Homoploid F1 hybrids and segmental allotetraploids of rice subspecies are similarly more tolerant to N-deficiency than are parental lines

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Highlight

This work revealed that the merger of two divergent genomes of subspecies *japonica* and *indica* by hybridization at homoploid level or allopolyploidy can bestow higher nitrogen use efficiency in rice.

Abstract

Whether merger of two divergent genomes by hybridization at the homoploid level or coupled with WGD (allopolyploidy) can bestow plants better tolerance to stress conditions remains understudied. In this study, two diploid rice (*Oryza sativa* L.) subspecies, *japonica*, and *indica*, their reciprocal F1 hybrids and segmental allotetraploids were compared for phenotypic performance and gene expression under normal and nitrogen (N)-deficient conditions. We found that F1 hybrids and tetraploids showed higher tolerance at similar levels than did either parent. In parallel, total expression levels of 18 relevant functional genes were less perturbed by nitrogen deficiency in F1 hybrids and tetraploids than in the parents. This is consistent with stable intrinsic partitioning of allelic/homoeologous expression defined by parental legacy in the homoploid F1 hybrids/tetraploids between the two conditions. Our results suggest that genetic additivity at both the homoploid level or allopolyploidy may lead to similar beneficial phenotypic responses to nitrogen stress compared with their parents. The lack of synergistic responses to nitrogen limitation concomitant with WGD, relative to that exhibited by F1 hybrids, adds new empirical evidence in support of the emerging notion that hybridization by itself may play a significant role in plant adaptive evolution in times of stress.

Keywords: hybridization, allopolyploidy, nitrogen (N)-deficient stress, gene expression, allele/homeologue partitioning, robustness, population

Introduction

Hybridization is increasingly recognized as common in all organismal lineages, especially in plants. It is estimated that at least 25% of plants have undergone hybridization and introgressive hybridization between related species (Mallet, 2005; Abbott *et al.*, 2013). The combination of divergent genomes within a common hybrid nuclear/cytoplasm can lead to immediate as well as prolonged genetic and epigenetic instabilities with profound phenotypic consequence and evolutionary relevance (Shen *et al.*, 2005; Doyle *et al.*, 2008; Hegarty and Hiscock, 2008; Soltis and Soltis, 2009; Tang *et al.*, 2009; Chen *et al.*, 2010; Jiang *et al.*, 2011; Abbott and Rieseberg, 2012; Abbott *et al.*, 2013; Xu *et al.*, 2014; Cheng *et al.*, 2015). Hybrid vigor or heterosis is often an immediate phenotypic manifestation in interspecific hybrids as in intraspecific hybrids (e.g., F1 hybrids of maize inbred lines) (Birchler *et al.*, 2006; Chen, 2010). In addition, hybridization itself may lead to new lineages, as noted in the many reported cases of homoploid hybrid speciation (Mallet, 2007; Feliner *et al.*, 2017; Schumer *et al.*, 2018; Taylor and Larson, 2019; Feliner *et al.*, 2020).

Interspecific hybridization includes two major forms, one is at the homoploid level, i.e., maintaining the same ploidy level of parental species, and the other is coupled with whole genome duplication (WGD), i.e., allopolyploidy. Allopolyploidization occurs more frequently than homoploid hybridization in plants, suggesting possible evolutionary advantages of allopolyploids over homoploid hybrids in the plant kingdom (Jiao *et al.*, 2011; Wendel, 2015; Van de Peer *et al.*, 2017; Huang *et al.*, 2018; Baniaga *et al.*, 2020). Indeed, an apparent advantage of allopolyploidy over homoploid hybrid is to overcome sterility that occurs in many (though not all) homoploid interspecific hybrids. Another major advantage of allopolyploids over homoploid hybrids is the potential additive or synergistic beneficial effects of hybridization and WGD; however, few studies directly compare qualitatively isogenic homoploid hybrids vs. allopolyploids (Fort *et al.*, 2016). As a matter of fact, allopolyploids are not without apparent disadvantages compared with homoploid hybrids on condition both are fertile. A major drawback of allopolyploidy, which contrasts with homoploid hybrids, concerns evolvability, i.e., the capacity or potentiality to generate heritable adaptive phenotypic variations (Mallet, 2007; Runemark *et al.*, 2019). This is largely due to the dual effects of genome stabilization in newly formed allopolyploids: to ensure genome stability and hence evolutionary persistence on the one hand, and to constrain recombination between homoeologous chromosomes on the other (Alix *et al.*, 2017). However, recent studies have shown that in some cases these conflicting properties of allopolyploidy can be resolved where homoeologous recombination can occur to variable extents, which do not necessarily compromise genome stability (Ozkan *et al.*, 2001; Chester *et al.*, 2012; Mason and Wendel, 2020). In light of these findings, an interesting question to ask is: when both homoploids and allopolyploids can be generated from the same pair of parental species, to what extent are they different in terms of phenotypic performance, and whether the differential performance (if exists) is environmental context-dependent and relevant to adaptation.

Nitrogen (N) as an integral element in the synthesis of amino acids, chlorophyll, phytohormones and many other organic compounds is essential for normal plant growth and development (Xu et al., 2012; Leghari *et al.*, 2016). As the most important fertilizer, N has dramatically boosted agricultural production (Qiao *et al.*, 2012; Hawkesford, 2014). However, excessive use of N fertilizers in agriculture has multiple negative consequences for ecosystems. N loss from volatilization and denitrification is frequent and recurrent, which inevitably drives more excessive use of N fertilizer in order to maintain the same or further

increase of agricultural productions, leading to a vicious circle (Shi *et al.*, 2009). Therefore, generating more N-deficiency tolerant crops is desirable for both agricultural production and environmental protection. Various pathways relating to plant growth and development are involved in response to N-deficiency, of which N metabolism and chlorophyll biosynthesis are major pathways because the final product glutamine of the former is the initial substrate of the latter (Masuda and Fujita, 2008). Thus, modulating expression or functional innovation of key genes involved in these pathways are promising strategies in generating crops that are more tolerant to N-deficient condition and higher N fertilizer use efficiency.

Asian cultivated rice (*Oryza sativa* L.) comprises of two subspecies, *japonica* and *indica*, and is a staple food crop for over half of the world population. The two subspecies are substantially differentiated genome-wide as a result of long-term selection under cultivation (Sang *et al.*, 2007). As a result, they are adapted to contrasting Agro-ecological habitats ranging from tropical to temperate zones (Kovach *et al.*, 2007; Huang *et al.*, 2012). For example, *indica* rice has higher N use efficiency (NUE) than *japonica*, while *japonica* has better tolerance to cold stress than *indica* (Jiang *et al.*, 2004; Ma *et al.*, 2015; Gao *et al.*, 2019). Therefore, reciprocal F1 hybrids and segmental allotetraploids constructed from these two rice subspecies represents an ideal system to address the question of whether hybridization alone or hybridization combined with WGD differentially respond to N limitation, as well as the molecular underpinnings of the effects.

