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MicroRNA164 Regulates Perennial Ryegrass (*Lolium perenne* L.) Adaptation to Changing Light Intensity

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Abstract: Plants especially need to adapt to all different light environments (shade, high light, etc.) due to the essential role of light in plant life. Either shade or high-light microenvironmental conditions are common for cool-season turfgrasses, such as perennial ryegrass (*Lolium perenne* L.). In order to study how a plant highly conserves microRNA, miR164-affected perennial ryegrass were studied under different light intensities. *OsmiR164a*-overexpression (OE164), target mimicry *OsmiR164a* (MIM164), and CRES-T (chimeric repressor gene-silencing technology) *OsNAC60* (NAC60) transgenic plants and wild-type (WT) plants were evaluated in both field (shade and full sun) and growth chamber conditions (low, medium, and high PAR at 100, 400, and 1200 $\mu\text{mol s}^{-1} \text{m}^{-2}$). Morphological and physiological analysis showed *miR164* could fine-tune perennial ryegrass adaptation to changing light intensity, possibly via the regulation of target genes, such as *NAC60*. Overall, OE164 and NAC60 plants were similar to each other and more sensitive to high light, particularly under the field condition, demonstrated by smaller size and much poorer grass quality; MIM164 performed more like WT plants than either the OE164 or NAC60 plants. This study indicates the potential of genetic manipulation of *miR164* and/or its targeted genes for turfgrass adaptation to changing light environments, and future research to further investigate the molecular mechanism beneath would be warranted.

Keywords: light intensity; microRNA; turfgrass; mitochondria; Fv/Fm



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1. Introduction

Sunlight is the dominant energy source for plants and all other life in the world by far, which varies from time to time (e.g., daily, seasonally), place to place, in intensity, and also in spectral composition (such as red, blue, and ultraviolet). Light is essential for plant life, and as primary producers on earth, plants need to adapt to all different light environments. Too little light, such as severe shade, means a negative energy balance, and the gain from photosynthesis cannot cover the basic energy requirements. However, the light response curves of most plant leaves saturate between 500 and 1000 $\mu\text{mol s}^{-1} \text{m}^{-2}$, which is well below full sun light (about 1900–2000 $\mu\text{mol s}^{-1} \text{m}^{-2}$). In addition, excess light entering the chloroplast leads to oxidative stress and photoinhibition, particularly in C_3 plants, including perennial ryegrass (*Lolium perenne* L.) [1,2]; this causes a reduction in photosynthetic efficiency due to damage caused by excessive light to the photosystem II, affecting the overall photosynthetic process.

Given the crucial role of light in plant life, adaptation to each light environment is present at all levels from the morphological and structural to the molecular. For example, sun leaves are usually smaller and thicker than shade leaves, along with better-developed mesophyll [3]. In addition, plants have evolved an array of receptors to monitor the light

environment, such as phytochrome A-E (PHYA-E), cryptochrome 1-3 (CRY1-3), and UVR8 (UV RESISTANCE LOCUS 8). After perceiving light quantity and quality changes, light signals will be transduced and regulated by a number of genes, including *COPs*, *PIFs*, *HY5*, hormone biosynthesis as well as transduction-related genes, microRNAs, etc. [4,5]. These allow plants to time and adjust their development such that they can cope with various light environmental circumstances.

MicroRNAs (miRNAs) are a type of endogenous small non-coding RNA, typically 20 to 24 bases long, that play a crucial role in the negative regulation of target gene expression in both plants and animals [6]. Research into plant miRNAs has revealed their pivotal roles in regulating diverse facets of plant growth and development, as well as interactions with the environment, encompassing responses to abiotic and biotic factors, and mutualistic or parasitic associations [7–9]. For example, miRNAs participate in modulating phenotypic plasticity in response to a range of environmental cues, including temperature and light. These findings underscore the multifaceted involvement of miRNAs in orchestrating plant physiological processes and their adaptation to environmental challenges [9]. Of which, miR156—as one of the most evolutionarily conserved miRNAs—forms a regulation module (miR156-SPLs) with its targeted SPL (SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE) genes to act as a negative regulator of plant shade-avoidance syndrome. The module is further regulated by shade-activating transcription factor PIFs (PHYTOCHROME INTERACTING FACTORS), which bind to the promoters of miR156s and suppress their expression in *Arabidopsis* [10].

The microRNA164 (miR164) family is another highly conserved class of miRNAs in plants [11]. In *Arabidopsis*, the miR164 family consists of three members (miR164a, miR164b, miR164c), which mediate five NAC (NAM, ATAF1/2, and CUC2) transcription factor genes; in rice, it contains six members of miR164a/b/f, miR164c, miR164d, and miR164e, which mainly target six NAC transcription factors (*OMT1-6*, *Oryza* miR164-targeted NAC), and some non-NAC genes, such as *PSK5* (phytosulfokines precursor gene) and *PM27* (seed maturation protein gene) [12–15]. These target genes are mainly involved in plant development, senescence-mediated cell death, and biotic/abiotic stresses [13,14]. For example, a number of studies have reported the regulation of miR164 by different environmental stimuli, particularly abiotic stresses, such as salt, cold, high temperature, drought, and heavy metals [12,16–18]. However, other than one study that showed the expression of miR164b was down-regulated under the treatment of high light ($1500 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$) in *Phyllostachys edulis* [17], no study has reported the relationship between miR164 and the plant response to diverse light intensity. In addition, the relationship between miR164 and plant shade/high-light stress tolerance has not yet been found, and the specific response and tolerance mechanisms need to be further explored.

