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ARTICLE

Lack of Tocopherol Inhibits Rice Growth by Triggering an Ectopic Stress Response and the Accumulation of DELLA Protein

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ABSTRACT

Although tocopherols are essential for rice development, the molecular details by which their absence affects development remain to be determined. To study how tocopherols function during rice development, we performed a transcriptome deep sequencing (RNA-seq) analysis of the rice cultivar Nipponbare (Nip) and the tocopherol-deficient mutant *small grain and dwarf 1-2* (*sgd1-2*). We identified 563 differentially expressed genes that were enriched in Gene Ontology categories associated with metabolism, stress, cellular responses, and transcriptional regulation. We determined that the total fatty acid composition of Nip and *sgd1-2* was comparable, although cell membrane penetrability in *sgd1-2* was significantly higher than in Nip under optimal growth conditions, indicating that tocopherol deficiency induces cell membrane damage. The expression levels of *dehydration-responsive element binding 1* (*DREB1*) genes and free proline content in *sgd1-2* were also higher than those in Nip. We also showed that the DELLA protein SLENDER RICE1 (SLR1) accumulated in *sgd1-2*, resulting in significant changes in the global transcriptome. Our study confirms that the lack of tocopherol accumulation in rice induced ectopic stress responses and limited growth by enhancing SLR1 abundance through increasing *SLR1* transcript levels. These results provide new insights into tocopherol during rice development.

KEYWORDS

Tocopherol deficiency; ectopic stress response; gibberellin; rice

1 Introduction

One of the vital functions of vitamin E, which is an integral part of the human diet, is as an antioxidant that protects membranes and helps prevent the onset of cardiovascular diseases [1,2]. Tocopherols (α, β, γ, and δ) and tocotrienols (α, β, γ, and δ) are two of the eight forms of vitamin E. Tocopherols are found in cell membranes and are linked to highly polyunsaturated fatty acids (PUFAs), through which they influence membrane characteristics such as permeability and stability [3–6]. Only certain cyanobacteria and plants can naturally produce tocopherols. The single distinguishing feature between tocopherols and tocotrienols is the presence of isoprenoid side chains in tocotrienols, which can be produced from either

phytyl-diphosphate (PDP) or geranylgeranyl-diphosphate (GGDP) [7]. The second step in tocopherol biosynthesis is the condensation of homogentisate (HGA) and PDP by homogentisate phytyltransferase (HPT/VTE2) [8]. The subsequent methylation and cyclization reactions that lead to the biosynthesis of γ- and δ-tocopherols, respectively, are catalyzed by VITAMIN E DEFECTIVE 3 (VTE3) and VTE1 [9,10]. VTE4 then catalyzes the conversion of both γ- and δ-tocopherols into α- and β-tocopherols, respectively [11].

Numerous plant tocopherol biosynthesis mutants have been identified and characterized [12–14]. The tocopherol-deficient *vte2* mutants in Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) are susceptible to cold stress, despite the different phenotypes displayed by these mutants when grown under ideal growth conditions [15,16]. Under non-freezing low-temperature conditions, Arabidopsis *vte2* mutants exhibit substantial growth retardation [17]. Linoleic acid desaturation in the *vte2* mutant is lower, primarily in membrane lipids produced in the endoplasmic reticulum (ER) rather than in chloroplasts before low-temperature treatment. Exposure to low temperature worsens this ER membrane lipid phenotype, which can be fully rescued by the introduction of mutant alleles of the ER-resident oleate desaturase genes *FATTY ACID DESATURASE2* (*FAD2*) and the ER-to-plastid lipid transporter genes *TRIGALACTOSYLDIACYLGLYCEROL1-4* (*TGD1-4*) [18,19]. Tocopherols may directly interact with ER-resident enzymes, as demonstrated by trans-organellar complementation, and may therefore play a role in determining membrane composition [20]. After exposure to cold stress, the survival rate of the rice mutant *small grain and dwarf1* (*sgd1*, also named *rice tocopherol deficiency 1* [*rtd1*]) lacking the function of a VTE2-like enzyme, was much lower than that of the wild type [15,16]. These findings suggested that tocopherols may influence cold stress tolerance by changing membrane composition. However, the chemical mechanisms underlying the link between low-temperature sensitivity and tocopherol deficiency need to be better understood.

