Nitrate reductase-mediated Nitric Oxide regulates the leaf shape in Arabidopsis by mediating the homeostasis of reactive oxygen species

Citation for published version:
Pan, Q, Geng, C-C, Li, D-D, Xu, S-W, Mao, D-D, Umbreen, S, Loake, GJ & Cui, B-M 2019, 'Nitrate reductase-mediated Nitric Oxide regulates the leaf shape in Arabidopsis by mediating the homeostasis of reactive oxygen species', *International Journal of Molecular Sciences*, vol. 20, no. 9.  
https://doi.org/10.3390/ijms20092235

Digital Object Identifier (DOI):
10.3390/ijms20092235

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
*International Journal of Molecular Sciences*

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Nitrate Reductase-Mediated Nitric Oxide Regulates the Leaf Shape in Arabidopsis by Mediating the Homeostasis of Reactive Oxygen Species

Qiao-Na Pan 1,2, Chen-Chen Geng 1, Dan-Dan Li 3, Shi-Wen Xu 1, Dan-Dan Mao 1, Saima Umbreen 2, Gary John Loake 2,4 and Bei-Mi Cui 2,5,*

1 The Key Laboratory of Biotechnology for Medicinal Plant of Jiangsu Province, School of Life Science, Jiangsu Normal University, Xuzhou 221116, China; youyouwel@163.com (Q.-N.P.); gengcc1314@163.com (C.-C.G.); shiwenxu97@163.com (S.-W.X.); 15150080176@163.com (D.-D.M.)
2 Institute of Molecular Plant Sciences, School of Biological Sciences, Edinburgh University, Edinburgh EH9 3BF, UK; saima.umbreen@ed.ac.uk (S.U.); g.loake@ed.ac.uk (G.J.L.)
3 Haikou custom, Haikou 570311, China; 108074182@qq.com
4 Transformational Centre for Biotechnology of Medicinal and Food Plants, Jiangsu Normal University—Edinburgh University, Xuzhou 221116, China
5 Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China
* Correspondence: Beimi.cui@gmail.com

Received: 16 April 2019; Accepted: 3 May 2019; Published: 7 May 2019

Abstract: As a gaseous biological signaling molecule, nitric oxide (NO) regulates many physiological processes in plants. Over the last decades, this low molecular weight compound has been identified as a key signaling molecule to regulate plant stress responses, and also plays an important role in plant development. However, elucidation of the molecular mechanisms for NO in leaf development has so far been limited due to a lack of mutant resources. Here, we employed the NO-deficient mutant nia1nia2 to examine the role of NO in leaf development. We have found that nia1nia2 mutant plants displayed very different leaf phenotypes as compared to wild type Col-0. Further studies have shown that reactive oxygen species (ROS) levels are higher in nia1nia2 mutant plants. Interestingly, ROS-related enzymes ascorbate peroxidase (APX), catalases (CAT), and peroxidases (POD) have shown decreases in their activities. Our transcriptome data have revealed that the ROS synthesis gene RBOHD was enhanced in nia1nia2 mutants and the photosynthesis-related pathway was impaired, which suggests that NO is required for chloroplast development and leaf development. Together, these results imply that NO plays a significant role in plant leaf development by regulating ROS homeostasis.

Keywords: nitric oxide; reactive oxygen species; antioxidant enzymes; leaf development; nitrate reductase (NR)

1. Introduction

Leaves are critical organs for the survival of plants as they are the primary source of photosynthesis-derived energy and also provide a large area for direct interaction with the environment [1,2]. Most importantly, the development of leaf shape and size contributes to the world food security. As settled organisms, plants have evolved a sophisticated molecular mechanism for the development of leaf shape and size [3,4]. Despite a huge diversity in shape and size, early leaf developmental modules are almost conserved in angiosperms [5,6]. In this context, leaf shape and size are initially determined by the meristematic cell proliferation and then by cell expansion. Co-ordination between cell proliferation and cell expansion is critical for the development of the
final size of the leaves. Mutations in the genes that control polar cell proliferation and cell expansion have yielded short leaves [7,8]. In contrast, transgenic lines expressing ICK1 (cyclin-dependent kinase inhibitor 1) or KRP2 (Kip-related protein 2), both of which inhibit the proliferation of leaf cells by interacting with CDKA–cyclin complexes, resulted in reduced cell number and smaller leaf size [9]. Therefore, plant leaf shape is dependent on temporal and spatial distributions of cell proliferation and expansion, both of which are regulated by multiple molecular pathways [3]. For example, phytohormones such as DEL1 regulate plant growth and development by modulating cell proliferation [10]. In addition to phytohormones, redox signaling molecules, such as ROS (reactive oxygen species) and NO [11], have been shown to play crucial roles in various physiological processes including plant leaf development [12–14]. Although leaf development has been the subject of numerous studies, the molecular mechanism that controls it remains far from understood.