In this study, we used reciprocal F1 hybrids and segmental allotetraploids constructed by hybridization followed by colchicine-mediated chromosome doubling between two standard laboratory genotypes, Nipponbare and 93-11, representing the two rice subspecies, *japonica* and *indica*, respectively, along with their parents, to assess the effects of hybridization alone and hybridization coupled with WGD on responses to a moderate N-deficient stress. We quantified morphological and physiological phenotypes and analyzed transcriptional expression of a set of key genes involved in N metabolism, chlorophyll biosynthesis and growth pathways under N-deficient vs. normal condition. Our results indicate that hybridization alone and hybridization coupled with WGD manifest similar degrees of enhanced tolerance to N-deficiency compared with both pure-line parents. Our results suggest that the enhanced abiotic stress tolerance by hybrids/tetraploids might be rooted in their more robust genome regulation under adverse conditions.

Materials and methods

Plant Materials

Reciprocal F1 hybrids (9N and N9) were made by crossing two diploid cultivars, Nipponbare and 93-11, representing the two subspecies, *japonica* and *indica*, respectively, of Asian rice (*Oryza sativa* L.). Tetraploids (99NN and NN99) were generated by colchicine treatment on tillers of the reciprocal F1 hybrids, and were then selfed consecutively for five generations. Authenticity of hybrids and tetraploids was confirmed by chromosome counting and Oligo-FISH (Xu *et al.*, 2014; Wu *et al.*, 2020), and for tetraploids, only euploid individuals were used. Seeds of both parents, their reciprocal F1 hybrids and the 5th-selfed generation (S5)-old reciprocal tetraploids (each direction originated from a single S0 tetraploid individual)

were germinated at 28°C in dark for 3 days. Germinated seeds were transferred into seeding trays floating on a culture solution (Yoshida, 1976) with slow rotation-mediated aeration under a 16-h light/8-h darkness regime in a standard greenhouse. When the seedlings reached the three-leaf stage, deficient nitrogen (N) treatment was performed.

N-deficient treatment

For each set of materials, two groups were separated for either control or N-deficient treatment. The concentration of NH_4NO_3 in the N-deficient nutrient solution was 0.048 mM, which was 1/30 of the amount supplied to the control. The N-deficient treatment lasted for three weeks and ended when distinct phenotypes were apparent. When the N-deficient treatment was completed, plants were harvested for measurement of biomass, chlorophyll content and gene expression assay. Tissues used for gene expression assay were immediately frozen in liquid nitrogen and stored at a -80°C freezer.

Measurement of biomass

The whole plant, shoot and root fresh weights were measured respectively from nine to 12 seedlings for each genotype under both normal and N-deficient conditions, respectively, when the N-deficient treatment was done.

Measurement of photosynthetic pigments

The 2nd and 3rd leaves of another three to nine individuals were collected for chlorophyll measurement, which included Chl a, Chl b and Chl a/b. The sampled tissues were the same part of the leaves as used for RNA extraction. Whole chlorophyll measurement procedures are as reported (Zhu *et al.*, 1993) and expressed in $mg \bullet g^{-1}$ FW. The data analyses were tested for statistical significance by Student's t-test.

RNA extraction, cDNA preparation and quantitative (q)RT-PCR amplification

Total RNAs were extracted by TRIzol (Invitrogen) from the leaves (the same part with chlorophyll assay) and roots for each individual plant. Quality and quantity of RNA samples were assessed by the Agilent 2100 Bioanalyzaer (Agilent Technologies, Waldbronn, Germany). Equal quantities of RNAs of the parental individuals (three plants from each parent) were mixed in a pairwise manner to construct three independent *in vitro* "hybrids", as reported (Buggs *et al.*, 2011). First-strand DNA was synthesized from ~500 ng RNA using one-step genomic (g) DNA Removal & cDNA Synthesis kit (QIANGEN) following the manufacturer's protocol. The total expression of each gene was quantified using a real-time reverse transcriptase (RT) PCR amplification kit (SYBR qPCR mix from TOYOBO) in randomly chosen samples, in which the gene-specific primers were designed with the conserved CDS region (Supplementary Table S1). Real-time quantitative (q)RT-PCR was performed under the amplification conditions as follows: 95°C for 1 min, followed by 40 cycles of 2-step cycle of 95°C for 5 s and 60°C for 1 min. Amplification of the rice *UBQ5* (LOC_Os01g22490) and *GAPDH* (LOC_Os08g03290) genes were used as internal quantitative controls (Jain *et al.*, 2006, Wang *et al.*, 2010). The relative expression of the target genes was calculated using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen 2001).

Gene selection and SNPs identification between parental orthologues for Sequenom assay

A set of 18 genes involved in four major pathways in rice growth and development were chosen for this study according to the database of KEGG (http://www.genome.jp/kegg/kegg1.html), which includes chlorophyll synthesis (Chl, eight genes), nitrogen metabolism (N, five genes), growth-rhythm (R, two genes), DNA mismatch repair (DR, MR, two genes) and a housekeeping gene *Ubi-1*. Single nucleotide polymorphisms (SNPs) for distinguishing parental (Nipponbare and 93-11) orthologues and Sequenom MassARRAY platform based genotyping assay were carried out as we previously reported (Sun *et al.*, 2017). Over 2000 data points in total for all individuals were obtained for the 18 assayed genes for each of the two tissues (leaf and root).

Data processing and statistical analysis

Expression and gene content data retrieved from the MassARRAY were filtered according to previously reported criteria (Chaudhary *et al.*, 2009). The relative expression of orthologues/alleles/homoeologues based on MassARRAY-based Sequnom platform was calculated as the proportion of Nip [Nip% = Nip / (Nip + 9311)] and used for subsequent analyses, in accordance with our previous study (Sun *et al.*, 2017). All statistical analyses and graphical illustrations were performed in the R and Python computing environments (version 3.1.3).

Results

Both homoploid F1 hybrids and tetraploids showed better tolerance to N-deficiency than their parental lines

Plant populations used in this study have been previously described in detail (Sun *et al.*, 2017). Specifically, the reciprocal rice tetraploids at the 5th selfed (S5) generation (designated as 99NN and NN99, 2n = 4x = 48), homoploid F1 hybrids (9N and N9, 2n = 2x = 24) and the diploid parents *Oryza sativa* ssp. *japonica* (cv. Nipponbare, 2n = 2x =24) and *indica* (cv. 93-11, 2n = 2x = 24) were compared under nitrogen (N)-deficiency relative to normal condition. Identities of all F1 hybrids and tetraploids (euploidy) used in this study were identified by chromosome counting and confirmed by whole-genome resequencing (Wu *et al.*, 2020). The N-deficient treatment we chose is a moderate abiotic stress that might be frequently experienced by crops grown in less fertile fields or with inefficient/insufficient N fertilizer input. Given that *indica* rice is known to have better nitric N use efficiency (NUE) than *japonica* rice (Gao *et al.*, 2019), we sought to investigate how the reciprocal inter-subspecific homoploid F1 hybrids and tetraploids would respond to the N-deficient condition. We assayed six phenotypic and physiological traits: whole-plant fresh weight (WFW), shoot fresh weight (SFW), root fresh weight (RFW), chlorophyll (Chl) a content, Chl b content, and Chl a/b ratio (a parameter for photosynthetic efficiency) (Penuelas *et al.*, 1993; Boegh *et al.*, 2002) three weeks after the N-deficient treatment.

