO_2, 900 ppm). For each combination of nitrate and Fe supply levels, 3 representative rosettes are shown under aCO_2 and eCO_2. (b) Quantitative RT-PCR showing the relative expression of marker genes of nitrate and Fe nutrition in the roots, in response to the combination of CO_2 and nitrate, or CO_2 and Fe supply. High nitrate (10 mM), Low nitrate (0,5 mM), +Fe (50 μM), -Fe (0 μM), Amb.(ambient CO_2, 420 ppm), Elev. (elevated CO_2, 900 ppm). Data represent mean ± SD of 5 biological replicates from a representative experiment. Statistical significance was computed using an unpaired two-tailed Student's t test (*, P≤0.05; **, P≤0.01, ***,P≤0,001).

Figure 2. Elevated CO2 reprograms root genome expression under nutrient deficiency. (a) Principal Component Analysis of the normalized root transcriptomes under the eCO_2 and nutrient limitations combinatorial design. Contributions (i.e correlation) of each experimental condition to the 6 first principal components. Each color stands for an experimental condition. Genes are shown as dots. (b) Percentage of variance explained by each of the principal components determined by the analysis. (c) Number of genes differentially expressed by eCO_2 under different conditions of nitrate and Fe provision.

Figure 3. Elevated CO_2 disrupts the expression of key nitrate and Fe marker genes specifically under nitrate limitation and Fe starvation. Heatmap representation showing the Z-score of normalized expression levels of genes important for nutrition in the transcriptomic

dataset. (a) Regulation by eCO_2 of genes involved in nitrate transport and assimilation and in their regulation, under high or limiting nitrate conditions. (b) Regulation by eCO_2 of genes involved in Fe transport and in their regulation, under Fe supply or Fe starvation.

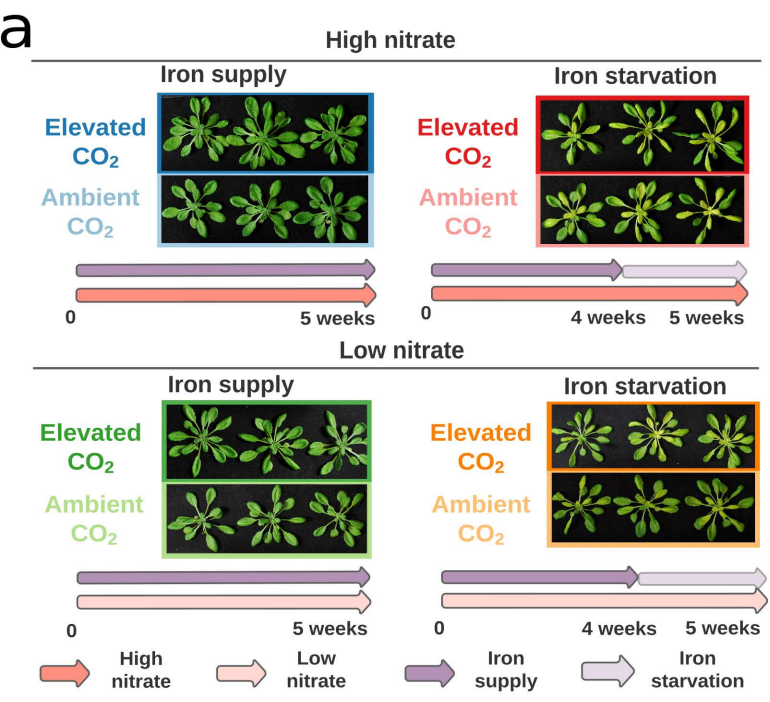
Figure 4. Elevated CO_2 modifies plant physiology depending on nutrient supply. Shoot biomass (a), N concentration (b) and Fe content (c) were measured on individual rosettes from plants grown for 5 weeks in hydroponics under contrasted levels of CO_2, nitrate and Fe. (d) Nitrate uptake measured on plants grown in hydroponics under contrasted levels of CO_2 and nitrate. Statistical tests for comparison between aCO_2 and eCO_2 were performed with the Wilcoxon test. High nitrate (10 mM), Low nitrate (0.5 mM), Fe supply (50 μM), Fe starvation (0 μM), aCO_2 (˜420 ppm), eCO_2 (900 ppm).

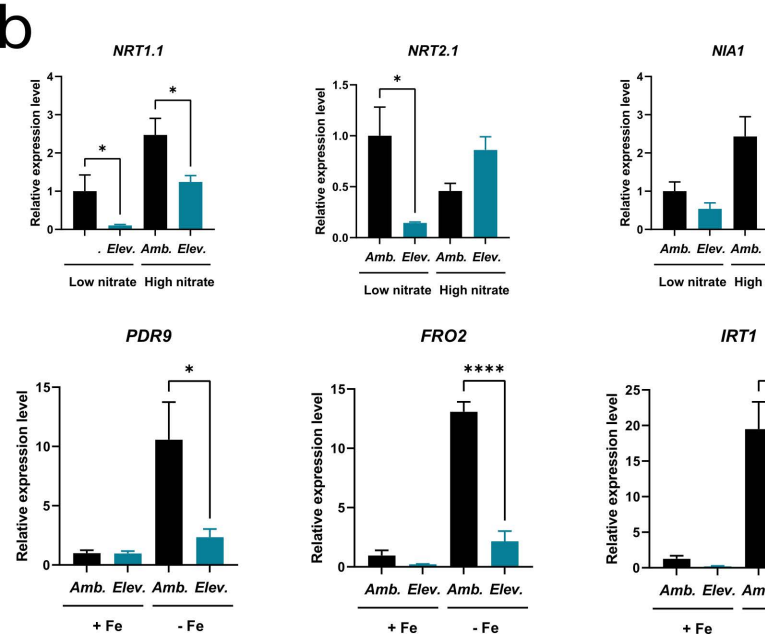
Figure 5. Clustering analysis of the 1550 DEGs by eCO_2 under low nitrate, on the 4 combinations of aCO_2 or eCO_2, and high or low nitrate. Expression profiles are defined as the normalized expression, divided by the mean normalized expression in all conditions. Genes were partitioned between 9 clusters. In some clusters, gene names previously identified as key actors of nitrate nutrition are highlighted, either in green (for actors or positive regulators) or red (for negative regulators). We identified 3 cluster categories based on the interpretation of their expression changes, as represented by the color of the expression profiles. Green, cancels/lessens starvation regulations. Orange, reverses starvation regulations. Blue, specific regulations.