Perennial ryegrass is a popular cool-season (C_3) turfgrass/forage grass species worldwide. Cool-season turfgrasses can potentially endure with as little as 5% of full sunlight. Nevertheless, the typical requirement for healthy growth is usually between 25% and 35% of full sunlight, varying based on the species and the intensity of management practices [19,20]. For perennial ryegrass, its light compensation point and saturation point were reported at 150 and $867 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$, respectively [21]. Either shade or high-light microenvironmental conditions are common for cool-season turfgrasses, including perennial ryegrass, and the growth and development of plants are greatly influenced by the quantity of light available for photosynthesis [22,23]. Therefore, the objective of this study aimed to elucidate the mechanisms by which miR164 and its target genes, such as NAC60, influence light adaptation. The findings from this study are expected to provide valuable insights into the molecular pathways governing plant adaptation to changing light environments, potentially informing genetic strategies for improving turfgrass resilience and performance under diverse light conditions.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions, and Experiment Treatments

Perennial ryegrass “Citation Fore” (Pure Seed, Canby, OR, USA) was used in this study. Overexpressing (OE164), target mimicry (MIM164) rice *miR164a*, and CRES-T (chimeric repressor gene-silencing technology) rice *NAC60* (NAC60) transgenic plants were developed as described previously [24–28]. Control plants of the wild type (WT) and one representative line from each transgenic perennial ryegrass variety (OE164, MIM164, and NAC60) were reproduced clonally from tillers within plastic cone tubes (18.5 cm deep and 5 cm wide), containing a soil blend of sand and peat (1:1 ratio). This propagation took place in a greenhouse maintained at China Agricultural University (Beijing, China), with temperature ranges of 15 ± 3 °C during the night and 25 ± 5 °C during the day. Plants with comparable growth statuses were carefully chosen for further experimentation. A total of 32 tubes of plants (8 tubes per type of ryegrass plants) were transplanted to the field in Experiment 1. Half of the plants were planted in a part shade plot (daily light integral, $15\text{--}19 \text{ mol m}^{-2} \text{ d}^{-1}$, LIGHTSCOUT DLI100 Daily light meter, Spectrum Technologies, Inc. Aurora, IL, USA), and another half were grown in a full sun conditions ($35+$ $\text{mol m}^{-2} \text{ d}^{-1}$). In Experiment 2, 72 plant tubes, with 18 tubes per type of ryegrass, were moved to plant growth chambers (RXZ, Jiangnan Instrument and Equipment Company, Ningbo, China). These chambers were equipped with LED white light, providing photosynthetically active radiation (PAR) at a rate of $400 \mu\text{mol s}^{-1} \text{ m}^{-2}$, and maintaining a dark/light cycle of 16/22 °C, 10/14 h, and 70% relative humidity. The plants received watering as necessary, were trimmed to a height of 6 cm weekly, and fertilized biweekly with Miracle-Gro (N-P-K 24-12-14, Scotts, Wuhan, China) at a rate of 5 kg N ha^{-1} per month to sustain normal plant growth. After one week of acclimation to chamber conditions, the plants (six tubes per light intensity treatment) were randomly assigned to three different light intensities for a duration of 28 days (with day/night temperatures set at 22/16 °C and 14/10 h, respectively). These light intensities were categorized as low, medium, and high, with PAR values of 100, 400, and $1200 \mu\text{mol s}^{-1} \text{ m}^{-2}$, respectively. To mitigate microenvironmental effects, tube positions within the chamber were rotated every 24 h throughout the experiment.

2.2. Sampling and Measurements

2.2.1. Morphological Comparison of Transgenic Plants Grown under Different Light Intensity

In Experiment 1, the grass quality was visually evaluated as an integral of color, uniformity, and density on a scale of 1 (poorest or dead leaves) to 9 (a perfect or ideal grass) with 6 or above being acceptable according to the NTEP (National Turfgrass Evaluation Program), 3 months after transplanting to the field. In Experiment 2, plant height was measured from the soil surface to the top of the plants, and the width of the ryegrass leaves was measured using a vernier caliper. This was performed by determining the width of the widest section of the first fully developed leaf on a tiller, measured from the top to the bottom of the plant. Three measurements of randomly selected leaves were taken for each tube of plants.

2.2.2. Plant Leaf Electron Microscopy

Both scanning electron microscopy (SEM) analysis and transmission electron microscopy (TEM) observation were prepared according to Fowke [29]. Leaf samples were collected from identical relative positions on the longest tillers of OE164, MIM164, and WT ryegrass plants, and then sectioned into segments of 1–2 cm. These segments were subsequently immersed in a phosphate buffer (pH 7.2) containing 2.5% (*v/v*) glutaraldehyde for fixation. After dehydration, some of the samples were coated with gold particles and examined under a scanning electron microscope (SU8010, Hitachi, Tokyo, Japan) to analyze the coated surfaces [30]. The other samples were then washed and immersed in 1% osmium acid for one day. Later the samples were dehydrated and polymerized. Ultrathin

sections with an ultramicrotome (EM UC7, Leica, Wetzlar, Germany) were photographed using a transmission electron microscope (JEM1230, Jeol, Tokyo, Japan).