In general, both reciprocal homoploid F1 hybrids (N9 and 9N) showed clear growth vigor, especially in roots, compared with both the diploid parents and the calculated mid-parent values (MPVs) under normal condition. Between the reciprocals, we noted that N9 was slightly but consistently larger than 9N in overall plant status at the final seedling stage

under both conditions, although the difference was not statistically significant (ANOVA followed by Tukey HSD tests, Fig. 1). Unexpectedly, both reciprocal S5 tetraploids showed overall similar sizes of aerial parts to their diploid parents but significantly decreased sizes in roots, although there was variability among individuals within each tetraploid population (Fig. 1A). This suggests there exists an overall antagonistic effect of hybridization and WGD with respect to heterosis manifestation at the seedling-stage, and which was not influenced by the contrasting N-condition. After 3 weeks under the N-deficient condition, the most obvious and general phenotypic response across all treated rice populations was significant overall growth retardation and leaf chlorosis compared with the corresponding populations under normal condition (ANOVA followed by Tukey HSD tests, Fig. 1), suggesting our treatment was in effect and deficiency in N affected the growth and development of all rice plants.

Quantification of the six measured phenotypic traits not only supported the overall phenotypic features, described above, but also revealed differential responses to N-deficiency among the six rice populations (Fig. 1B). For each trait, we calculated the mean difference between the two growing conditions (N-deficient minus N-normal) to quantify the degree of response of each population to N-deficiency. We observed the following: (*i*) 93-11 has better tolerance than NPB, as reflected in significant lower reductions of WFW, SFW, contents of both Chl a and b, Chl a/Chl b ratios, and a greater increase of RFW, in 93-11 than in NPB ($P < 0.05$; ANOVA followed by Tukey HSD tests) - this is consistent with previous findings (Li *et al*., 2006; Gao *et al.*, 2019) that *indica* rice has better NUE than *japonica* rice (Fig. 1B); (*ii*) both reciprocal F1 hybrid and reciprocal S5 tetraploid populations showed significantly better tolerance to N-deficiency compared with the MPV in WFW trait, which were -0.35 to -1.27 in the F1 hybrids and tetraploids, respectively, vs. -1.44 in MPV, while they were positively transgressive compared with both diploid parents in the SFW trait (-0.62 to -0.81 in the F1 hybrids and tetraploids vs. -1.15 and -2.14 in NPB and 93-11, respectively) (Fig. 1B); (*iii*) differences in response to N-deficiency between the reciprocals were detected in both F1 hybrid and S5 tetraploid populations, with populations having 93-11 as the maternal parent (9N and 99NN) showing better N-deficiency tolerance than populations having NPB as the maternal parent (N9 and NN99) in traits of WFW, SFW, and contents of both Chl a and b (Fig. 1B); (*iv*) in general, N-deficiency induced root elongation with increased RFW, however, N9 F1 hybrids showed decreased RFW in response to N-deficiency and this feature was also inherited to the NN99 tetraploid population, suggesting a heritable role of maternal effect and/or cytonuclear interaction under N-deficient condition (Fig. 1B).

Extensive phenotypic diversification is a prominent feature of these segmental allotetraploid populations, primarily due to the rampant occurrence of homeologous recombinations or homoeologous exchanges, dubbed HEs (Sun *et al.*, 2017; Wu *et al.*, 2020). We thus asked if the phenotypic ranges of the six measured traits in the tetraploids could be altered under N-deficiency relative to those under N-normal condition. Three parameters that reflect differences among individuals within a population, including standard deviation (SD), range (R, maximum value minus minimum value) and coefficient of variation (CV), were calculated for each trait (Supplementary Dataset S1). Results showed that for most traits (WFW, SFW, RFW and Chl a content) the tetraploids manifested dramatically larger within-population phenotypic variations than the diploid parental and F1 hybrid populations under both normal and N-deficient conditions ($P < 0.05$, by F-test) but no

significant difference between the two conditions ($P > 0.05$, Tests for the Equality, Marwick and Krishnamoorthy 2019, Supplementary Dataset S1).

Both homoploid F1 hybrids and tetraploids showed more robust expression of critical genes involved in nitrogen metabolism-related pathways

To gain insights into the molecular basis underlying the tolerance differences in biomass and Chl contents among different populations under N-deficiency, transcript-level expression of 18 critical genes known to be involved in four plant growth and development-related pathways were analyzed by real-time qRT-PCR in both leaf and root tissues with a housekeeping gene *Ubi-1* as an internal control. Selected genes included those involved in chlorophyll synthesis (Chl, eight genes), nitrogen metabolism (N, five genes), growth-rhythm (R, two genes) and DNA mismatch repair (DR, MR, two genes), For each gene, the mid-parent values (MPVs) of expression level were calculated under both normal and N-deficient conditions and used as references to quantify gene expression levels of the F1 hybrids and S5 tetraploids under each condition. Because the tetraploid populations contain genomically heterogeneous individuals (Sun *et al.*, 2017; Wu *et al.*, 2020), 12 randomly chosen individuals of each tetraploid population were used to investigate gene expression levels while three individuals were employed for each of the genetically homogeneous parental and F1 hybrid populations.

Under normal condition, expression level differences for many of the 18 studied genes were observed between the two diploid parents (NPB and 93-11) in both tissues but especially in roots (Fig. 2A), suggesting there was intrinsic expression differentiation between the two rice subspecies, and for some genes the differentiation is tissue-specific. Specifically, there were seven and eight significantly up- and down-regulated genes in 93-11 relative to NPB, respectively, in leaves, while16 of 17 differentially expressed genes showed significantly lower levels in 93-11 than in NPB, in roots (Supplementary Table S2). In addition, a majority of the 18 analyzed genes showed nonadditive expression in both F1 hybrids and S5 tetraploids relative to the corresponding MPVs in both leaves and roots under normal condition (Fig. 2A and Supplementary Table S3), suggesting a strong hybridization-associated “transcriptome shock” (Hegarty *et al.*, 2006). Specifically, compared with the MPVs, 13 (72.22%) and 12 (66.67%) of the 18 genes showed significantly lower expression in 9N and N9, respectively, in leaves; and 10 (55.56%) and 11(61.11%) of the 18 genes showed significantly lower expression in 9N and N9, respectively, in roots. Likewise, 99NN tetraploids had similar higher- and lower-expressed genes compared with the MPVs (four higher- and five lower-expressed genes) in leaves under normal condition, while mainly higher-expressed genes in NN99 tetraploids (all of the nine nonadditive expression genes were expressed at higher levels relative MPV); in roots, however, both 99NN and NN99 tetraploids had more genes showing significantly lower expression levels relative to MPVs (Supplementary Table S3), which may partially explain why the root size and fresh weight of both tetraploid populations were significantly smaller and less than those of parents and F1 hybrids (Fig. 1).