Figure 6. Inferred GRN of the root response to eCO_2 under low nitrate. In each network panel, round nodes are target genes, square nodes are regulator genes, and large square nodes are regulator genes grouped together because of a high correlation. (a) Network view with a

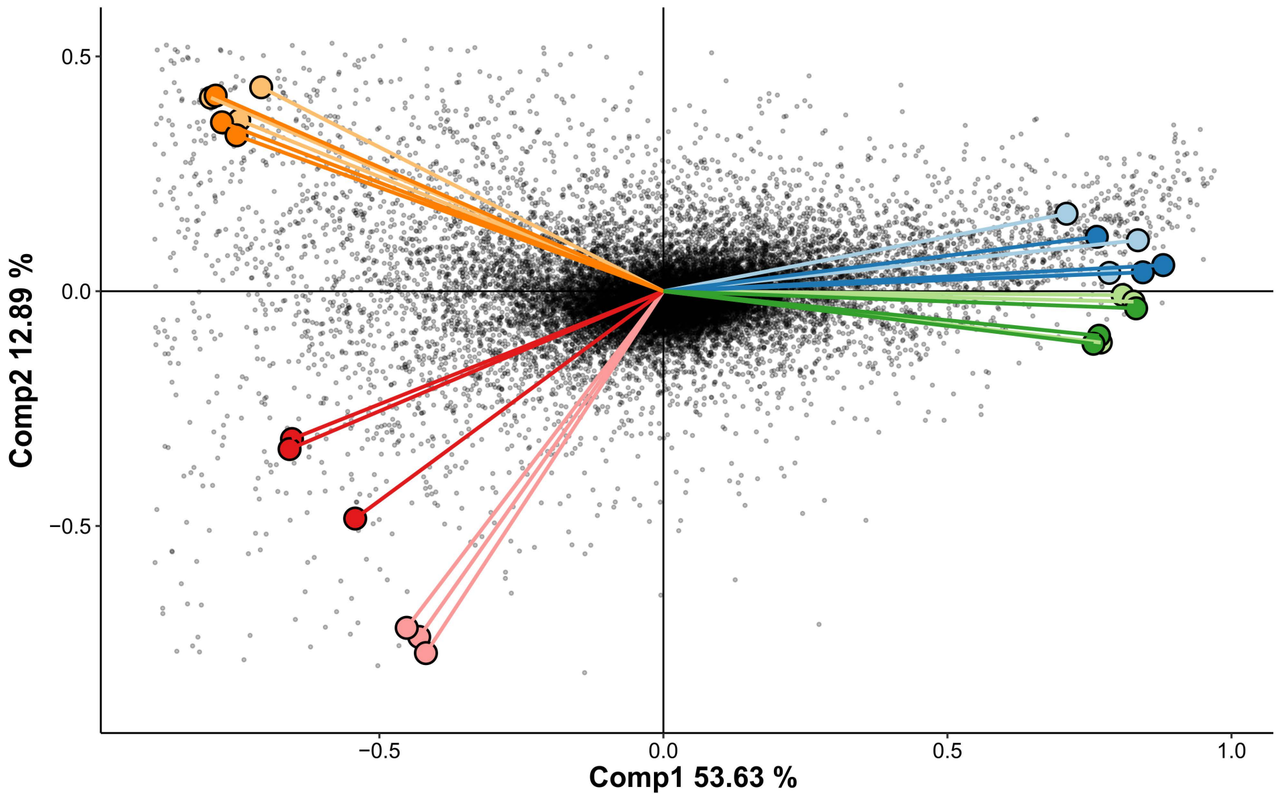
highlight on actors of nitrate acquisition, signaling and metabolism and their regulators. (b) Topological clustering in the inferred GRN. Each color represents a community of highly connected genes. (c) Network view with a highlight on the 12 most influencing candidate regulators identified on the basis of their overall degree. (d) Ranking of these the 12 most influencing candidate regulators in the inferred GRN, with their TAIR AGI, common name, topological community and overall degree.