2.2.3. Measurement of Physiological Parameters

In Experiment 1, fully expanded leaves were harvested 3 months after transplanting to the field. Leaf chlorophylls and carotenoids were extracted using dimethyl sulphoxide (DMSO) and quantified as described previously [31]. The absorbance of extracts was read at 665, 649, and 480 nm with a UV-Vis spectrophotometer (UH5300, Hitachi, Tokyo, Japan). Chlorophyll a, b, and carotenoids were calculated as milligrams per gram dry weight (DW) using the equations below: Chlorophyll a (Chla) = $12.19 \times A_{665} - 3.45 \times A_{649}$; Chlorophyll b (Chlb) = $21.99 \times A_{649} - 3.52 \times A_{665}$; and Carotenoids = $(1000 \times A_{480} - 2.14 \times \text{Chla} - 70.16 \times \text{Chlb}) / 220$. In Experiment 2, the SPAD index value (representing leaf total chlorophyll content) was recorded 21 d after light treatments using a chlorophyll meter according to the user guide (SPAD-502Plus, Konica Minolta, Tokyo, Japan). The leaf maximum (Fv/Fm) and effective (Y(II)) photochemical quantum yields of PS II were measured 14 d after light treatments using a MINI PAM chlorophyll fluorescence meter (Walz, Effeltrich, Germany) according to the user manual.

Enzyme extracts were prepared following the method described by Wang et al. [32]. Leaf samples weighing 0.2 g were rapidly frozen in liquid nitrogen and then ground in 4 mL of a solution containing 50 mM phosphate buffer (PBS) at pH 7.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 1% polyvinylpyrrolidone (PVPP). The resulting homogenate was centrifuged at $12,000 \times g$ for 15 min at 4 °C, and the supernatant was used to assay superoxide dismutase (SOD) activity. To measure SOD activity, the reaction mixture without the enzyme extract developed the maximum color after illumination, representing the maximum reduction in p-Nitro-Blue tetrazolium chloride (NBT), while the mixture without illumination served as the control. The absorbance at 560 nm (A_{560}) of the reaction mixture was measured using a Hitachi UH5300 spectrophotometer.

2.3. Experimental Design and Statistical Analysis

Experiment 1 and Experiment 2 were a split-plot design with four and six replications, respectively. The light treatments (shade/full sun or low/medium/high light) were the main plot and four genotypes of ryegrass plants (OE164, NAC60, MIM164, and WT) were the split plot. The analyses of the two experiments were conducted individually. Unless specified otherwise, all measurements were scrutinized utilizing the samples gathered according to the previously described sampling time. The data acquired were subjected to analysis employing SPSS software (Version 25.0, IBM, Armonk, NY, USA). Distinctions among treatments were discerned through the application of Fisher's least-significant-difference (LSD) test, with a significance threshold set at 0.05.

3. Results and Discussion

3.1. OE164, NAC60, MIM164, and WT Plants Performed Differently under Shade or Full Sun Field Conditions

3.1.1. Grass Quality and Photosynthetic Pigments

Unlike animals, light stands out as a pivotal environmental element that significantly influences the growth and development of plants. It plays a crucial role in processes like photosynthesis and photomorphogenesis. Just like all biological conditions, too much light can be as detrimental as too little light [33,34]. In Experiment 1, the grass quality data showed that all the ryegrass plants performed better under shade than those under full sun conditions, regardless of the genotype. Among them, the WT plant was overall the best with a quality rating of about 6 and 8 under full sun and shade, respectively. The grass quality of MIM164 was better than those of both OE164 and NAC60, which were poor and lower than acceptable, particularly under full sun conditions (Figure 1A,B,F). Nearly all four replications of NAC60 plants were dead under full sun, for which NAC60 was not included in the chlorophyll measurements nor electron microscopy analysis later.