Under N-deficient condition, except for the significantly up-regulated expression (fold change > 2) of nine genes in root of 93-11, the remaining populations showed a general down-regulated expression for most genes studied in both leaves and roots (including leaf of 93-11 population) when compared with their corresponding populations under normal condition; however, the extent of downregulation differed substantially across the

populations (Fig. 2 and Supplementary Fig. S1). We quantified the change of gene expression under N-deficient vs. normal condition by dividing the gene expression levels (N-deficient vs. normal) (N-deficient/normal) with Log2 transformation (Fig. 2B). We found that NPB showed the most down-regulation in all 18 tested genes in roots, while 93-11 showed up-regulation of nine genes and down-regulation of only three genes with a lesser extent than those in NPB. Compared with the corresponding parental MPVs, the reciprocal F1 hybrids and 99NN tetraploids all showed lower extents of gene downregulation or even upregulation in most tested genes (13/18); in contrast, NN99 tetraploids showed similar and greater down-regulations in four and nine genes, respectively, compared with MPVs, suggesting clear positive maternal (NPB) effect. In addition, although the 18 tested genes did not show consistent expression changing trend in leaf when comparing all of the tested populations in response to N-deficient condition, both reciprocal F1 hybrids and tetraploids showed lower extents of downregulation or even upregulation compared with the parental MPVs in most of the studied genes (Fig. 2 and Supplementary Fig. S1). This suggests that the differences in the extent of downregulation of critical genes under N-deficient vs. normal condition might be an important determinant that contributed to the tolerance differences to N-deficiency across the populations, with both F1 hybrids and tetraploids showing higher gene expression robustness than the parents.

The relative allelic/homoeologous expression levels are highly stable in homoploid F1 hybrids/tetraploids in response to N-deficiency

To further explore whether the lower level of down-regulation in gene expression in the F1 hybrids and tetraploids under N-deficient vs. normal condition were due to differential or equal stability of alleles/homoeologues, we quantified the relative allelic and homoeologous expression for the 18 assayed genes at the population level. The diagnostic single nucleotide polymorphisms (SNPs) of the 18 genes between the two parental genomes enabled this analysis by the MassARRAY-based Sequenom platform in both tissues, leaf and root (Zhao *et al.*, 2018). Multiple individuals of the reciprocal F1 hybrids and tetraploids were compared to 10 independently constructed parental mixes (MPVs). The relative expression levels of the 18 gene transcripts from the parental alleles/homoeologs in the two tissues of each hybrid/tetraploid individual were computed and summarized in heatmaps (Fig. 3). Conspicuous changes were observed in parental orthologous expression in root, where the NPB orthologues were down-regulated while the 93-11 orthologues were greatly up-regulated under N-deficient vs. normal condition, consistent with the qRT–PCR results of total expression levels, described above. Opposing effects on allelic and homoeologous expression in F1 hybrids and tetraploids were observed where intrinsic parental difference was strongly attenuated by hybridization (mainly due to upregulation of 9311 alleles) but augmented by WGD (due both to upregulation of NPB homoeologous and down-regulation of 9311 homoeologues) (Fig. 3). No discernible difference on overall allelic and homoeologous expression patterns was observed between the reciprocal F1 hybrids (equal allelic expression in almost all tested genes) and between the reciprocal tetraploids (all showed dramatic homoeologous expression differentiation in most genes across the individuals) for both leaf and root under N-deficient vs. normal condition (Fig. 3). Notably, however, three genes (N-9, MR-1 and UBI-1) showed a highly consist homoeologous

expression pattern across individuals within the NN99 in both leaf and root and under both normal and N-deficient conditions (Fig. 3).

To further investigate the allelic and homoeologous expression in both the reciprocal F1 hybrids and S5 tetraploids under N-deficient vs. normal condition, three additional analyses were conducted: (*i*) principal component analysis (PCA) for the 18 genes of all populations together, (*ii*) standard deviation of each relative gene expression among individuals within each population, and (*iii*) distribution of relative gene expression by density plot to test for possible directional shifting in response to N-deficient in each population. PCA results showed that the MPVs, F1 hybrids and S5 tetraploids were clustered into distinct groups in both leaves and roots (Fig. 4A). The data points from the reciprocal F1 hybrid populations of both tissues were highly condensed under normal condition; in contrast, the relative homoeologous expression of the 18 genes in the tetraploid populations were generally clustered into two groups, i.e., in line with the direction of the cross (Fig. 4A). Thus, homoeologous expression divergence within each of the tetraploid populations were much greater than both orthologous expression divergence between parents and allelic expression divergence in the F1 hybrids (Fig. 4A). The N-deficient treatment did not cause changes in the clustering patterns for both F1 hybrids and S5 tetraploids in both tissues, but it did for the parental populations (reflected by MPVs with changes greater in root than in leaf (Fig. 4A). Interestingly, the overall allelic expression status in the parental populations was more similar to that in the reciprocal F1 hybrid populations of both tissues under N-deficient condition (many genes showed 93-11 allelic preferential expression) compared with normal condition (Fig. 2 and 4A).

To quantify the extents of orthologous/allelic/homoeologous expression divergence in the MPVs, and the F1 hybrid and tetraploid populations, we calculated the standard deviation (SD) among individuals within a given population for each of the 18 genes (Fig 4B). The overall divergence level of relative homoeologous expression in the reciprocal tetraploid populations was significantly higher than both parental orthologues and F1 hybrid alleles under both normal and N-deficient conditions (Fig. 4B). N-deficient treatment increased the data range of MPVs compared with the normal condition, but did not for the F1 hybrids and tetraploids (Fig. 4B). Allelic or homoeologous expression bias refers to the preferential expression towards one parental copy or only one copy expressed in a F1 hybrid or allopolyploid. The density distribution curves of the two parental populations in leaf shifted from slight 93-11 orthologous expression bias under normal condition to NPB bias under N-deficient condition (Fig. 4C). The opposite trend was observed in roots which shifted from biased NPB orthologous gene expression to that of 93-11 from normal to N-deficient conditions (Fig. 4C). Notably, no bias shifting of allelic or homoeologous expression was found in both F1 hybrids and tetraploids in either tissue (Fig. 4C). Given that relative homoeologous expression ratio was highly DNA copy number dependent (in > 90% tested genes and tetraploids) in this rice tetraploid system under normal condition, and at least 60% genomic regions were heterozygous at the S5 generation according to our previous studies (Sun *et al.*, 2017; Wu *et al.*, 2020), it is likely that the homoeologous expression robustness in the reciprocal tetraploid populations under N-deficient vs. normal condition observed in this study was DNA copy number independent. These results indicate that there was no

alteration in expression partition of alleles or homoeologs in the F1 hybrids or tetraploid populations in response to the N-deficient treatment. This suggests that, in contrast to parental orthologues, the allelic/homoeologous partition in F1 hybrids and tetraploids is highly robust, i.e., insensitive to the N-deficient treatment. This is consistent with the significantly less down-regulation of total gene expression levels in the F1 hybrids and tetraploids than orthologues in the parental lines under N-deficiency.