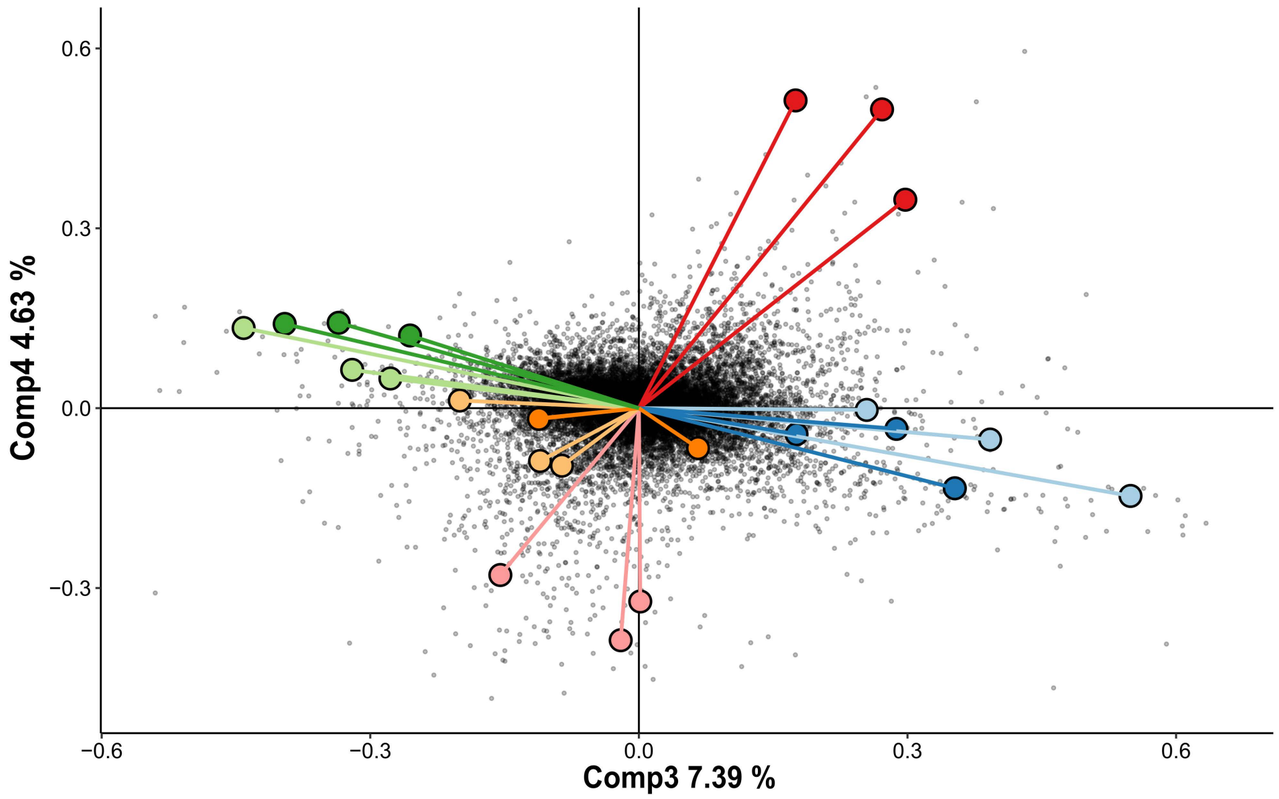
Figure 7. *MYB15*, *WOX11* and *EDF3* control the stimulation of biomass production by eCO_2 under nitrate limitation. Phenotypic response to eCO_2 of plants with mutations for the candidate regulators *rsl5*, *myb15*, *myb85*, *wox11*, *wrky59* and *edf3*, as compared to their relative wild type (*Col*, Columbia; *WS*, Wassilewskija). Plants were grown for 5 weeks in hydroponics under contrasted levels of CO_2. The significance levels refer to the p-values relative to the genotype by environment interactions (i.e., the effect of the mutation on the effect of eCO_2), using a linear model. a, b. Leaf N concentration measured in WT and in candidate mutant lines. c, d. Shoot biomass measured in WT and in the candidate mutant lines. aCO_2, ambient CO_2. eCO_2, 900 ppm.