The cellular redox status plays an important role in the regulation of cell fate and organ development, and changes in redox status are known to occur during cell proliferation and expansion [15,16]. Increasing reports suggest that leaf shape development is regulated through the modification of redox status within plant cells [17]. In this context, Arabidopsis mutants with higher ROS production (such as kua1) and higher NO generation (such as nox1 and gsnor1-3) have smaller leaves as compared to wild type plants [8,18], which strongly suggests that redox signals play a central role in plant leaf development. Many biochemistry studies have suggested that redox molecules, including RNS (reactive nitrogen species) and ROS, modulate the activities of proteins by oxidation of amino acids such as Cysteine (Cys), Tryptophan (Trp), and Serine (Ser), which is a prominent feature of redox signaling networks [16]. For example, TCP1 (TEOSINTE BRANCHED1-CYCLOIDEA-PROLIFERATING CELL FACTOR1) transcription factors are well known to regulate plant leaf development associated with cell proliferation and growth [19]. Specifically, a conserved Cys20 within TCP1s could be a target of H2O2- and NO-derived oxidation, which inhibits its DNA binding activity during plant development [19]. Numerous redox-sensitive proteins have been identified, which shows the role of redox regulation in plant development [14]. Since the redox status plays a central role in plant development, imbalance of redox homeostasis will always result in unfavorable conditions. To cope with this, plant cells have developed enzymatic and non-enzymatic systems to maintain the appropriate redox status. Furthermore, high levels of NO could provide a feedback to inhibit NADPH oxidase activity through S-nitrosylation of AtRBOHD (Arabidopsis thaliana Respiratory Burst Oxidase Homologue D) at Cys890, and thus reduce production of reactive oxygen intermediates (ROIs) during immune response [20]. These evidences suggest that the oxidative status generated by reactive oxygen species seems to be alleviated by reducing ROS production. Therefore, we need more powerful genetic tools to further explore their roles in plant development.

So far, redox molecules such as H2S, ROS, CO, and NO have been shown to have functions in various physiological processes among plants and animals [13,21–24]. Moreover, H2S was shown to regulate ROS and NO levels in BV2 microglial cells [22]. In plants, RNS and ROS synthesis is a routine requirement for plant development. A major source of NO production in plants is nitrate reductase (NR), which also facilitates its homeostasis [25]. In Arabidopsis, NR is encoded by two genes NIA1 and NIA2. NR double mutant nia1nia2 failed to synthesize NO [26,27]. Interestingly, NR-dependent NO plays a crucial role in plant development and various stress responses. For example, NR-mediated NO is essential for abscisic acid (ABA)-induced stomatal closure, floral transition, and root hair development, and NR-dependent NO also plays a role in auxin-induced NO production [28–31]. Besides this, NR-dependent NO regulates various abiotic stresses such as freezing, hypoxic, and osmotic stress tolerance [32–36], as well as biotic stress responses to Pseudomonas syringae [37].

The accumulating evidence suggests that NO and ROS can function independently or synergistically to regulate development and stress responses [12,38–40]. However, until now, the cross-talk of NO and ROS signals in plant leaf development remains to be uncovered. In this study, we employed the NR-deficient mutant nia1nia2 to investigate how NO mediates leaf development, and how the cross-talk between ROS and NO regulates it. We found that NO is required for leaf development.
Further, ROS levels in nia1nia2 mutants were increased, but the activities of ROS-related enzymes APX (ascorbate peroxidase), CAT (catalases), and POD (peroxidases) were reduced as compared to wild type Col-0 (Columbia-0). Our findings emphasize the role of NO in leaf development and also the importance of ROS homeostasis to regulate it.

2. Results

2.1. Lack of NR-Mediated NO Production Affects the Leaf Shape and Size in Arabidopsis

In order to study the role of NR-mediated NO in Arabidopsis leaf development, we selected nia1nia2 mutant lines for conducting the research. We characterized the leaf shape and size at three various time points (3-, 5-, and 7-week-old plants) of the plant life cycles in order to understand the differences in leaf shape and size at various stages of leaf development. Our results showed that there is not much difference in leaf shape or size of 3-week-old nia1nia2 plants as compared to wild type Col-0 (Figure 1A). However, 5-week-old plants of nia1nia2 mutants have much narrower and smaller leaves as compared to wild type Col-0 (Figure 1B). Interestingly, leaf shape and size differences became even more obvious at 7 weeks with the development of rosette leaf shapes in nia1nia2 mutant plants, which were strikingly different to wild type Col-0 (Figure 1C).

![Figure 1](image_url)

Figure 1. nia1nia2 mutants showed altered leaf shape development. (A–C), Leaf phenotypes of Col-0 and nia1nia2 plants at 3, 5, and 7 weeks. Scale bar, 2 cm. (D), Measurement of average leaf surfaces of Col-0 and nia1nia2 plant. Leaves were detached from 3, 5, and 7-week-old plants and then measured. Eight plants of each ecotype were used for measurement, and error bars represent standard deviation (SD). (E), Leaf length-to-width ratios of 3, 5, and 7-week-old Col-0 and nia1nia2 plants were measured. Error bars represent SD from eight replicates. (F), Fresh weight of 3, 5, and 7-week-old plants of Col-0 and nia1nia2. Error bars represent SD from eight replicates. *, p < 0.001 (Student’s t-test). Experiment was repeated three times with similar results.

Next, we quantified the leaf shape and size by measuring leaf surface area, and calculated the leaf length-to-leaf width ratios at the same three time points. Our data have suggested that leaf surface area is significantly reduced in nia1nia2 mutants as compared to wild type Col-0 plants at all three time points (Figure 1D). However, leaf length-to-width ratios are significantly different between nia1nia2 mutants and wild type Col-0 plants at 5 and 7 weeks only (Figure 1E). Further, nia1nia2 showed significantly less fresh weight than Col-0 in 7-week-old plants (Figure 1F).

In summary, our data have clearly indicated that NR-mediated NO generation might be required for leaf development, especially during later stages of leaf development.