Discussion

Episodes of interspecific hybridization and polyploidization (or whole genome duplication, WGD) are abundant and recurrent in the evolutionary histories of many eukaryotic lineages, and more often than not, both events have occurred concomitantly, i.e., allopolyploidization, especially in plants. Apparent advantages are associated with allopolyploidization compared with hybridization and WGD alone. Relative to homoploid hybridization, allopolyploidy may confer instant diploid-like meiosis (due to availability of an identical partner for each chromosome) and hence high fertility in otherwise complete or highly sterile homoploid F1 hybrids, while compared with WGD alone (autopolyploidy), allopolyploidy has fixed heterosis and also more stable meiosis.

A conceptually useful goal is to deconvolute the biological effects of allopolyploidization into effects from two events, genome merger *per se* and WGD (Van de Peer *et al.*, 2017). However, most naturally formed homoploid hybrids and allopolyploids are not amenable to this type of analysis because only in very rare cases has the same pair of parental species given rise to both types of descendant species, and even so, both parental and derived species have undergone evolutionary changes often in distinct ecological settings. In addition, naturally formed F1 hybrids are ephemeral unless they can immediately convert to asexual reproduction, and which is rare too. However, there are exceptions, and which mainly concern extremely young natural hybrids and/or allopolyploids. For example, parental species, F1 hybrids and their derived allopolyploids are all extant in *Spartina* (Ainouche *et al.*, 2004). Likewise, in several other plant systems, including *Tragopogon*, *Senecio* (Hegarty *et al*., 2006) and *Mimulus* (Edger *et al*., 2017), both parental species pairs and allopolyploids are still extant, and hence both F1 hybrids and early generation allopolyploids can be resynthesized for comparison. These systems and several other synthetic systems (such as in *Arabidopsis*) have been used to study the separate effects of hybridization and WGD on gene expression, but highly variable results have been obtained (Soltis *et al.*, 2016); these ranged from large effects of hybridization but negligible effects of WGD in *Arabidopsis* (Wang *et al.*, 2006; Chen, 2007), similar effects of both hybridization and WGD in cotton (Flagel *et al.*, 2008), amelioration of hybridization-invoked transcriptomic shock by WGD in *Senecio* (Hegarty *et al.*, 2006), antagonistic effects of hybridization and WGD on tissue specific homeologous expression in *Tragopogon* (Buggs et al., 2011), variable effects of hybridization and WGD on different aspects of gene expression in *Spartina* (Chelaifa *et al.*, 2010), to largely leveling out the effects of hybridization by WGD in wheat (Qin *et al*., 2021).

To systemically dissect the separate effects of hybridization and WGD, synthetic plant materials are more suited because by using pure-line parental species, the derived homoploid F1 hybrids and polyploids are “qualitatively isogenic”, as they share identical parentage and differ only in ploidy levels. Thus, confounding effects of pre-exiting genetic

variations, which might be differentially partitioned among the F1 hybrids and segregating in the derived tetraploids, can be ruled out. Based on this line of thinking, we have constructed a rice system that involves pure-line parents representing the two subspecies (*indica* and *japonica*), their reciprocal homoploid F1 hybrids and derived segmental allotetraploids (Xu *et al.*, 2014). Our previous studies using this system have (*i*) revealed distinct and in some respects opposing effects of hybridization and WGD on gene expression in the hybrids and the immediate generation (S1) polyploids (Xu *et al.*, 2014); (*ii*) established relationships between relative homeolog copy number (resulted from homoeologous exchange, or HE) and homoeologous expression partitioning and phenotypes in more advanced generations of the tetraploids (Sun *et al.*, 2017); (*iii*) uncovered distinct and often exacerbating effects of hybridization, WGD and HE on DNA methylation repatterning (Li *et al.*, 2019), and (*iv*) depicted genome-wide landscape of HEs, delineated major genetic constraints of HEs, as well as assessed the fitness consequences of HEs (Wu *et al.*, 2020). However, all these prior studies using this system have been on plants that are grown under normal condition, rendering the roles of hybridization and WGD in response to stress conditions uninvestigated, which however is highly relevant to evolution, environmental adaptation and agricultural utility.

Here, we extend our previous studies by conducting a nitrogen (N)-deficiency stress experiment in this rice system. Given the clear significance of generating higher N-deficiency tolerant and N use efficient crops to meet the due requirements of maintaining agricultural production and protecting environment, knowledge gained with respect to the effects of hybridization and WGD on N-deficiency stress is of practical significance. We show that both homoploid F1 hybrids and tetraploids manifested significantly enhanced tolerance to the N-deficiency stress than their pure-line parents on all measured morph-physiological traits, but there is no evidence for an additive or synergistic effect by coupling hybridization and WGD. Moreover, we show that under normal condition, superimposing WGD on the F1 hybrids incurred an antagonistic effect with respect to heterosis manifestation of some traits at the seedling-stage, although both F1 hybrids and tetraploids are overall superior to both pure-line parents. It is conceivable that this might be due to the adverse effects of having doubled genomes with increased DNA contents and altered physiology in general and cellular metabolism in particular (Doyle and Coate, 2019). Notwithstanding, it should be noted that given the rampant HEs at genome-wide scale and their profound impacts on phenotypes in these rice tetraploids at the population level (Wu *et al.*, 2020) there is good reason to believe that outlier individuals and lines showing more adaptive phenotypes to a given stress could be obtained with progressive recombination across the generations, along with selection. In addition, considering the F1 homoploid hybrids may decrease overall N-deficiency tolerance at the population level due to breakdown in fertility and segregation in all traits, while the tetraploids would show more robust phenotypic stability, the tetraploids may have larger evolutionary potential if under natural settings and more agricultural utility under human care than the F1 homoploid hybrids.