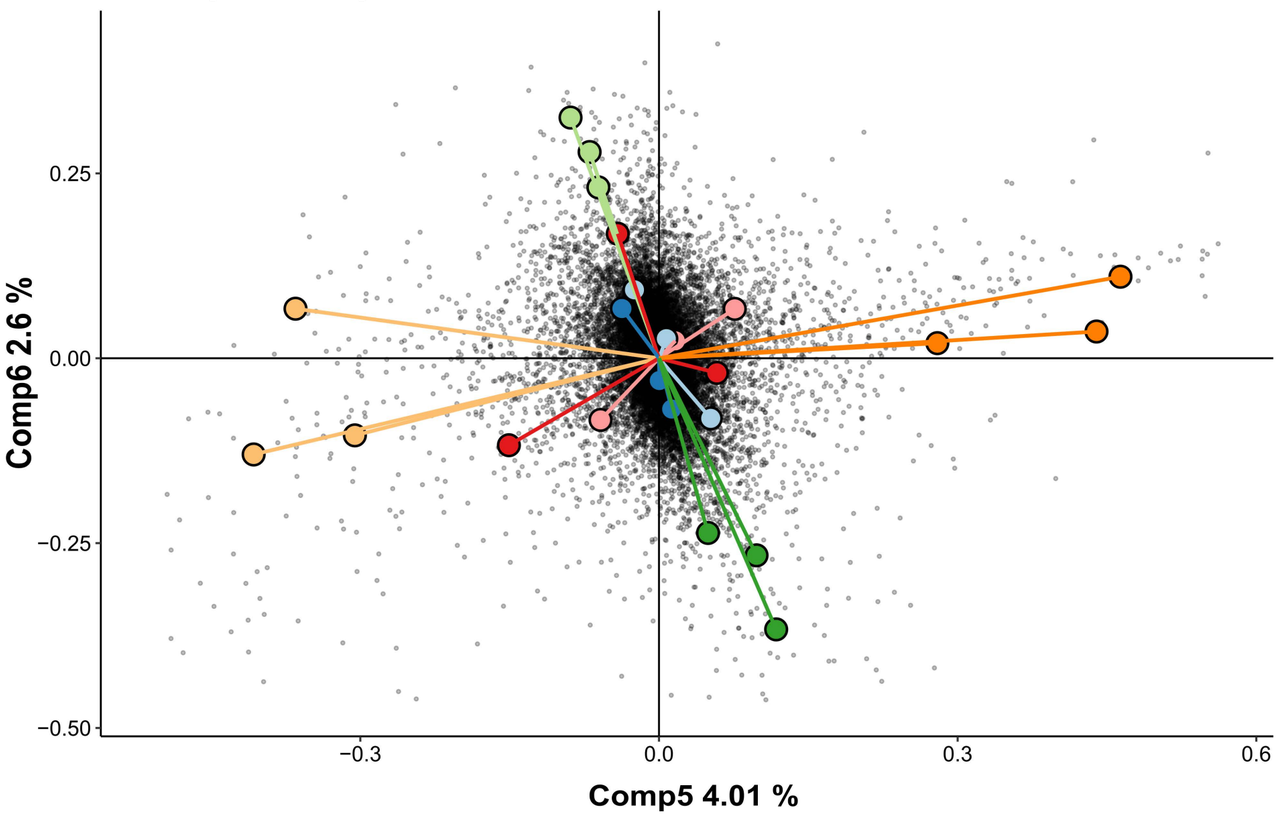




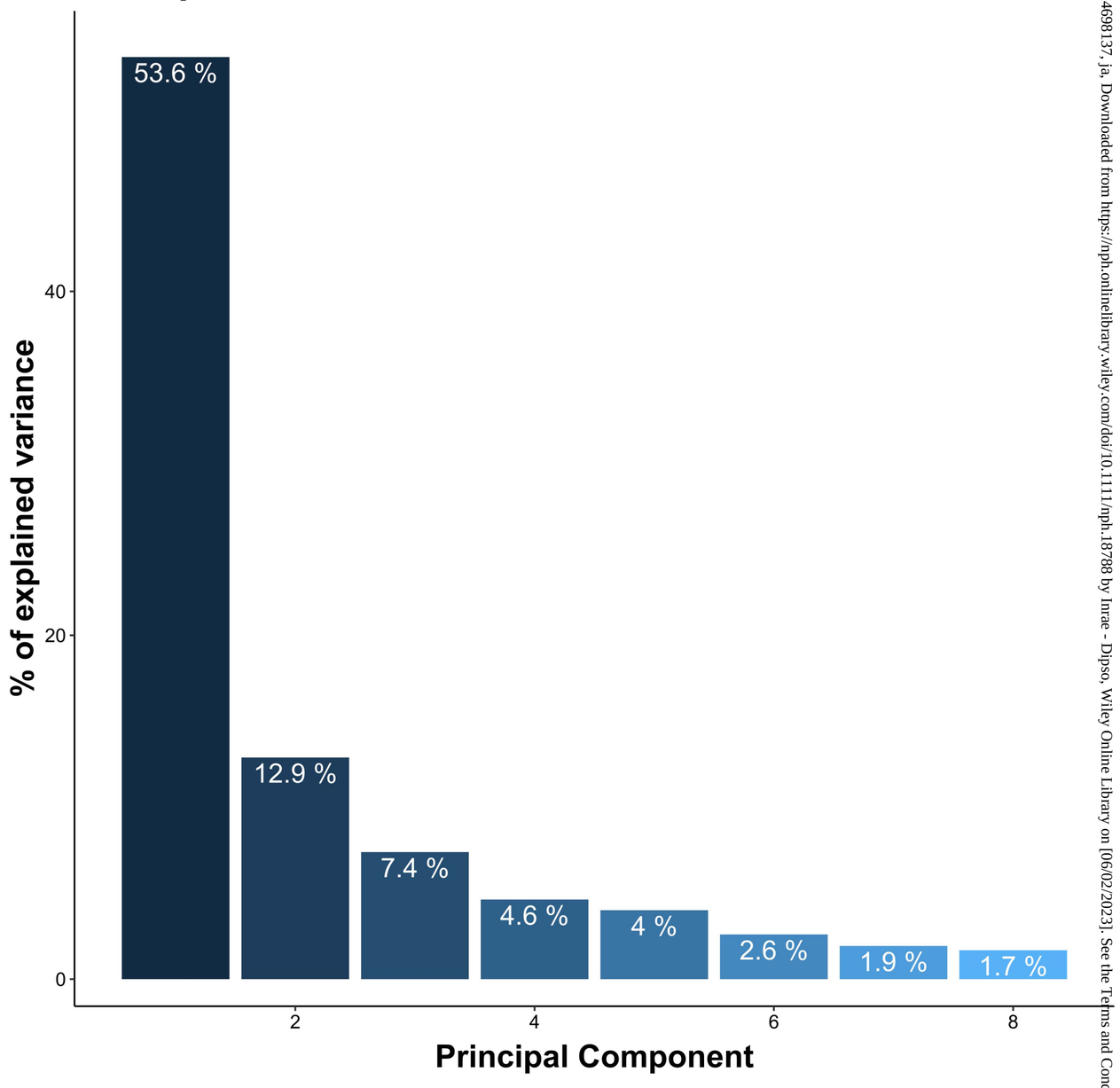
a



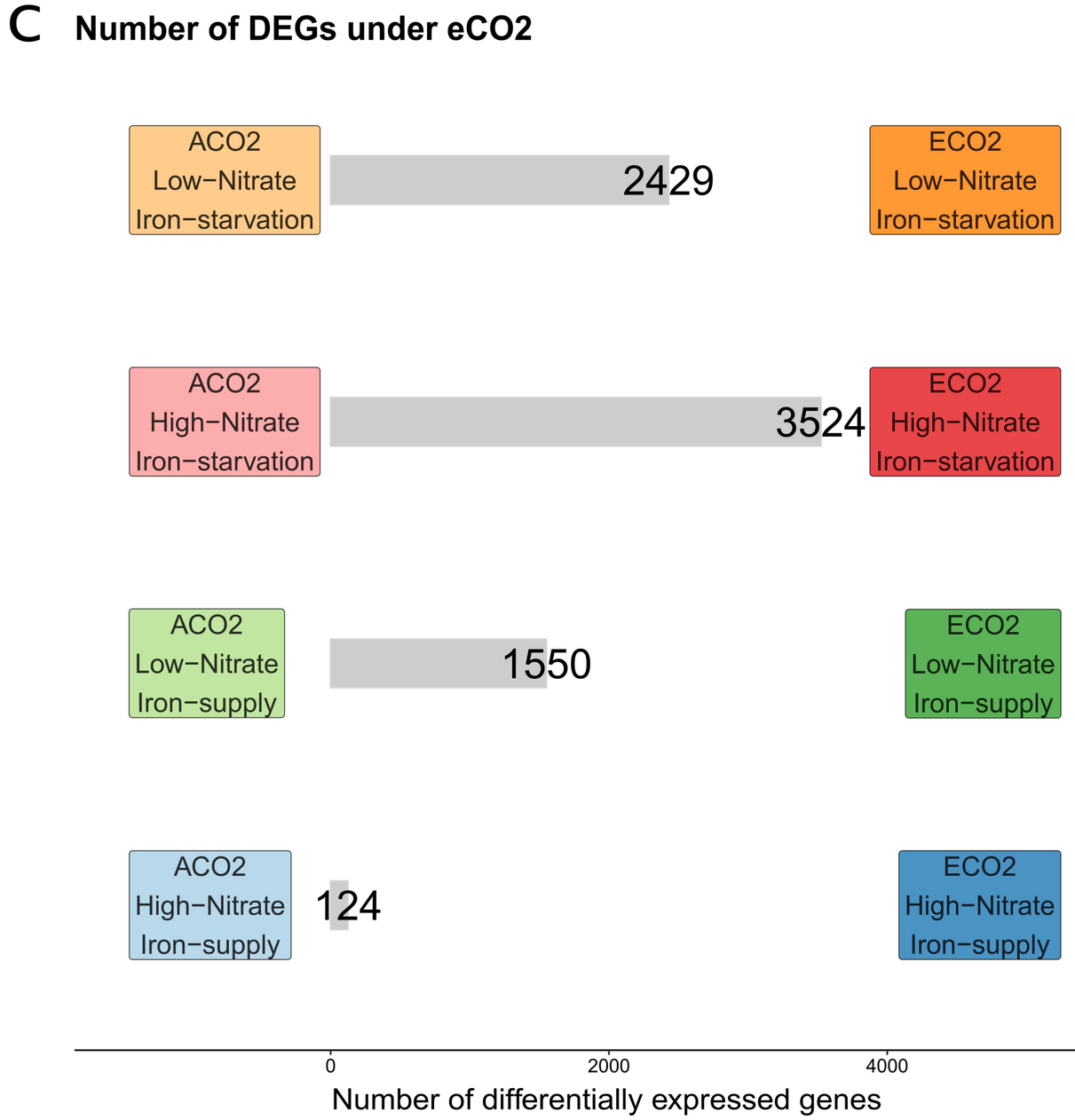


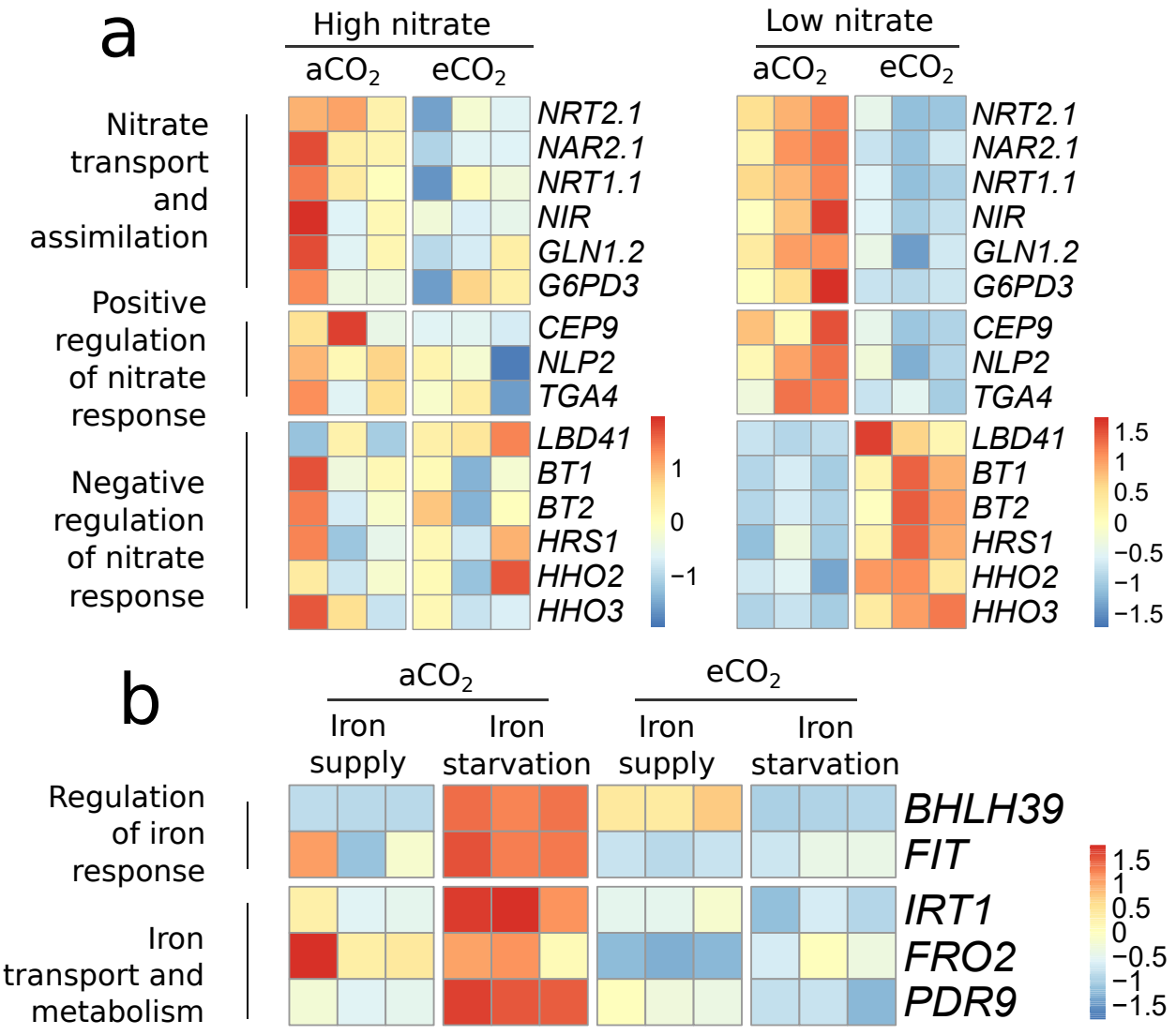


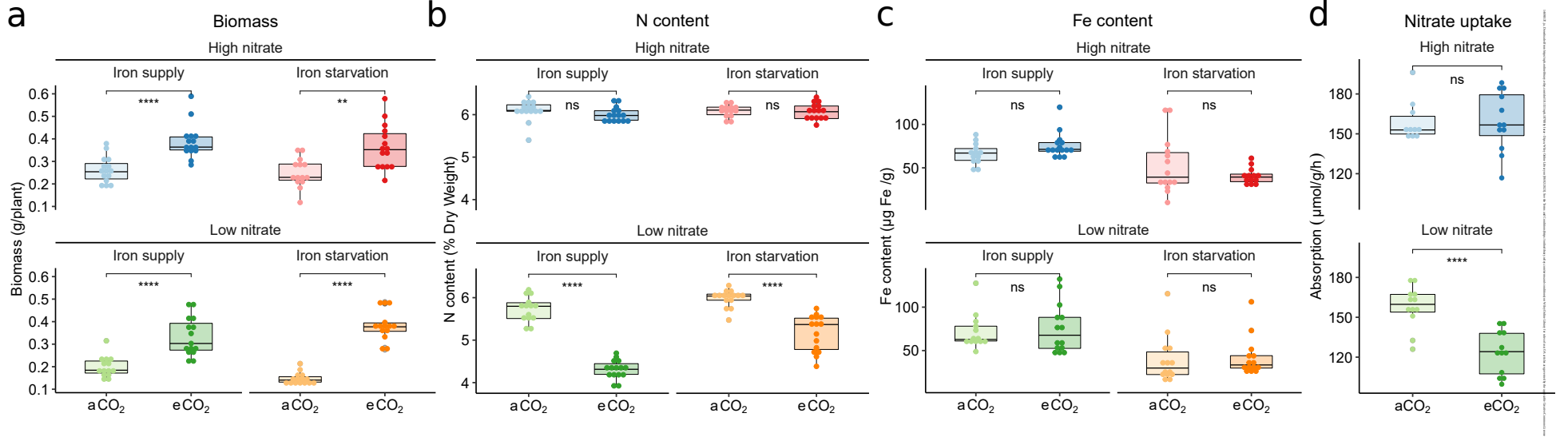
b

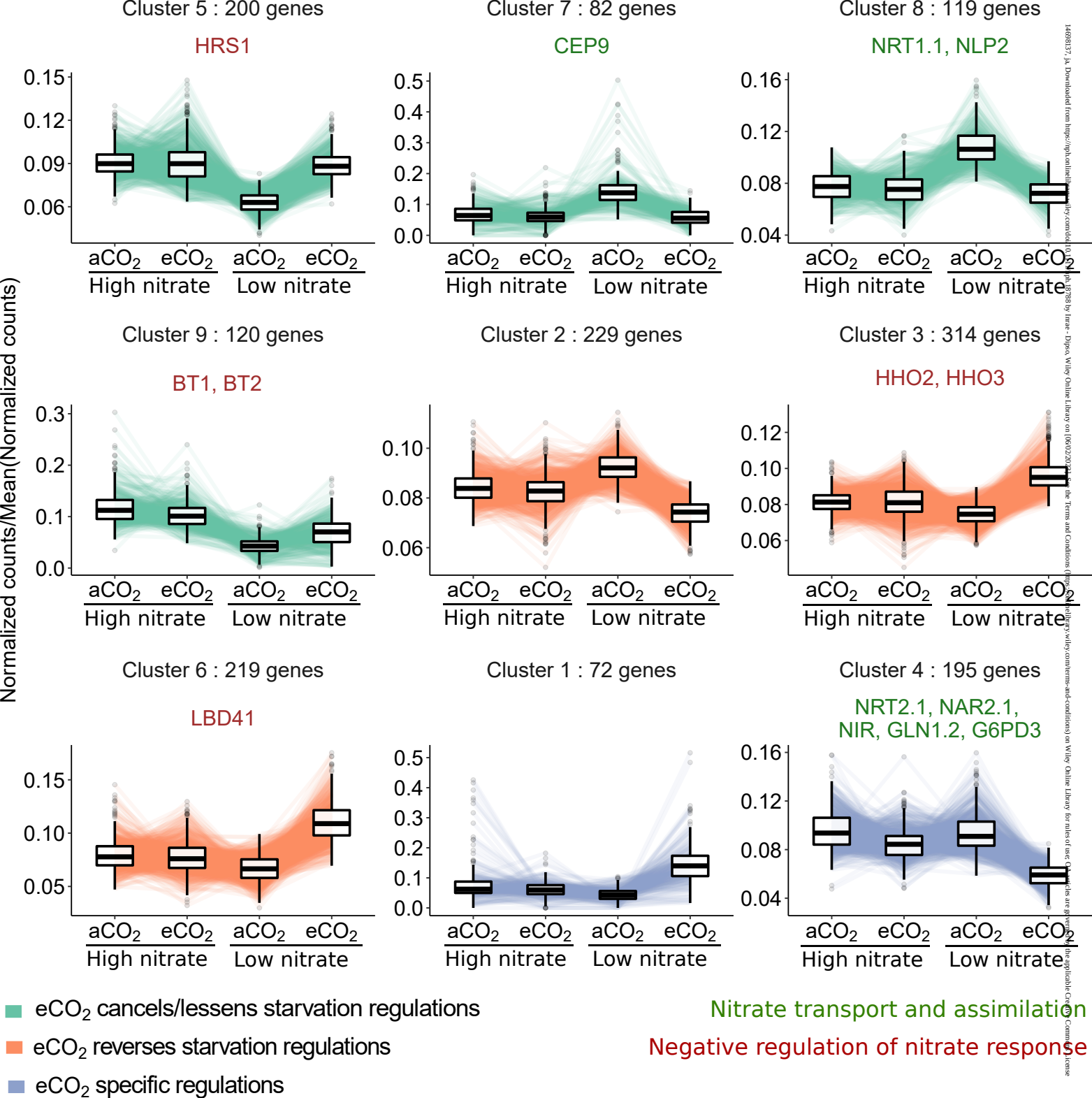