2.2. nia1nia2 Mutant Plants Have Smaller Leaves as Compared to Wild Type Col-0 due to Having Lesser Number of Cells and Reduced Cell Size in Their Leaves

Leaf size and shape is determined by the sum of cell number and cell size. In order to investigate whether differences in size and shape of NO-deficient mutant nia1nia2 leaves are due to cell proliferation, expansion, or both, we examined the cell number and cell size within adaxial and abaxial epidermal cells of the third and fourth rosette leaves of 3- and 5-week-old plants. Our results showed that the
cell size of adaxial epidermis in nia1nia2 plants was slightly smaller. However, the cell size of abaxial epidermis is significantly smaller as compared to Col-0 in 3-week-old plants (Figure 2A,C). Both abaxial and adaxial epidermis have more cells in nia1nia2 plants than Col-0 in 3-week-old plants (Figure 2A,D). Interestingly, in 5-week-old plants, the cell size of nia1nia2 was significantly larger than that of Col-0 in both upper and lower epidermal cells. However, the count of cell number in both adaxial and abaxial epidermis was less in nia1nia2 mutant plants as compared to Col-0 (Figure 2B,C).

![Figure 2. The nia1nia2 mutation affects cell size and cell number. (A,B) Adaxial and abaxial epidermal cells in 3-week- (A) and 5-week-old plants (B) of Col-0 and nia1nia2. Scale bar, 100 µm. (C,D) The size (C) and number (D) of epidermal cells in Col-0 and nia1nia2. Error bars represent SD from six replicates. *, p < 0.05 (Student’s t-test).](image)

Collectively, NO has affected both cell size and cell number during leaf development in nia1nia2 mutant plants as compared to Col-0, demonstrating that NO regulates leaf development in Arabidopsis by mediating cell number and size.

2.3. nia1nia2 Plants Have Lesser Chlorophyll a/b Contents as Compared to Col-0

NO is mostly produced inside the chloroplasts of the plants, which indicates that NO could regulate chloroplast development [14]. Interestingly, the leaf color of 5-week-old nia1nia2 mutant plants is strikingly different than wild type Col-0. This observation has led us to analyze the chlorophyll (Chl) and carotenoid (CAR) composition in these leaves. Consistent with the observed color phenotype, the levels of Chl a and Chl b decreased significantly in nia1nia2 mutants as compared to Col-0 in 5-week-old plants (Figure 3A,B). Consistently, the total Chl synthesis in nia1nia2 leaves was inhibited (Figure 3D). Strikingly, carotenoid contents were similar between nia1nia2 and Col-0 (Figure 3C), indicating that NR deficiency-derived NO production does not affect carotenoid synthesis. Collectively, contents of Chl a/b were changed in nia1nia2 plants as compared to Col-0 plants, which has yielded differences in the leaf color.
ROS have been reported to regulate cell expansion and cell number, which determine final leaf shape and size [41,42]. NO has been shown to prevent oxidative stress through regulating ROS levels [24]. In order to test if NO is regulating leaf shape through mediating ROS levels in nia1nia2 plants, we quantified ROS (hydrogen peroxide, H$_2$O$_2$ and superoxide, O$_2^-$) levels in 3-week- and 5-week-old plants. We stained 3-week-old plants’ leaves with 3,3-diaminobenzidine (DAB), which is a standard dye used to quantify the levels of H$_2$O$_2$. We found an increased intensity of DAB in 3-week-old plants of nia1nia2 mutants leaves as compared to Col-0 (Figure 4A), suggesting that nia1nia2 leaves contained slightly higher H$_2$O$_2$ levels than Col-0 at this stage of leaf development. Similar results were obtained when we quantified the intensity of DAB staining in 5-week-old plants (Figure 4A). Interestingly, the DAB staining showed that the H$_2$O$_2$ levels were markedly higher in nia1nia2 mutants as compared to Col-0 in 5-week-old plants. Quantification of H$_2$O$_2$, as shown in Figure 4C, also supported the observation that nia1nia2 mutant plants have higher production of H$_2$O$_2$ as compared to Col-0 in both 3-week- and 5-week-old plants.

In addition to the quantification of H$_2$O$_2$, we also quantified the superoxide (O$_2^-$) by nitro blue tetrazolium (NBT) staining, and as shown in Figure 4B, the O$_2^-$ levels were similar between nia1nia2 mutants and Col-0. Moreover, the O$_2^-$ levels were higher than that in Col-0 in 5-week-old plants (Figure 4B). The quantification of NBT staining also suggested that there is a significant increase of O$_2^-$ in 5-week-old nia1nia2 plants as compared to Col-0 (Figure 4D).

Taken together, these data suggest that the ROS levels (H$_2$O$_2$ and O$_2^-$) have increased in nia1nia2 mutants as compared to Col-0, implying that NR-dependent NO production is required for ROS homeostasis during leaf development.
2.5. *nia1nia2* Has Reduced Antioxidant Enzyme Activity

High levels of ROS are harmful to cells, and so plants have developed a protective system to reduce the oxidative stress and avoid damage. The enzymes POD, CAT, and APX are ROS scavengers in the anti-oxidant protection system. As shown above, the ROS levels increased in *nia1nia2* mutants as compared to Col-0, and we further assayed the activities of these three ROS scavengers. As shown in Figure 5, the activity of CAT in leaves of 5-week-old *nia1nia2* plants was extremely reduced as compared to that of Col-0 plants. Furthermore, the activities of APX and POD in *nia1nia2* plants were significantly lower than that of Col-0 plants. Thus, it appears that inhibition of these antioxidant enzymes’ activities in *nia1nia2* mutant plants leads to an increase in ROS levels.