In accordance with the better performance in morpho-physiological manifestations by the F1 hybrids and tetraploids, compared with their pure-line parents, in response to the N-deficiency stress, we demonstrate that key genes involved in nitrogen metabolism, chlorophyll synthesis and growth-rhythm pathways showed more robust overall expression levels, i.e., less perturbed by the stress, in both leaves and roots of F1 hybrids and tetraploids than in both parents. Further, quantification of allelic/homoeologous partitioning in

the F1 hybrids and tetraploids indicated invariability between normal vs. N-deficient condition, indicating high stability of inherent parental expression partitioning. Stable inheritance of parental legacy in homoeologous expression has been reported previously. For example, it was found in coffee (a natural allotetraploid) that homoeologous partitioning was highly insensitive to cold and heat stresses although the tetraploids showed higher tolerance to the stresses than diploid parental species (Combes *et al.*, 2013). Further studies are needed to unravel the molecular mechanisms underlying more robust overall gene expression, stable allelic and homoeologous expression partitioning, and enhanced stress tolerance in homoploid F1 hybrids and allopolyploids relative to their pure-line parents.

Data Availability Statement

The data that support the findings of this study are openly available in “Dryad” at https://orcid.org/0000-0001-5507-7070.

Acknowledgements

This study was supported by the National Key Research and Development Program of China (grant no. 2016YFD0102003), the National Natural Science Foundation of China (grant no. 31901076), the China Postdoctoral Science Foundation (grant no. 2019M651186) and the Fundamental Research Fund for Central Universities.

Author contributions

BL and DLW conceived and designed the study. YS, YW, YZW, SNW, XZ, ZDL, GL and JHL performed the laboratory experiments and analyses. YS, YW, BL, DLW, LG and JFW wrote the manuscript, with input from all authors. All authors read and approved the final manuscript.

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Figure legends

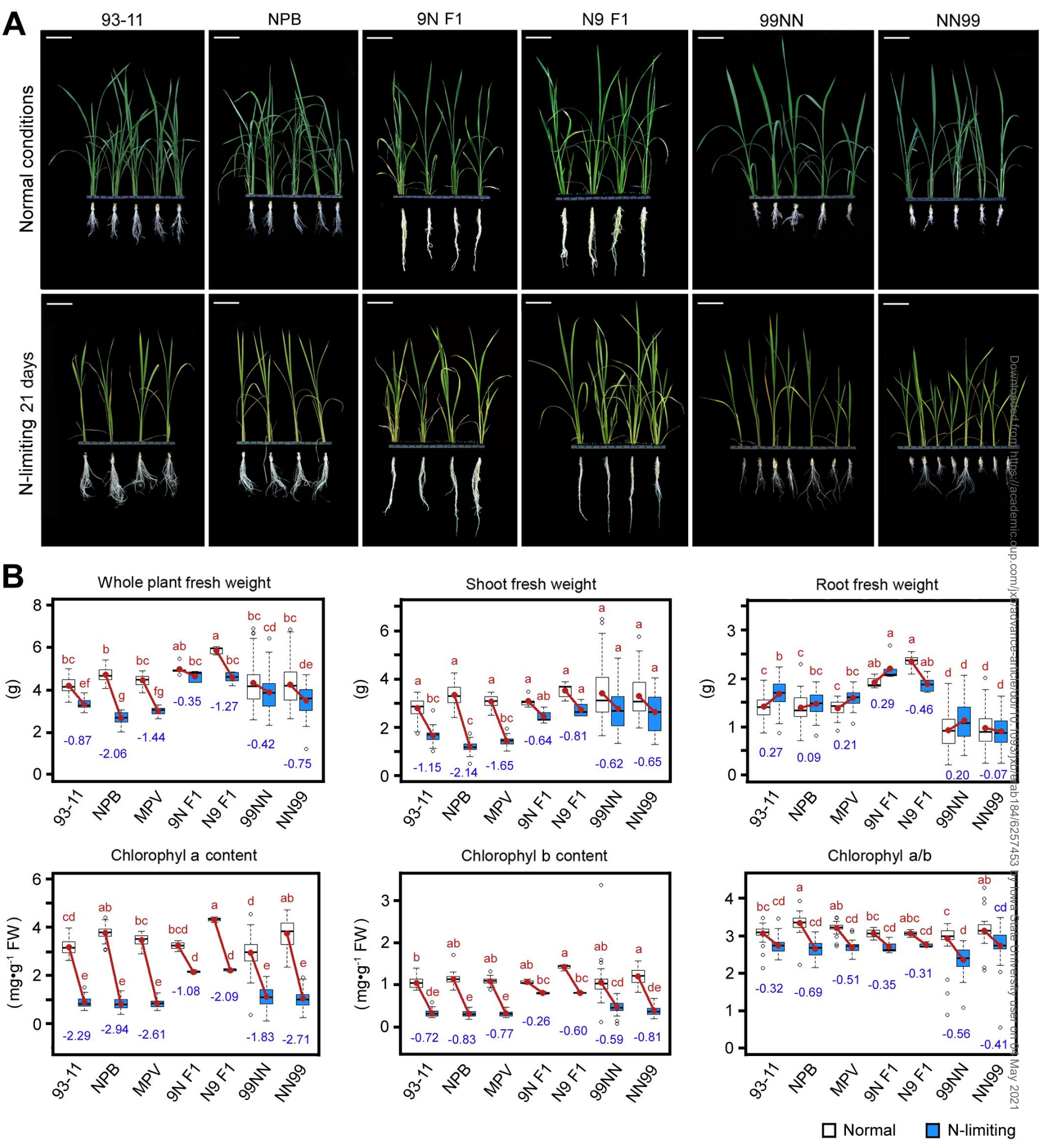
Fig. 1. Illustration and quantification of phenotypic traits of the diploid parents (NPB and 93-11), reciprocal F1 hybrids (N9 and 9N) and reciprocal S5 tetraploids (NN99 and 99NN) under both normal and N-deficient conditions. (A) Overall phenotypes after 21 days growing under normal or N-deficient condition. (B) Quantification of six phenotypic traits by boxplots. In (B), red letters above each box denote statistically different phenotypic distributions in each comparison of 14 plant groups (two conditions time seven rice populations) based on ANOVA followed by Tukey HSD tests, blue numbers below each box pair refer to the relevant data change between N-deficient and normal conditions (N-deficient condition minus normal condition).

Fig. 2. Total gene expression in leaf and root tissues of the diploid parents, the reciprocal hybrids and tetraploids under both normal and N-deficient conditions assessed by Real-time qRT-PCR. (A) Heatmaps illustrate the total transcript level of all studied 18 genes in all tested plant groups. For leaf and root, the expression level of each gene (row) in each individual (column) was quantified. For each gene, the original expression level of the first NPB individual of leaf tissue under normal condition (denoted with *) was used as a reference, to which the gene expression of all other individuals was computed. In the legend bar, $\log_2$-transformed changes of aforementioned relative gene expression in each individual were illustrated in a way that higher folds of expression increase and decrease are denoted in reddish purple and blue, respectively. (B) Histograms depicting the total gene expression change (up- or down- regulation) of selected four genes for each plant group under N-deficient vs. normal condition.