Plant adaptation to cold temperatures is mostly regulated by the *C-REPEAT BINDING FACTOR* (*CBF*)/*DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN1* (*DREB1*) cold-responsive pathway. The transcription of many stress-inducible genes is under the control of the transcription factors CBF/DREB1, which bind to the DRE/CRT *cis*-acting element [21]. The Arabidopsis genome encodes three CBF proteins: CBF1/DREB1B, CBF2/DREB1C, and CBF3/DREB1A; the rice genome harbors 10 potential *DREB1* homologs (*OsDREB1A* to *OsDREB1J*). Cold stress was previously shown to induce the expression of 6 of these 10 genes (*OsDREB1A*, *OsDREB1B*, *OsDREB1C*, *OsDREB1E*, *OsDREB1F*, and *OsDREB1G*). Moreover, transgenic rice plants overexpressing *OsDREB1* genes showed improved resistance to drought, high-salt, and low-temperature stressors [22,23]. The above examples underscore how *OsDREB1* genes are essential for the rice cold response pathway.

Gibberellins (GAs) are a class of tetracyclic diterpenoid plant hormones that have a variety of biological roles, including the promotion of flowering and seed germination, stem and leaf growth, and seed germination [24]. Aspartic Acid–Glutamic Acid–Leucine–Leucine–Alanine Protein (DELLA) proteins are crucial regulators of GA responses and essential components of the GA signaling cascade. DELLAs negatively regulate the GA response by preventing transcription factors from directly activating the expression of their downstream genes, while also indirectly regulating GA biosynthesis genes to increase GA responsiveness by promoting the transcription of the GA receptor gene *GIBBERELLIN INSENSITIVE DWARF1* (*GID1*). GA communicates extensively and establishes crosstalk with other plant hormones to regulate plant growth and development [25]. The Arabidopsis genome encodes five DELLAs: GA-INSENSITIVE (GAI), REPRESSOR of GA1-3 (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3 [26]. The GA response phenotype of the rice mutant *slender rice 1-1* (*slr1-1*) demonstrated that the single DELLA protein SLR1 of rice also regulates GA signaling [27]. The discovery that growth constraints caused by exposure to various types of abiotic stresses is at least partially mediated by DELLAs represents a significant advance in our understanding of the role of these proteins in regulating plant development. Moreover, Arabidopsis seedlings exposed to salt or cold stress displayed lower levels of

endogenous bioactive GAs and a concomitant accumulation of DELLAs [28,29]. CBF1 was also demonstrated to control plant growth via a DELLA-dependent signaling cascade [29].

We previously showed that tocopherols are crucial for rice growth, based on the characterization of the *sgd1-2* mutant. Indeed, the *sgd1-2* mutant displayed a pronounced dwarf phenotype and produced small grains relative to its wild-type Nipponbare (Nip). The *sgd1-2* mutant also showed hypersensitivity to cold stress [15]. However, how rice development is affected by the loss of tocopherol accumulation is unknown. To begin to answer this question, we explored the effects of the loss of tocopherol on global gene expression using transcriptome deep sequencing (RNA-seq) of the wild-type Nip and the *sgd1-2* mutant. We identified 563 differentially expressed genes (DEGs) that were enriched in Gene Ontology (GO) categories related to transcriptional control, stress, metabolism, and other cellular processes. We observed no differences in the total fatty acid contents of Nip and *sgd1-2*. However, cell membrane penetrability was much higher in *sgd1-2* than in Nip when grown under optimal growth conditions, indicating that the absence of tocopherol damaged the cell membrane. The expression levels of *CBF/DREB1* genes and free proline content were also higher in *sgd1-2* compared to Nip, indicating that an ectopic stress response was induced in *sgd1-2*. Finally, the accumulation of SLR1 in the *sgd1-2* mutant suggested that tocopherol deficiency affects rice growth via the GA signaling pathway.