Figure 5. Activities of ROS-related enzymes in mutant *nia1nia2* and its wild-type Col-0. Third rosette leaves from 5-week-old plants of the indicated genotypes were collected for catalase (CAT) (A), ascorbate peroxidase (APX) (B), and peroxidase (POD) (C) activity assays. Vertical bars represent SD (n = 5). *, p < 0.05 (Student’s t-test).

Collectively, this observation clearly suggests that APX-, CAT-, and POD-mediated ROS metabolism is relatively impaired in *nia1nia2* mutants, which has eventually altered their leaf morphology.

2.6. RNA Sequencing Showed Clear Differences in Key Gene Expression Levels in *nia1nia2* Mutant Plants

Once we have established the role of NR-mediated NO production in leaf development of *Arabidopsis* through the regulation of ROS levels, we further conducted RNA sequencing to investigate the differences in gene expressions between *nia1nia2* mutant plants and Col-0 which could possibly explain the differences in leaf shape between these two genotypes. For this purpose, we compared the transcriptomic profiles of third rosette leaves of 5-week-old *nia1nia2* mutant plants and wild type Col-0 plants using RNA sequencing (RNA-Seq). We sequenced the libraries on Illumina HisSeq 2000, and a total of 271,323,530 high-quality reads were generated. We found a difference in expression levels of a total of 1950 genes between these two genotypes. Interestingly, among these genes, 679 genes were up-regulated and 1271 got down-regulated (Figure 6A,B), and these genes were divided into two groups using a hierarchical clustering algorithm (Figure 6C). The global transcriptome changes in examined samples were further categorized based on their gene ontology (GO) and divided into groups of predicted or experimentally defined biological processes, molecular functions, and cellular components (Figure 6D). Functional categorization of differentially regulated genes revealed that a wide variety of biological processes are associated with pigment binding, and chloroplast and cell wall development.
Figure 6. Nitrate reductase (NR)-deficient mutant nia1nia2 plants have affected gene expression. (A) MA plot of RNA-seq data showing the up-regulated (red) and down-regulated (green) genes in nia1nia2 plants compared with Col-0. (B) A total of 1950 TFs were identified at a Q-value < 0.05 in the transcriptome. Among these, 679 were up-regulated and 1271 were down-regulated (≥log2-fold change). (C) Heat-map showing the expression patterns of transcriptome between nia1nia2 plants and WT. (D) Distribution of differentially expressed genes using pairwise comparisons in WT and nia1nia2 plants.

Interestingly, ROS synthesis key gene RBOHD, but not RBOHF, was highly induced in nia1nia2 mutants as compared to Col-0, (Figure 7), in agreement with the fact that ROS production is higher within nia1nia2 leaves as compared to Col-0. Additionally, mRNA levels of ROS response marker genes were increased in nia1nia2 compared with Col-0 (Figure 7), suggesting that these ROS-related genes are induced in nia1nia2 plants. Overall, transcriptome analysis gives more insight to understand the molecular mechanism underlying leaf shape in nia1nia2 plants.
lyC green

[0x0]-

we found that which had impaired NO production in stress responses, also showed similar phenotypes [52]. Further, Arabidopsis is important for plant leaf development. These findings strongly demonstrated that the endogenous NO level in nia1nia2 synthesis [18,60]. Our RNA sequence data have shown that photosynthetic genes are down-regulated NO-overproduction mutant nox1 in a nitrite-dependent NO manner [59]. Our results have shown that bananas [57]. Further, NO is produced in chloroplasts [58], most probably through arginine and in a Chl degradation [56]. By contrast, NO scavenger cPTIO reduced the SNP-enhanced Chl stability in nitrite-deficiency mutations in Arabidopsis or rice affect the leaf development [50–52]. However, the underlying mechanism was left to be explored.

We observed that nia1nia2 leaves were smaller in size as compared to Col-0 (Figures 1 and 2). Chloroplasts are the major source for free energy transduction (photophosphorylation) in plants, and NO has been shown to affect the function of chloroplasts through the inhibition of photophosphorylation [53]. NO affects photosynthesis and photorespiration in various plants [53,54]. For example, OsNOA1 loss-of-function mutations in rice reduced chlorophyll levels during low temperature [55]. It was also reported that the application of a NO donor, SNP, could enhance the photosynthetic activity and reduce the Chl degradation [56]. By contrast, NO scavenger cPTIO reduced the SNP-enhanced Chl stability in bananas [57]. Further, NO is produced in chloroplasts [58], most probably through arginine and in a nitrite-dependent NO manner [59]. Our results have shown that nia1nia2 mutant plants have lesser chlorophyll contents as compared to Col-0 (Figure 3). Consistently, another NO-related mutant, noa1, which had impaired NO production in stress responses, also showed similar phenotypes [52]. Further, NO-overproduction mutant nos1 (CUE1) and gsnor1-3par2-1 showed stunted phenotypes and Chl synthesis [18,60]. Our RNA sequence data have shown that photosynthetic genes are down-regulated in nia1nia2 mutant plants, which demonstrates that NO is required for Chl synthesis and, by extension, for proper leaf size development. These findings strongly demonstrated that the endogenous NO level is important for plant leaf development.