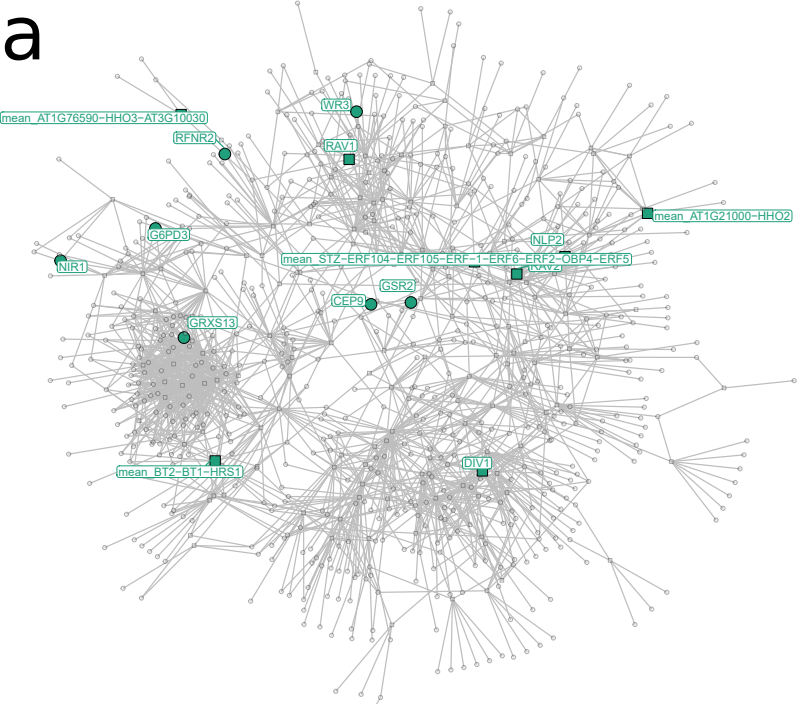
c

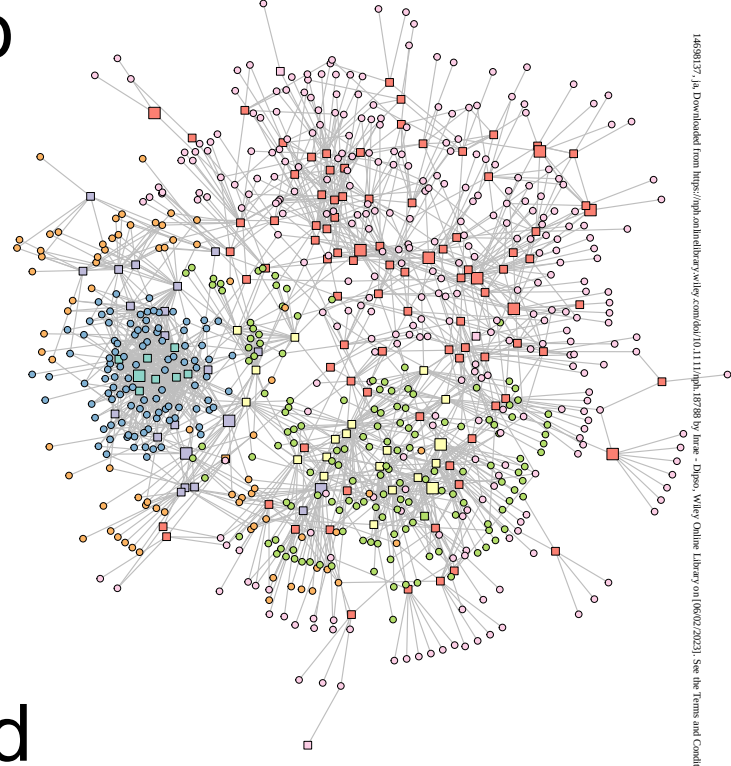


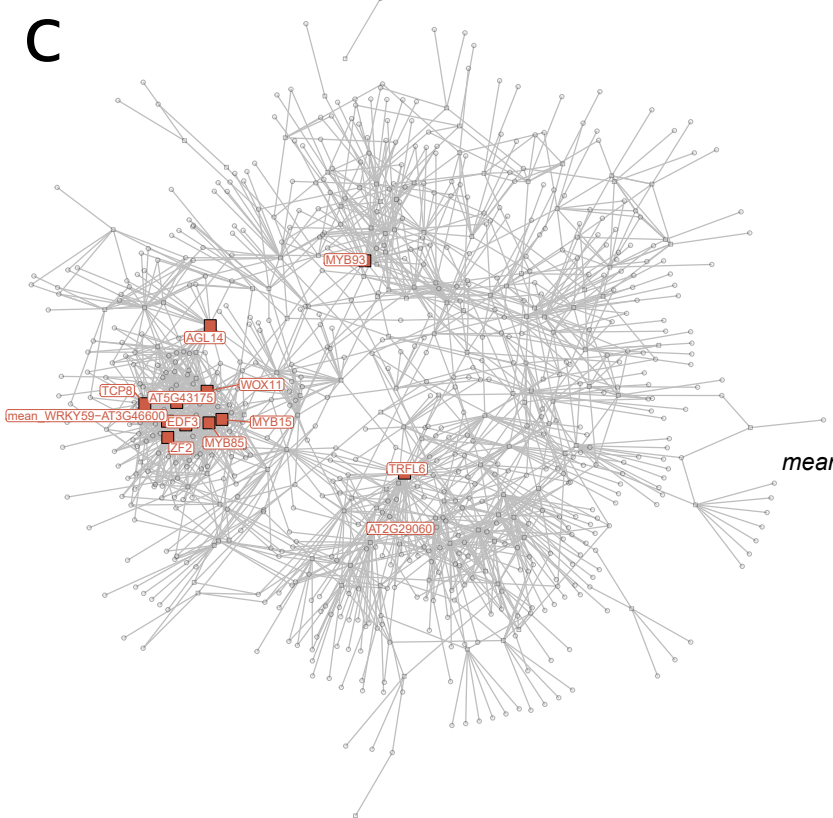



a
Biomass
High nitrate
Iron supply
Iron starvation
Low nitrate
Biomass (g/plant)
****
**
aCO2
eCO2
b
N content