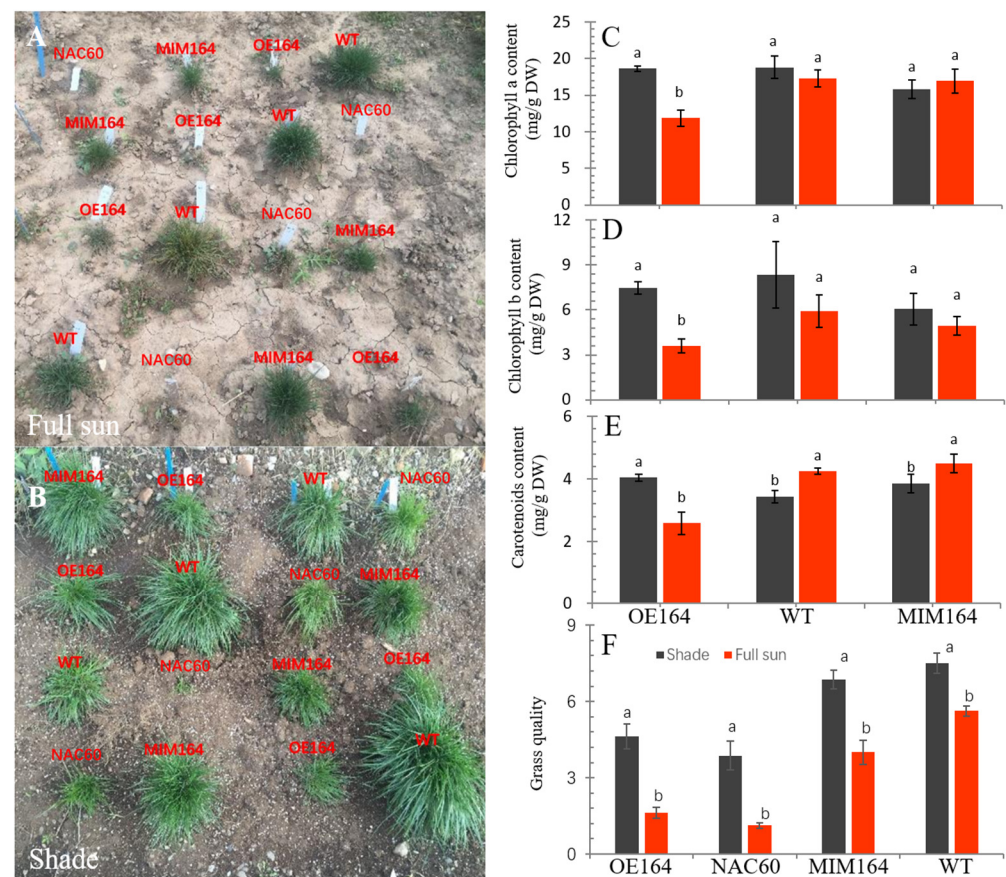


Figure 1. OE164, NAC60, MIM164, and WT perennial ryegrass plants under full sun (A) and shade (B) conditions in the field. (C–E) Chlorophyll a, b, and carotenoid contents of the *OsmiR164a* overexpression (OE164), target mimicry *OsmiR164a* (MIM164) transgenic and wild-type (WT) plants. (F) Grass quality of OE164, NAC60 (CRES-T *OsNAC60*), MIM164, and WT plants. Different letters indicate statistical differences ($p < 0.05$).

Photosynthetic pigments, including chlorophyll a, chlorophyll b, and carotenoids vary under different abiotic stresses, including shade and high light. Chlorophylls absorb light energy and are directly related to photosynthesis, and carotenoids function in transferring the light energy absorbed by chlorophylls and play a protective role in photosynthesis [2,35]. Here both chlorophyll a and b contents of OE164 decreased under full sun compared to the shade condition, and they showed an overall reducing trend but this was not significantly different between the light conditions in both WT and MIM164 plants (Figure 1C,D). In the meanwhile, the carotenoid content declined under full sun but they increased in both WT and MIM164 plants (Figure 1E). These generally agreed with the overall performance demonstrated by the grass quality data. Reduced chlorophyll content under high light is proposed as an indicator of chlorophyll destruction through excessive irradiation [36]. Zhang, Ge, et al. [37] reported that the lower chlorophyll content of *Heptacodium miconioides* seedlings under high light (full sun) was also a result of expression changes in chlorophyll metabolism-related genes, such as the higher level of a chlorophyll-degradation associated gene, *PAO* (pheophorbide a oxygenase) [38]. Previous studies indicated that a target gene of *miR164*, *ORE1/NAC2*, directly regulates chlorophyll catabolic genes, including *PAO* and *PPH* (pheophytin pheophorbide hydrolase), during ethylene-mediated senescence [38–41]. Under shade, OE164 maintained a higher level of chlorophyll content than MIM164 plants (Figure 1C,D), which might be related to the role of *miR164* in leaf senescence. Future analysis of the expressions of photosynthetic and chlorophyll metabolism-related genes in the four genotypes of ryegrass plants would help to understand more details of the regulatory role of *miR164* in plant responses to light.

3.1.2. Leaf SEM and TEM Analysis

Leaf scanning electron microscopy analysis found that a few siliceous papillae on the leaf surfaces were only observed in WT plants under full sun conditions. Further epidermal cell-length measurement found that the cells of OE164 and MIM164 plants under shade were longer than those under full sunlight conditions. A similar trend in the WT plants was observed, while the difference was not significant (Figure 2A–F,M). This was somehow consistent with the plant phenotype we observed. Under full sunlight, all four genotypes of plants, including OE164 and MIM164, were shorter and smaller than those under shade conditions (Figure 1A,B). Dunn et al. [42] reported four grass species increased cell length in shaded treatments. Other than the high light intensity, the increased blue light perceived by cryptochromes under full sun compared to shade conditions would also inhibit the elongation of stems and leaves [3,43]. Additionally, magnified pictures showed that the stomatal length of OE164 plants decreased under full sun in comparison to their length in shaded environments. On the contrary, it increased in MIM164 plants (Figure 2G–L,N). Stomatal characteristics are highly responsive to light variations due to their integral role in photosynthesis. This process, wherein plants convert carbon dioxide and water into energy and oxygen using light, occurs primarily in the stomata. Stomata intake carbon dioxide and release oxygen so their aperture size influences the plant's response to light intensity. In high light, stomata open wider to increase carbon dioxide intake, boosting photosynthesis. Under low or excessive light, stomata may close to prevent water loss via transpiration. Hence, analyzing stomatal traits like size, density, and aperture can reveal plant adaptations to light conditions. Plants with larger, denser stomata are suited to bright environments, while those with smaller, more regulated stomata thrive in low or fluctuating light. Stomatal traits thus indicate a plant's light adaptability [44,45]. Zhang et al. [34] reported the stomata of seedlings under high light had larger length and width than those under low light. Higher lighting conditions prompt the opening of stomata, facilitating greater CO₂ absorption [46]. Conversely, when light levels become excessive, stomata close swiftly to reduce water loss and prevent xylem damage by limiting transpiration, thereby protecting against photoinhibition [23,47]. Moreover, Phookaew et al. [48] found the expression levels of *miR164*/*MesNAC* in Cassava (*Manihot esculenta*) were found to vary in relation to stomatal closure changes due to water deficits; however, how exactly *miR164* was involved in the stomatal response difference among the three genotypes of ryegrass plants would need further investigation.