ROS regulate the leaf development by controlling cell proliferation and cell size [16]. In this study, we found that nia1nia2 mutant plants have smaller leaves due to reductions in cell number and cell size

3. Discussion

NO is a key redox-active molecule of cellular signal transduction networks, which regulates many physiological processes in plants, including development and immunity [14,18,44,45], and also in animals, including immunomodulatory responses and oncogenesis [21,46]. However, little is known about the role of NO in leaf development. In mammals, NO is generated by nitric oxide synthase (NOS), a NADPH-dependent enzyme [47,48]. NOS catalyzes the conversion of L-arginine to citrulline and NO [48]. Despite several completed genome projects, the gene for NOS has not been uncovered in plants yet [11,49]. NR has been the main source of NO production in plants to date [30–32]. In Arabidopsis, NR occurs in the cytosol and is encoded by two genes: NIA1 and NIA2 [33]. In this study, we found that the NO-deficient mutant nia1nia2 displayed distinguished leaf phenotype compared with Col-0, as shown in previous reports where noa1/nos1 NO-deficiency mutations in Arabidopsis or rice affect the leaf development [50–52]. However, the underlying mechanism was left to be explored.

Leaf photosynthesis ability is mainly associated with Chl contents and leaf development [43]. Our RNA-seq data have shown a reduction in the expression of photosynthetic genes such as Lhca and Lhcb, and also Psb and Psa genes. This clearly indicates that the chloroplast and photosynthesis systems are severely compromised in nia1nia2 mutant plants. Collectively, these data clearly suggest that NO might have a role in the reprogramming of chloroplast-related gene expression to alter Chl contents and, by extension, leaf development.
as compared to wild type plants. Interestingly, our data have suggested that NO is regulating leaf size through controlling ROS homeostasis, as ROS synthesis gene RBOHD was highly induced in nia1nia2 plants as compared to Col-0, as revealed by RNA-seq. We further confirmed this by qPCR studies (Figure 7). Hence, NO regulates ROS homeostasis through dual mechanisms: First, by enhancing ROS production (Figures 4 and 7), and secondly by inhibition of ROS-scavenger enzymes, such as APX, CAT, and POD (Figure 5).

Past reports have shown that NR-dependent NO production is required for abiotic stress-induced antioxidant defense in wheat and bean plants [61,62]. It was shown that NO scavengers reduced the activities of all antioxidant enzymes under abiotic stresses, while SNP reduced the production of ROS, consistent with our results. Hence, our results have confirmed that NR-dependent NO mediates the leaf development and stress response by regulating ROS hemostasis.

In conclusion, our results presented here have demonstrated that NO-deficient mutant nia1nia2 plants showed visibly different leaf phenotypes compared with WT Col-0 due to compromised cell proliferation. We have uncovered the mechanism behind this difference in leaf phenotype between nia1nia2 mutant plants and wild-type plants: NR-dependent NO regulates leaf development by controlling ROS homeostasis in these plants. Indeed, this study sheds light on the critical role of NO in regulating plant leaf development.

4. Materials and Methods

4.1. Plant Growth and Morphological Analysis

*Arabidopsis thaliana* Columbia-0 (Col-0) and nia1nia2 mutant plants used in this study [26] were grown on soil after cold stratification for 2–3 days under 10 h photoperiod conditions at 22 °C. To measure the leaf area, and width and length of indicated leaves, leaves were cut and photographed, and then stated measurements were performed with image J software.

4.2. Cell Number and Size Analysis

Leaves of indicated genotypes were bleached with clearing solution (8 g chloral hydrate, 3 mL water, and 1 mL glycerol), and then observed under microscopy [63]. Photos were taken at the same area of each leaf for both the abaxial and adaxial sides, and then the cell size and number were scored. At least 10 leaves of each genotype at given developmental stages were observed.

4.3. Staining and Analysis of H$_2$O$_2$ and O$_2^-$

For DAB and NBT staining, dyes were prepared following a previously described method with some modifications [64]. Briefly, the third rosette leaf of each genotype at a particular developmental stage was cut, and then stained in the DAB solution (1 mg/mL) for 8 h in dark for detection of H$_2$O$_2$, or NBT solution (0.5 mg/mL in PBS) for 3 h in dark for detection of O$_2^-$, followed by destaining of the leaves with ethanol until there was proper visualization of the stain. The final staining intensity was recorded by photographing the leaves. The H$_2$O$_2$ and O$_2^-$ contents were assayed as described previously [65].

4.4. Antioxidant Enzyme Extraction and Quantification

Enzymes were extracted from 0.2 g of leaf tissues of each genotype at a particular developmental stage using a mortar and pestle with 2 mL of extraction buffer containing 50 mM K-phosphate buffer (pH 7.6) and 0.1 mM Na$_2$-EDTA. The homogenate was centrifuged at 15,000 g for 15 min at 4 °C, and the supernatant fraction was used to assay various enzymes according to the method described elsewhere [66].
4.5. Quantification of Chl Contents

For chlorophyll determination, the first two true leaves of each genotype were collected, and their fresh weights were determined individually. Leaves were extracted with 80% ethanol at 4 °C overnight with agitation under dark. Chl contents were determined as described in reference [67].