Fig. 3. Heatmaps illustrate the expression profile of orthologs, alleles and homeologs for all studied 18 genes in the diploid parents (93-11 and NPB), MPV, reciprocal F1 hybrid (9N and N9) and reciprocal S5 tetraploid (99NN and NN99) populations under normal and N-deficient conditions. The MPV population was constructed by mixing of equal amount of total RNAs extracted from independent individuals of the NPB and 93-11 diploid parents, and used as a referential group. Within each population, the relative expression status of each gene (row) in each individual (column) is denoted in a color-filled rectangle. As denoted in the legend bar, the relative expression proportion occupied by NPB allele is colored in increasing redness from right (dark blue) to left (dark red) to represent a higher expression of NPB alleles/homeologs in total expression of a given gene for a given individual. The number of individuals sampled in each population was shown in parenthesis.

Fig. 4. Orthologous, allelic and homeologous expression changes of the MPV, reciprocal F1 hybrid (9N and N9) and corresponding S5 tetraploid (99NN and NN99) populations in both leaf and root tissue to response N-deficient condition. (A) PCA plots showing orthologous, allelic and homeologous expression comparisons among the MPV, reciprocal F1 hybrids (9N and N9) and corresponding S5 tetraploids (99NN and NN99) and between normal and N-deficient conditions in both leaf and root tissues. A different colored circle as per the legend of the figure represents each plant group under either normal or N-deficient condition. (B) Boxplots of relative orthologous, allelic and homeologous expression in leaf and root tissues of all tested plant groups using standard deviation of gene expression among independent

individuals under both normal and N-deficient conditions. Each data point in the boxplot represents the standard deviation of each relative gene expression proportion (proportion of NPB orthologs, alleles and homoeologs) among individuals within the corresponding population. Each box was composed of 18 data points representing 18 genes. (C) Density distribution of orthologous, allelic and homoeolog expression in both leaf and root tissues of the MPV, reciprocal F1 hybrids (9N and N9) and corresponding S5 tetraploids (99NN and NN99) under normal and N-deficient conditions. X-axis represents the proportion of NPB orthologs, alleles and homoeologs.



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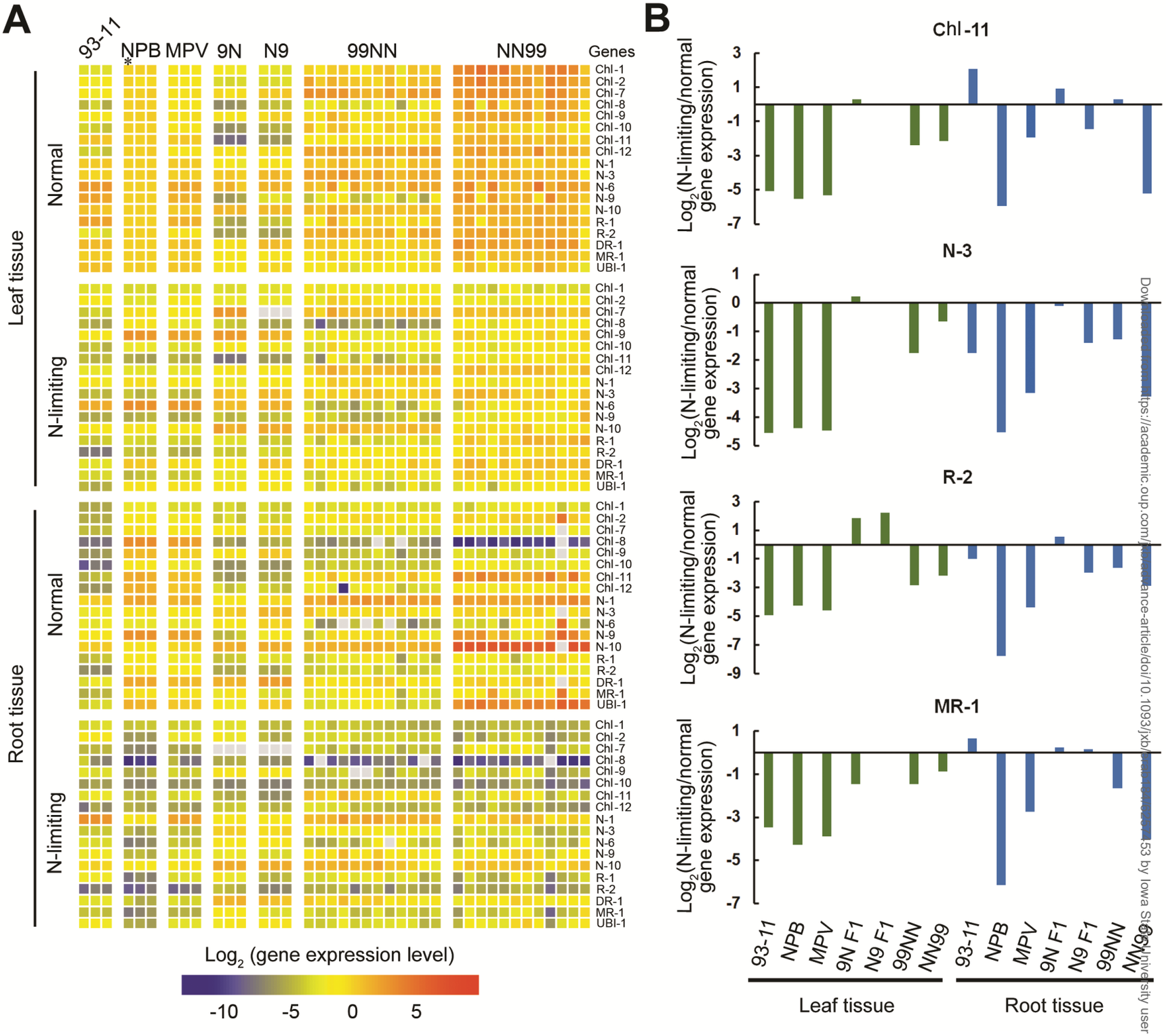