Based on the TEM observation, the number of chloroplasts and peroxisomes of the three genotypes of plants under full sunlight conditions increased compared with shade (Figure 3). Under high light conditions, the rate of photorespiration increases, necessitating a greater peroxisomal activity to manage the resultant oxidative stress. Enhanced photosynthesis leads to the accumulation of larger starch granules as more glucose is converted into starch for storage. In contrast, under low light conditions, photosynthetic activity diminishes, resulting in smaller starch granules due to the reduced conversion of glucose into starch. Thus, the size of starch granules can reflect the photosynthetic capacity of ryegrass under different light intensities. Under the shading condition, the starch grains produced by chloroplasts in the OE164 plants were significantly bigger than those under the full sunlight condition, which indicated that the photosynthetic yield of OE164 plants under the shading condition is possibly higher than that under full sun. This was in accordance with the better performance of OE164 under shade (Figure 1). In the meanwhile, in high-light environments, excessive light energy can damage the photosynthetic machinery. To mitigate this, plants increase the extent of thylakoid stacking to enhance non-photochemical quenching, which dissipates excess energy as heat. Conversely, in low light conditions, thylakoid stacking is reduced to maximize the efficiency of light capture and utilization. The OE164 plants showed less thylakoid stacking than MIM164 and WT under shade (Figure 3C,F,I), whereas they had larger and more swelling in the mitochondria than MIM164 and WT under full sun, particularly when compared to the WT (Figure 3L,O,R). Chloroplasts from shade leaves often show substantial thylakoid

stacking, and small granal stacks are typical of chloroplasts from high-light leaves [3]. Lin, Zhu, et al. [49] reported the loss of function of tomato (*Solanum lycopersicum*) miR164a by CRISPR/Cas9-mediated mutagenesis resulted in enhanced chloroplast development. Under abiotic stresses, such as temperature and oxidative stresses, mitochondrial swelling and ultrastructural disorganization occur [50,51]; moreover, changes in mitochondrial morphology including mitochondrial swelling and aggregation have been observed during programmed cell death (PCD) progression in vivo [52].

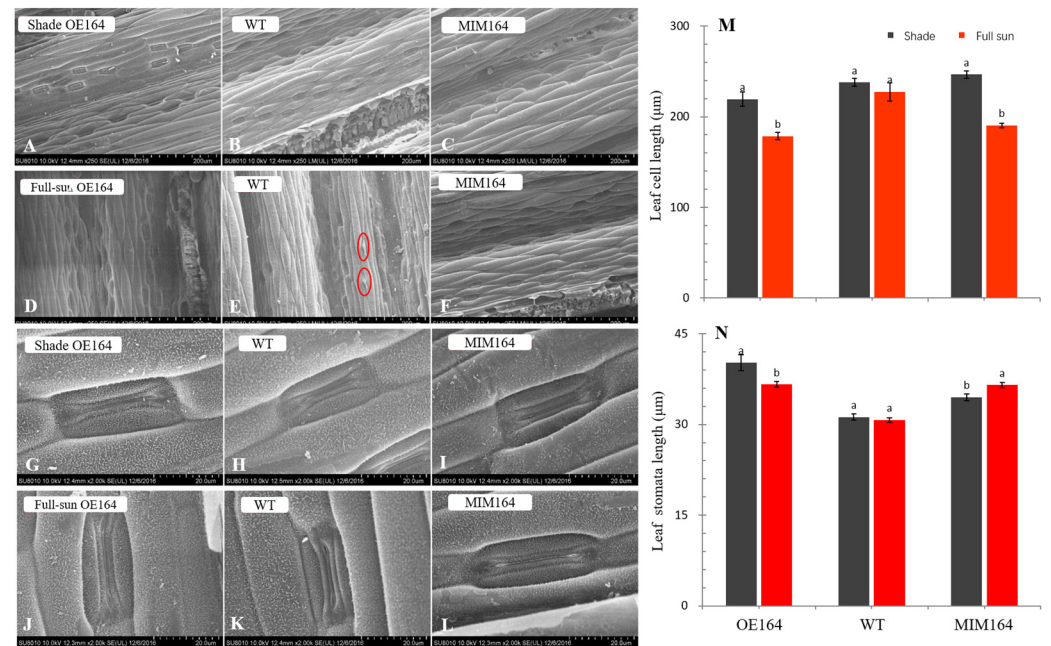


Figure 2. Scanning electron microscopy (SEM) analysis of leaf surface of OE164, MIM164, and WT perennial ryegrass plants in full sun and shade conditions in the field. (A–F) Representative SEM images of leaf epidermal cells, the red circle represented siliceous papillae. (G–L) Representative SEM images of leaf stomata. (M) Leaf cell length. (N) Leaf stomata length. Different letters indicate statistical differences ($p < 0.05$).