4.6. RNA Sequencing and Gene Ontology (GO) Enrichment Analysis

For transcriptome analysis, 5-week-old leaves from Col-0 and nia1nia2 plants were grown in growth room. Total plant RNAs were extracted using the plant RNA isolation kit (Agilent, Santa Clara, CA, USA). A total of 3 ug of high-quality RNA per sample was used for sequencing on an Illumina HisSeq2500 platform, and 125 bp paired-end reads were generated. Reference genome and gene model annotation files were downloaded from a genome website directly (ftp://ftp.arabidopsis.org/home/tair) using TopHat v2.0.12. Cuffquant and cuffnorm (v2.2.1) were used to calculate the FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) of genes in each sample [68].

Gene ontology (GO) enrichment analysis of differentially expressed genes was implemented by the GOseq R package, in which gene length bias was corrected. GO terms with corrected p value < 0.05 were considered significantly enriched by differentially expressed genes.

Author Contributions: Conceptualization, Q.-N.P and B.-M.C.; formal analysis, Q.-N.P and C.-C.G.; funding acquisition, Q.-N.P; investigation, Q.-N.P., C.-C.G., D.-D.L., S.-W.X., and D.-D.M.; methodology, C.-C.G.; writing—original draft, C.-C.G. and S.U.; writing—review & editing, S.U., G.J.L. and B.-M.C.

Funding: This research was funded by the Science and Technology Support Program of Jiangsu Province of China, grant number SBK2017040656, and the National Natural Science Foundation of China, grant number 31700241.

Acknowledgments: We kindly thank Steven Spoel (Institute of Molecular Plant Sciences, University of Edinburgh) for providing the nia1nia2 knockout mutant seeds.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chitwood, D.H.; Sinha, N.R. Evolutionary and environmental forces sculpting leaf development. Curr. Biol. 2016, 26, R297–R306. [CrossRef] [PubMed]
2. Afzal, A.; Duiker, S.W.; Watson, J.E. Leaf thickness to predict plant water status. Biosyst. Eng. 2017, 156, 148–156. [CrossRef]
3. Rodriguez, R.E.; Debernardi, J.M.; Palatnik, J.F. Morphogenesis of simple leaves: Regulation of leaf size and shape. Wires Dev. Biol. 2014, 3, 41–57. [CrossRef]
4. Kessler, S.; Sinha, N. Shaping up: The genetic control of leaf shape. Curr. Opin. Plant Biol. 2004, 7, 65–72. [CrossRef]
5. Kidner, C.A.; Umbreen, S. Why is leaf shape so variable. Int. J. Plant Dev. Biol. 2010, 4, 64–75.
6. Townsley, B.T.; Sinha, N.R. A new development: Evolving concepts in leaf ontogeny. Ann. Rev. Plant Biol. 2012, 63, 535–562. [CrossRef] [PubMed]
7. Narita, N.N.; Moore, S.; Horiguchi, G.; Kubo, M.; Demura, T.; Fukuda, H.; Goodrich, J.; Tsukaya, H. Overexpression of a novel small peptide ROTUNDIFOLIA4 decreases cell proliferation and alters leaf shape in Arabidopsis thaliana. Plant J. 2004, 38, 699–713. [CrossRef] [PubMed]
8. Lu, D.; Wang, T.; Persson, S.; Muellerroeber, B.; Schippers, J.H.M. Transcriptional control of ROS homeostasis by KUODA1 regulates cell expansion during leaf development. Nat. Commun. 2014, 5, 3767. [CrossRef]
9. Verkest, A.; Manes, C.L.D.; Vercruysse, S.; Maes, S.; Van der Schueren, E.; Beeckman, T.; Genschik, P.; Kuiper, M.; Inze, D.; De Veylder, L. The cyclin-dependent kinase inhibitor KRP2 controls the onset of the endoreduplication cycle during Arabidopsis leaf development through inhibition of mitotic CDKA;1 kinase complexes. Plant Cell 2005, 17, 1723–1736. [CrossRef]
10. Berckmans, B.; Vassileva, V.; Schmid, S.P.C.; Maes, S.; Parizot, B.; Naramoto, S.; Magyar, Z.; Kamei, C.L.A.; Koncz, C.; Bogre, L.; et al. Auxin-Dependent Cell Cycle Reactivation through Transcriptional Regulation of Arabidopsis E2Fa by Lateral Organ Boundary Proteins. Plant Cell 2011, 23, 3671–3683. [CrossRef]
11. Santolini, J.; André, F.; Jeandroz, S.; Wendehenne, D.J. Nitric oxide synthase in plants: Where do we stand? *Nitric Oxide* 2017, 63, 30–38. [CrossRef]
12. del Rio, L.A. ROS and RNS in plant physiology: An overview. *J. Exp. Bot.* 2015, 66, 2827–2837. [CrossRef]
13. Mittler, R. ROS Are Good. *Trends Plant Sci.* 2017, 22, 11–19. [CrossRef]
14. Yu, M.D.; Lamattina, L.; Spoel, S.H.; Loake, G.J. Nitric oxide function in plant biology: A redox cue in deconvolution. *New Phytol.* 2014, 202, 1142–1156. [CrossRef]
15. Foyer, C.H.; Noctor, G. Redox Signaling in Plants. *Antioxid. Redox Signal.* 2013, 18, 2087–2090. [CrossRef]
16. Schmidt, R.; Schippers, J.H.M. ROS-mediated redox signaling during cell differentiation in plants. *Biochim. Biophys. Acta Gen. Subj.* 2015, 1850, 1497–1508. [CrossRef]
17. Kocsy, G.; Tari, I.; Vankova, R.; Zechmann, B.; Gulyas, Z.; Poor, P.; Galiba, G. Redox control of plant growth and development. *Plant Sci.* 2013, 211, 77–91. [CrossRef]
18. Kwon, E.; Feechan, A.; Yun, B.W.; Hwang, B.H.; Pallas, J.A.; Kang, J.G.; Loake, G.J. AtGSNOR1 function is involved in water stress-induced subcellular anti-oxidant defense in maize plants. *J. Integr. Plant Biol.* 2008, 50, 231–243. [CrossRef]
33. Zhao, M.-G.; Chen, L.; Zhang, L.-L.; Zhang, W.-H. Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in Arabidopsis. *Plant Physiol.* **2009**, *151*, 755–767. [CrossRef]