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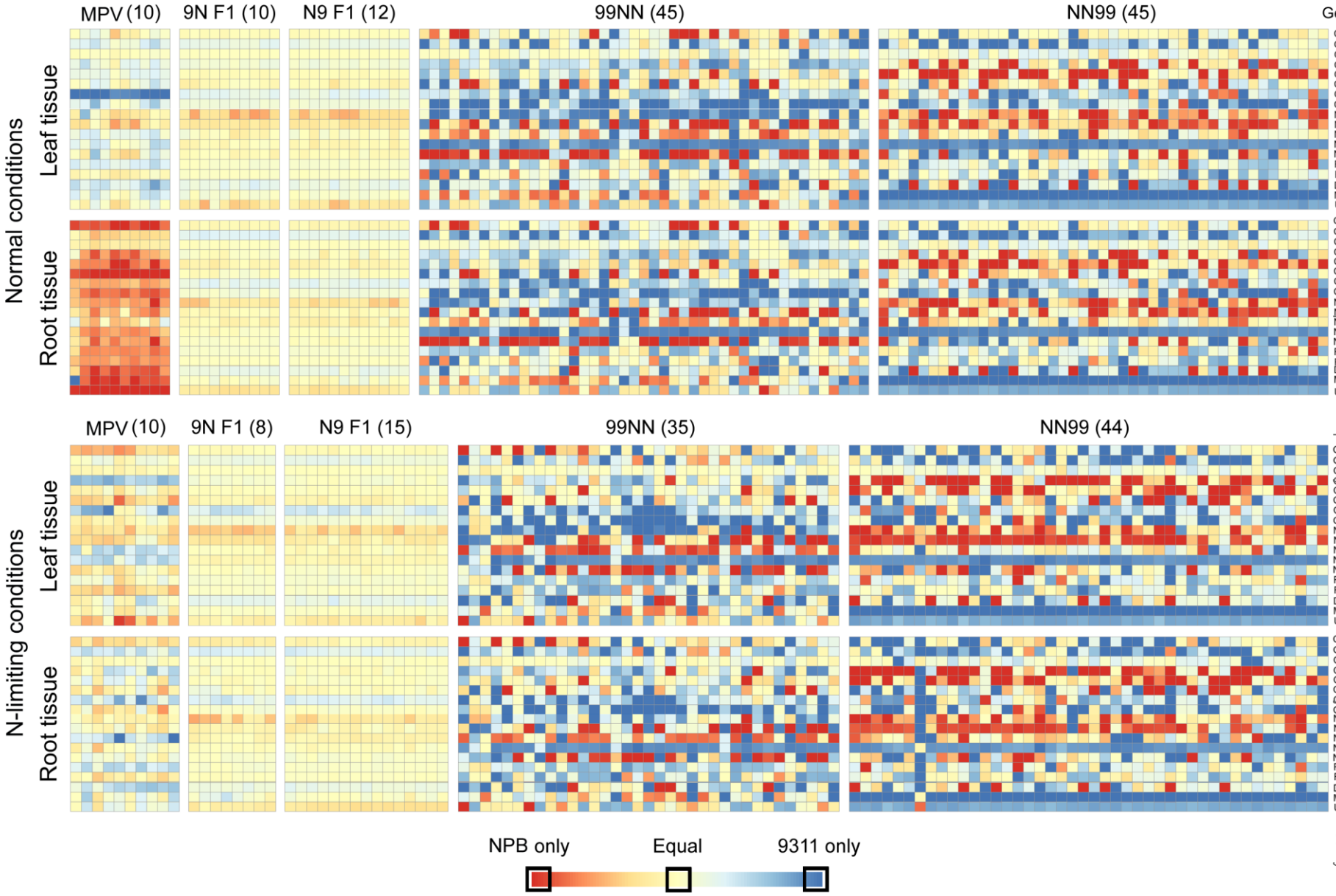

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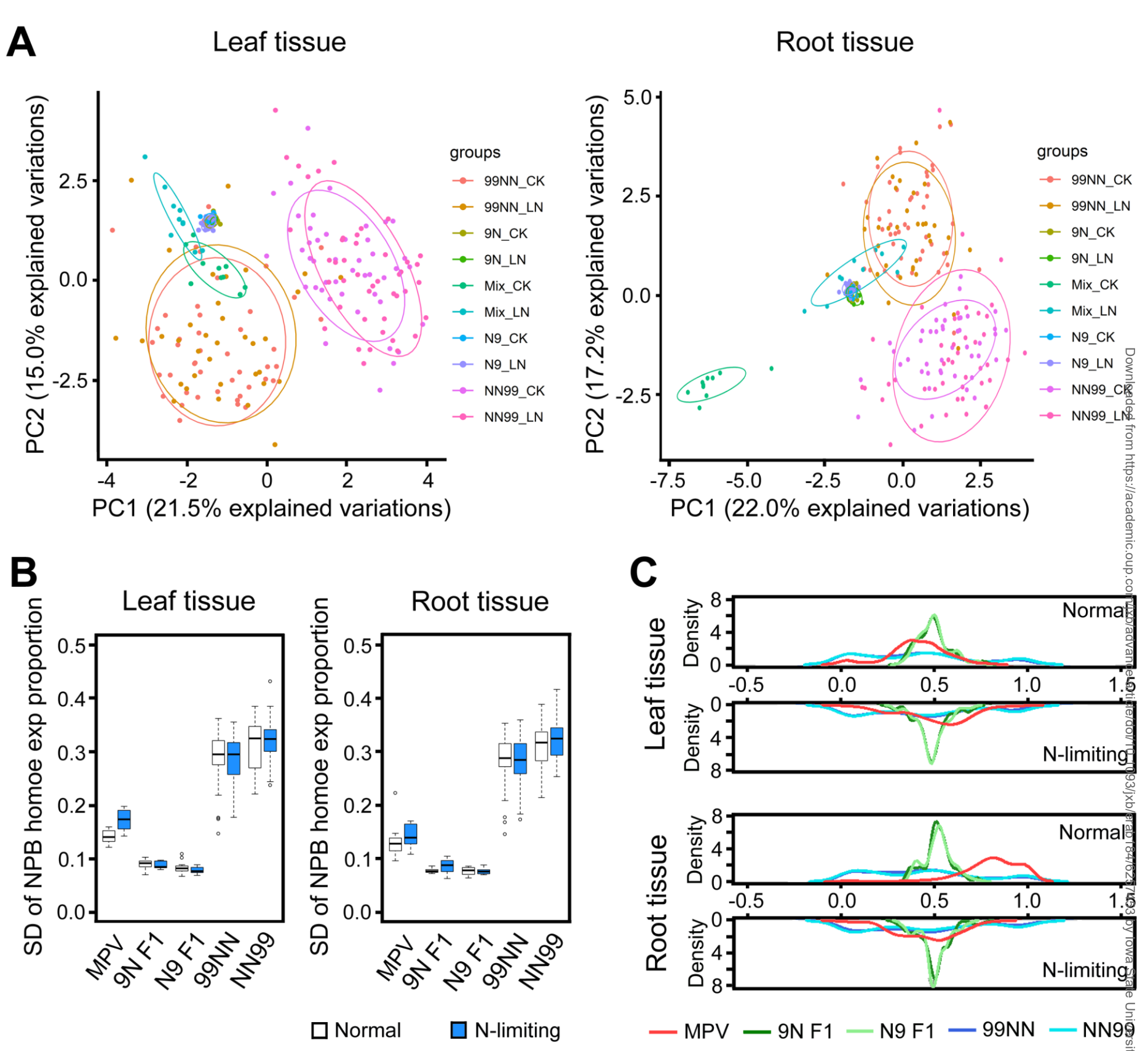

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