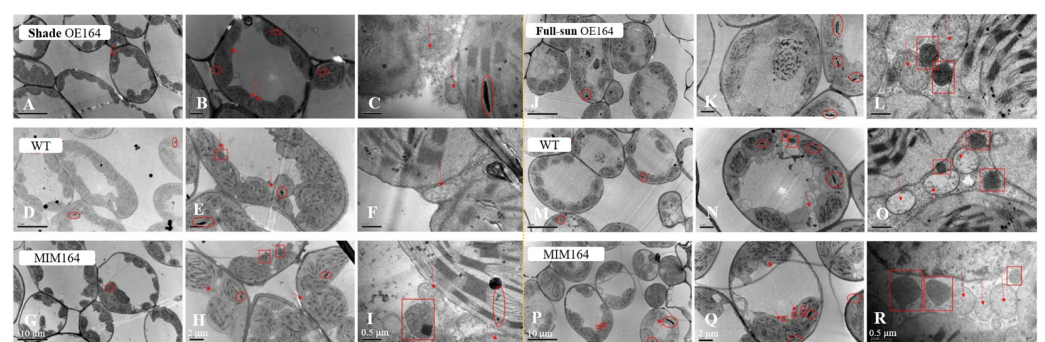


Figure 3. Transmission electron microscopy (TEM) observation of leaf cells of OE164, WT, and MIM164 perennial ryegrass plants in shade (A–I) and full sun (J–R) conditions in the field. The red circle, arrow, and rectangle indicated starch grains in chloroplast, mitochondria, and peroxisomes, respectively.

3.2. OE164, NAC60, MIM164, and WT Plants Responded Similarly to Various Light Intensities in Growth Chambers but to Different Extents

3.2.1. The Morphology of OE164, NAC60, MIM164, and WT Plants

Similar to the observation in Experiment 1, plant height decreased with the increase in light intensity, regardless of the genotypes (Figure 4). Among them, OE164 and NAC60

looked most alike. For example, from low light to high light treatment, the heights of OE164 and NAC60 decreased from 17.0, 11.6, and 7.8 cm, and 16.2, 12.4, to 8.7 cm, respectively; MIM164 decreased from 17.8, 16.2, to 11.2 cm (Figure 4D–F). The leaf width of MIM164 was the narrowest, and the leaf widths of all four genotypes of plants were relatively unchanged across the light treatments (Figure 5). However, different from Experiment 1, the overall dwarf phenotype under high light intensity in Experiment 2 was not as severe as that under full sun field conditions, especially for the OE164 and NAC60 plants. With the global environmental changes, increasing UV and temperature are becoming increasing threats to plant growth and development [53–55]. As a cool-season grass, perennial ryegrass is known to be UV and high-temperature sensitive [56,57]; thus, we assumed that in addition to high light intensity, the increased UV radiation and summer high temperature under full sun conditions could also account for the differences between Experiment 1 and Experiment 2.

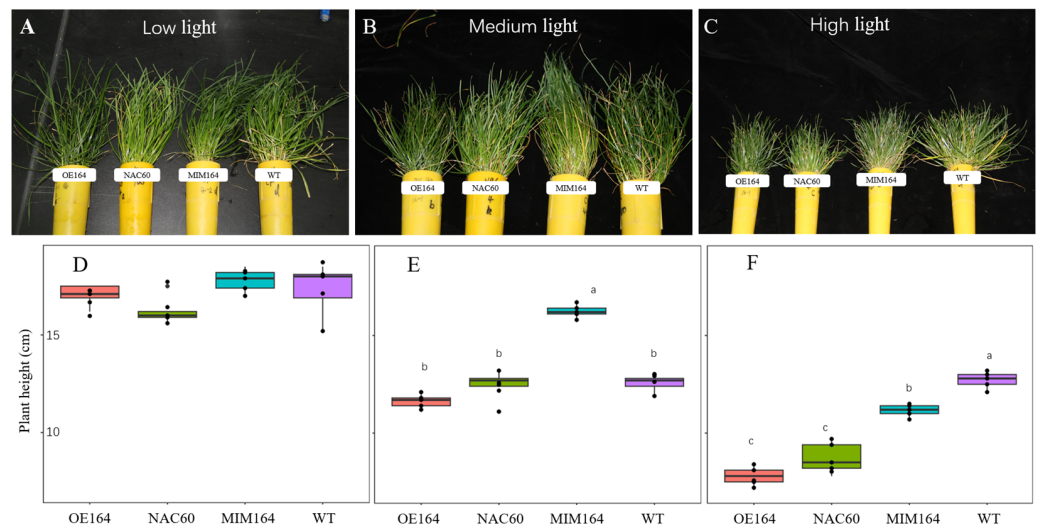


Figure 4. Morphology of OE164, MIM164, NAC60, and WT perennial ryegrass plants under different light intensities in growth chamber: (A,D) plants under low light; (B,E) plants under medium light; (C,F) plants under high light. Different letters indicate statistical differences ($p < 0.05$).