34. Kolbert, Z.; Ortega, L.; Erdei, L.J. Involvement of nitrate reductase (NR) in osmotic stress-induced NO generation of Arabidopsis thaliana L. roots. *J. Plant Physiol.* **2010**, *167*, 77–80. [CrossRef]

35. Xie, Y.; Mao, Y.; Lai, D.; Zhang, W.; Zheng, T.; Shen, W. Roles of NIA/NR/NOA1-dependent nitric oxide production and HY1 expression in the modulation of Arabidopsis salt tolerance. *J. Exp. Bot.* **2013**, *64*, 3045–3060. [CrossRef]

36. Blokhina, O.; Fagerstedt, K.V. Oxidative metabolism, ROS and NO under oxygen deprivation. *Plant Physiol. Biochem.* **2010**, *48*, 359–373. [CrossRef]

37. Vitor, S.C.; Duarte, G.T.; Saviani, E.E.; Vincentz, M.G.; Oliveira, H.C.; Salgado, I.J. Nitrate reductase is involved in the metabolic regulation of Arabidopsis thaliana against Pseudomonas syringae. *Acta Physiol. Plant.* **2013**, *328*, 475–486. [CrossRef]

38. Romero-Puertas, M.C.; Sandalio, L.M. Nitric oxide level is self-regulating and also regulates its ROS partners. *Front. Plant Sci.* **2016**, *7*, 316. [CrossRef]

39. Niu, L.; Liao, W. Hydrogen peroxide signaling in plant development and abiotic responses: Crosstalk with nitric oxide and calcium. *Front. Plant Sci.* **2016**, *7*, 230. [CrossRef]

40. Nabi, R.B.S.; Tayade, R.; Hussain, A.; Kulkarni, K.P.; Imran, Q.M.; Mun, B.-G.; Yun, B.-W. Nitric oxide regulates plant responses to drought, salinity, and heavy metal stress. *Environ. Exp. Bot.* **2019**, *161*, 120–133. [CrossRef]

41. Schippers, J.H.M.; Foyer, C.H.; van Dongen, J.T. Redox regulation in shoot growth, SAM maintenance and flowering. *Curr. Opin. Plant Biol.* **2016**, *29*, 121–128. [CrossRef]

42. Maldonado-Alcornada, A.M.; Echevarría-Zomeño, S.; Lindermayr, C.; Redondo-López, I.; Durner, J.; Jorrín-Novó, J.V. Proteomic analysis of Arabidopsis protein S-nitrosylation in response to inoculation with Pseudomonas syringae. *Acta Physiol. Plant.* **2011**, *33*, 1493–1514. [CrossRef]

43. Bhagsari, A.S.; Brown, R.H. Leaf Photosynthesis and Its Correlation with Leaf-Area. *Crop Sci.* **1998**, *36*, 127–132. [CrossRef]

44. Wang, Y.Q.; Feechan, A.; Yun, B.W.; Shaﬁei, R.; Hofmann, A.; Taylor, P.; Xué, P.; Yang, F.Q.; Xie, Z.S.; Pallas, J.A.; et al. S-Nitrosylation of AtSABP3 Antagonizes the Expression of Plant Immunity. *J. Biol. Chem.* **2009**, *284*, 2131–2137. [CrossRef] [PubMed]

45. Kaya, C.; Sönmez, O.; Ashraf, M.; Polat, T.; Tuna, L.; Aydemir, S. Exogenous application of nitric oxide and thiourea regulates on growth and some physiological processes in maize (*Zea mays* L.) plants under saline stress. *Toprak Su Der. 2015*, 61–66. [CrossRef]

46. Paskas, S.; Mazzon, E.; Basile, M.S.; Cavalli, E.; Al-Abed, Y.; He, M.; Rakovec, S.; Nicoletti, F.; Mijatovic, S.; Maksimovic-Ivanic, D. Lopinavir-NO, a nitric oxide-releasing HIV protease inhibitor, suppresses the growth of melanoma cells in vitro and in vivo. *Investig. New Drugs* **2019**, 1–15. [CrossRef]

47. Alderton, W.K.; Cooper, C.E.; Knowles, R.G. Nitric oxide synthases: Structure, function and inhibition. *Biochem. J.* **2001**, *357*, 593–615. [CrossRef] [PubMed]

48. Guo, F.-Q.; Okamoto, M.; Crawford, N.M. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* **2003**, *302*, 100–103. [CrossRef] [PubMed]

49. Jeandroz, S.; Wipf, D.; Stuehr, D.J.; Lamattina, L.; Melkonian, M.; Tian, Z.; Zhu, Y.; Carpenter, E.J.; Wong, G.K.-S.; Wendeheine, D.J. Occurrence, structure, and evolution of nitric oxide synthase–like proteins in the plant kingdom. *Sci. Signal.* **2016**, *9*, re2. [CrossRef]