N content (% Dry Weight)
ns
c
Fe content
Fe content (μg Fe /g)
d
Nitrate uptake
Absorption (μmol/g/h)





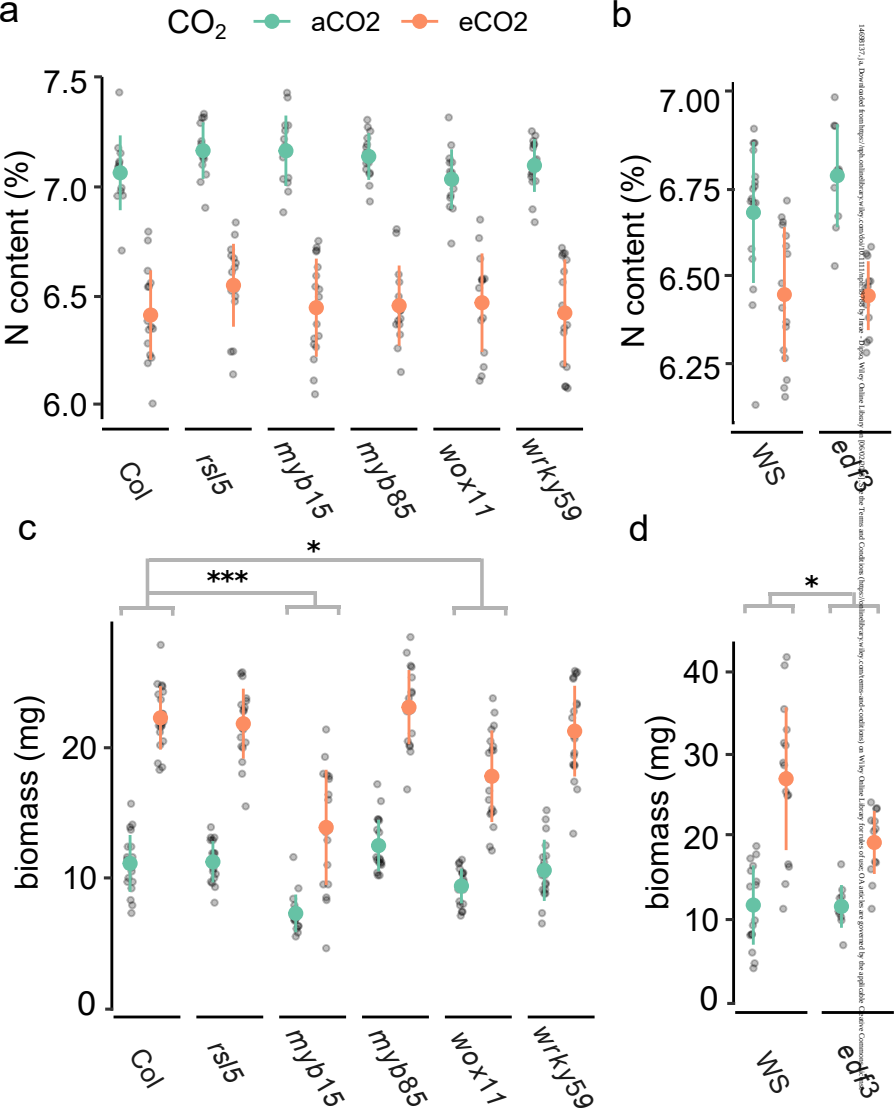




d

Community membership 1 2 3 4 5 6 7 8

	label	community	degree	rank
AT4G22680	MYB85	1	69	1
AT3G23250	MYB15	1	65	2
AT3G03660	WOX11	1	57	3
AT3G25730	EDF3	1	54	4
AT1G72650	TRFL6	2	40	5
AT3G19580	ZF2	1	38	6
mean_AT2G21900−AT3G46600	mean_WRKY59−AT3G46600	1	37	7
AT5G43175	RSL5	1	36	8
AT1G58100	TCP8	1	35	9
AT1G34670	MYB93	4	27	10
AT2G29060	AT2G29060	2	27	10
AT4G11880	AGL14	3	27	10


a
CO2
aCO2
eCO2
N content (%)
7.5
7.0
6.5
6.0
Col
rsl5
myb15
myb85
wox11
wrky59
b
N content (%)
7.00
6.75
6.50
6.25
WS
edf3
c
*
***
biomass (mg)
20
10
0
Col
rsl5
myb15
myb85
wox11
wrky59
d
*
biomass (mg)
40
30
20
10
0
WS
edf3