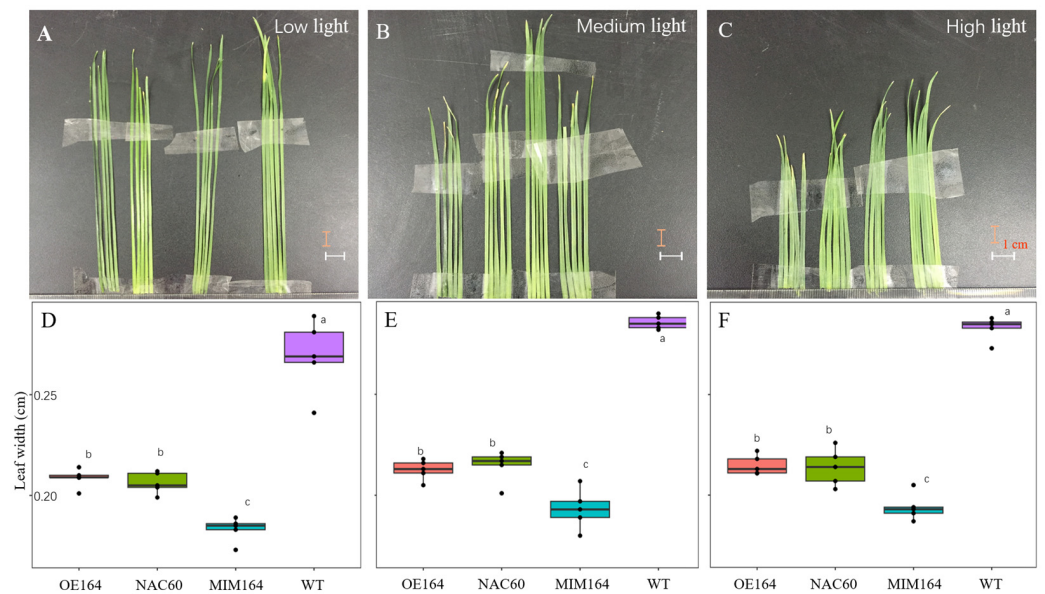


Figure 5. Leaf characteristics of OE164, MIM164, NAC60, and WT perennial ryegrass plants under different light intensities in growth chamber: (A,D), plants under low light; (B,E) plants under medium light; (C,F) plants under high light. Different letters indicate statistical differences ($p < 0.05$).

3.2.2. The Physiology of OE164, NAC60, MIM164, and WT Plants

The intensity of light has a direct impact on the process of light absorption in plants, resulting in alterations in the quantities of chlorophyll pigments and variations in the operational condition of photosystem II (PSII) [58]. Interestingly, a trend of increase in the SPAD index along with light intensity increase was observed (Figure 6C). The rate of photosynthesis is determined by light intensity. At low intensity, the rate of photosynthesis rises with increasing light intensity, peaking at the light saturation point. However, excessive light can damage the photosynthetic machinery, leading to photoinhibition [2,59]. Under strong light, the photosystem II (PSII) is readily inactivated, which is referred to as the photoinhibition of PSII [60]. Overall, the Fv/Fm increased and then decreased slightly with the increase in the light intensity across all four genotypes (Figure 6A). Consequently, the observed decrease in Fv/Fm under intense light reflects the impact of photoinhibition on PSII efficiency; the changing trend of Y(II) was similar to Fv/Fm (Figure 6B). Additionally, the WT plants performed generally better than the other three genotypes regardless of light conditions (Figure 6A,B). Previous studies suggest an increased Fv/Fm and Y(II) in plants grown under normal light compared to shade conditions [33]. Interestingly, the SOD activities showed a trend opposite to Fv/Fm and Y(II) under different light intensities (Figure 6A,B,D). Intense light triggers the generation of reactive oxygen species (ROS), leading to the direct deactivation of the photosystem II (PSII) photochemical reaction center [61]. To avoid/reduce photoinhibition, plants have developed various photoprotection mechanisms, including the reactive oxygen species (ROS) scavenging system [62]. Within a cell, the superoxide dismutases (SODs) serve as the initial defense against reactive oxygen species (ROS). They achieve this by catalyzing the dismutation of the superoxide anion free radical ($O_2^{\cdot-}$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) [63]. Hence, the increase in SOD activity under intense light conditions serves as a photoprotective mechanism against photoinhibition and ROS-induced damage to the PSII. The trend of higher leaf SOD activities at both low and high light conditions than medium light might reflect a stress-induced antioxidant response, which is in accordance with the lower Fv/Fm and Y(II). Like high light stress, shade/low light could result in increased SOD activity, along with accumulation of the malondialdehyde content due to ROS damage [64]. The relationship between Fv/Fm, photoinhibition, and SOD activity reflects the dynamic response of the photosynthetic apparatus to varying light intensities. Decreased Fv/Fm values indicate the onset of photoinhibition due to excessive light, while increased SOD activity signifies the activation of photoprotective mechanisms to mitigate ROS-induced damage and maintain PSII efficiency under high light stress; however, the higher SOD activity of WT plants under both low and medium light more likely indicates a more active growth and metabolic status than other ryegrass plants.