50. Frungillo, L.; Skelly, M.J.; Loake, G.J.; Spoel, S.H.; Salgado, I. S-nitrosothiols regulate nitric oxide production and storage in plants through the nitrogen assimilation pathway. *Nat. Commun.* **2014**, *5*, 5401. [CrossRef]

51. Liu, F.; Guo, F.Q. Nitric Oxide Deficiency Accelerates Chlorophyll Breakdown and Stability Loss of Thylakoid Membranes during Dark-Induced Leaf Senescence in Arabidopsis. *PLoS ONE* **2013**, *8*, e56345. [CrossRef]

52. Yang, Q.S.; He, H.; Li, H.Y.; Tian, H.; Zhang, J.J.; Zhai, L.G.; Chen, J.D.; Wu, H.; Yi, G.J.; He, Z.H.; et al. NOA1 Functions in a Temperature-Dependent Manner to Regulate Chlorophyll Biosynthesis and Rubisco Formation in Rice. *PLoS ONE* **2011**, *6*, e20005. [CrossRef]

53. Takahashi, S.; Yamashita, H. Reversible inhibition of photophosphorylation in chloroplasts by nitric oxide. *FEBS Lett.* **2002**, *512*, 145–148. [CrossRef]
Yamasaki, H.; Sakihama, Y. Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: In vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett.* 2000, *468*, 89–92. [CrossRef]

He, H.; Yang, Q.S.; Shen, B.R.; Zhang, S.; Peng, X.X. OsNOA1 functions in a threshold-dependent manner to regulate chloroplast proteins in rice at lower temperatures. *BMC Plant Biol.* 2018, *18*, 44. [CrossRef]

Dong, Y.J.; Xu, L.L.; Wang, Q.H.; Fan, Z.Y.; Kong, J.; Bai, X.Y. Effects of exogenous nitric oxide on photosynthesis, antioxidative ability, and mineral element contents of perennial ryegrass under copper stress. *J. Plant Interact.* 2014, *9*, 402–411. [CrossRef]

Wang, Y.S.; Luo, Z.S.; Du, R.X. Nitric oxide delays chlorophyll degradation and enhances antioxidant activity in banana fruits after cold storage. *Acta Physiol. Plant* 2015, *37*, 74. [CrossRef]

Cooney, R.V.; Harwood, P.J.; Custer, L.J.; Franke, A.A. Light-mediated conversion of nitrogen dioxide to nitric oxide by carotenoids. *Environ. Health Perspect.* 1994, *102*, 460–462. [CrossRef]

Jasid, S.; Simontacchi, M.; Bartoli, C.G.; Puntarulo, S. Chloroplasts as a nitric oxide cellular source. Effect of reactive nitrogen species on chloroplastic lipids and proteins. *Plant Physiol.* 2006, *142*, 1246–1255. [CrossRef]

He, Y.K.; Tang, R.H.; Hao, Y.; Stevens, R.D.; Cook, C.W.; Am, S.M.; Jing, L.F.; Yang, Z.G.; Chen, L.G.; Guo, F.Q.; et al. Nitric oxide represses the Arabidopsis floral transition. *Science* 2004, *305*, 1968–1971. [CrossRef]

Wang, H.-H.; Huang, J.-J.; Bi, Y.-R. Nitrate reductase-dependent nitric oxide production is involved in aluminum tolerance in red kidney bean roots. *Plant Sci.* 2010, *179*, 281–288. [CrossRef]

Wu, S.; Hu, C.; Tan, Q.; Xu, S.; Sun, X. Nitric oxide mediates molybdenum-induced antioxidant defense in wheat under drought stress. *Front. Plant Sci.* 2017, *8*, 1085. [CrossRef] [PubMed]

Li, S.; Liu, Y.; Zheng, L.; Chen, L.; Li, N.; Corke, F.; Lu, Y.; Fu, X.; Zhu, Z.; Bevan, M. The plant-specific G protein γ subunit AGG3 influences organ size and shape in Arabidopsis thaliana. *New Phytol.* 2012, *194*, 690–703. [CrossRef]

Pan, Q.N.; Cui, B.M.; Deng, F.Y.; Quan, J.L.; Loake, G.J.; Shan, W.X. RTP1 encodes a novel endoplasmic reticulum (ER)-localized protein in Arabidopsis and negatively regulates resistance against biotrophic pathogens. *New Phytol.* 2016, *209*, 1641–1654. [CrossRef] [PubMed]

Wang, F.; Liu, J.; Zhou, L.; Pan, G.; Li, Z.; Cheng, F. Senescence-specific change in ROS scavenging enzyme activities and regulation of various SOD isozymes to ROS levels in psf mutant rice leaves. *Plant Physiol. Biochem.* 2016, *109*, 248–261. [CrossRef] [PubMed]

Cakmak, I.; Strbac, D.; Marschner, H. Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. *J. Exp. Bot.* 1993, *44*, 127–132. [CrossRef]

Datt, B. Remote sensing of chlorophyll a, chlorophyll b, chlorophyll a+b, and total carotenoid content in eucalyptus leaves. *Remote Sens. Environ.* 1998, *66*, 111–121. [CrossRef]

Trappnell, C.; Williams, B.A.; Perteza, G.; Mortazavi, A.; Kwan, G.; Van Baren, M.J.; Salzberg, S.L.; Wold, B.J.; Pachter, L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* 2010, *28*, 511–515. [CrossRef]