3.3. MiR164 Plays a Role in Regulating Plant Adaptation to Changing Light Intensity

The miR164 family is well-known to play important roles in various abiotic stresses. For instance, the miR164-TaNAC14 module regulates drought tolerance in wheat seedlings, with tae-miR164 reducing drought and salinity tolerance by downregulating the expression of *TaNAC14* [12]. Fang et al. [13] also suggest that the conserved miR164-targeted NAC genes may be negative regulators of drought tolerance in rice. However, Lu et al. [65] reported that overexpression of the *peu-miR164* target *PeNAC070* gene in *Arabidopsis* promoted sensitivity of transgenic plants to drought and salt stresses. More recently, the miR164 mutants presented heat-sensitive phenotypes, while 164OE transgenic plants showed better heat tolerance than WT plants [66]. Overall, whether miR164 is a negative or positive regulator of plant tolerance to abiotic stresses is not determined. Wang et al. [17] found high light down-regulated the expression of miR164b in *Phyllostachys edulis* but did not further investigate how miR164 affected the plant responses to light. Here we used OE164, NAC60, MIM164, and WT perennial ryegrass plants to further functionally analyze the role of miR164 in regulating plant adaptation to changing light environments. In this study, the WT plants generally performed the best across different light environments

among all four types of ryegrass plants, and MIM164 was more similar to WT than to either OE164 or NAC60. In addition, OE164 and NAC60 were like each other, and were both sensitive to high light, particularly in the field full sun conditions. These results indicated that a certain/suitable range of miR164 level could be important for plants to maintain a wide adaptation to changing light environments, which were most likely through the regulation of target genes, such as *NAC60*, a putative target gene of miR164 in rice [27]. One possible explanation could be that wild-type plants or wild plants are those that have been naturally selected and adapted in natural environments for hundreds of thousands of years. For example, wild plants are generally thought to have higher plant plasticity or greater phenotypic homeostasis than crops [67,68]. Further experiments would be needed to investigate how the regulation module of miR164-NACs works under varying light intensity conditions (e.g., shade, high light).

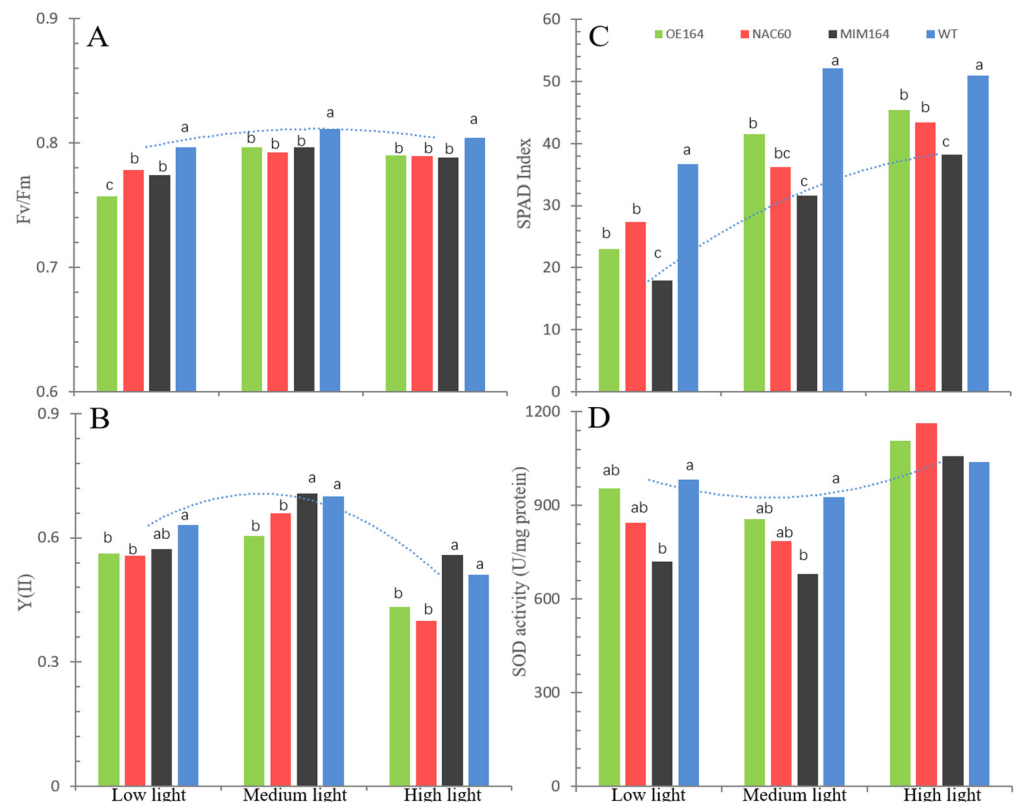


Figure 6. Effects of light intensity on leaf maximum ((A), Fv/Fm) and effective ((B), Y(II)) photochemical quantum yields of PS II, SPAD index (C), and superoxide dismutase ((D), SOD) activity in OE164, NAC60, MIM164, and WT perennial ryegrass plants. Different letters indicate statistical differences ($p < 0.05$).

4. Conclusions

In summation, morphological and physiological analysis of OE164, NAC60, MIM160, and WT plants under different light intensity conditions showed that the *miR164* gene affected perennial ryegrass responses to changing light environments in both field and growth chamber experiments. Particularly, both OE164 and NAC60 plants presented high-light sensitive phenotypes in the field full sun condition, such as much smaller plants, lower grass quality, and more mitochondrial swelling. This study indicates that miR164 and/or its targeted genes can serve as new genetic resources for plant light adaptation breeding in the future. Future research will focus on further elucidating the molecular mechanisms underlying miR164-mediated light sensitivity and exploring the potential for genetic modification to enhance light tolerance in perennial ryegrass and other crop species. These efforts aim to develop new plant varieties better suited to varying light

conditions, which is increasingly important in the context of climate change and the need for sustainable agricultural practices. The successful application of these genetic resources could lead to significant improvements in turfgrass yield and quality, thereby contributing to global food security and agricultural sustainability.

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