2 Materials and Methods

2.1 Mutant Materials

The previously characterized *sgd1-2* mutant in the rice (*O. sativa* L.) Nip background was used in this study [15]. The wild-type cultivar Nip and the *sgd1-2* mutant were grown in a growth chamber under a 16-h-light/8-h-dark photoperiod and 30/22°C day/night temperature cycle.

2.2 RNA-Seq and RT-qPCR Analysis

Samples were collected from 2-week-old Nip and *sgd1-2* seedlings for RNA-seq. Total RNA extraction, library construction and sequencing, and subsequent analysis were carried out by Nanjing Genepioneer Institute (Nanjing, China). Three biological replicates were analyzed for each genotype. One-week-old Nip and *sgd1-2* seedlings were also collected for RT-qPCR analysis. The internal reference transcript was *OsACTIN1*. Primers used for qPCR of *DREB1* genes were reported in an earlier work [22]. Table S1 provides the list of other primers used in this investigation.

2.3 Fatty Acid Composition Analysis

One-week-old Nip and *sgd1-2* seedlings were harvested and their fatty acid methyl esters determined in accordance with a previously published method [30]. Briefly, Nip and *sgd1-2* seedlings were frozen in liquid nitrogen, crushed coarsely, and extracted in acidic methanol. The extracts were combined with 1 mL of hexane and 1 mL of 0.9% (w/v) NaCl after incubation at 80°C for 2 h. The top organic layer was collected and dried under nitrogen gas flow. Hexane (25 mL) was used to reconstitute the samples. The fatty acid methyl esters were evaluated by gas chromatography and quantified by flame ionization detection largely as previously reported, using internal heptadecanoic acid (C17:0) as standard [31,32].

2.4 Cell Membrane Penetrability Assay

Electrolyte leakage (EL) was determined as EL1/EL2 of the cell membrane as previously published with a few changes and was used to assess the permeability of the cell membrane [33].

2.5 Proline Content Analysis

Proline contents were measured using a proline assay kit according to the manufacturer's recommendations (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) from three biological replicates.

2.6 GA Sensitivity Assay

The α-amylase test was carried out essentially as previously described [34]. An assay based on second leaf sheath elongation was conducted to test the sensitivity to GA, largely according to the published method [35]. Surface-sterilized rice seeds were incubated for 2 days at 30°C in complete darkness. Germinated seedlings were then transferred to half-strength Murashige and Skoog medium contained various concentrations of GA_3 (0.01, 0.1, 1, 10, and 100 μM), an grew under a 16-h-light/8-h-dark photoperiod and a 28/22°C day/night temperature cycle, with 15 seeds per plate. After 7 days of growth, the lengths of the second leaf sheaths were measured.

2.7 GA Quantification

The leaves of 10-day-old seedlings (0.5 g) were frozen in liquid nitrogen, crushed finely, and extracted overnight at 4°C in 80% (v/v) methanol. Levels of endogenous GA_1 were quantified as described previously [36].

2.8 Immunoblot Analysis

A polyclonal anti-SLR1 antibody was produced by ABclonal (www.abclonal.com). Ten-day-old seedlings of Nip and *sgd1-2* were treated with 10 mM GA_3 or 10 mM PAC (paclobutrazol) for 6 h or with double distilled water as control. Samples were then collected and processed for immunoblot analysis with the anti-SLR1 antibody, using an anti-EF-1 (Agrisera) antibody as loading control.

3 Results

3.1 Expression Analysis of Nip and the sgd1-2 Mutant

We conducted an RNA-seq analysis of 2-week-old Nip and *sgd1-2* seedlings grown under optimal growth conditions to identify DEGs between the two genotypes. We selected this time point based on the clear dwarf phenotype exhibited by *sgd1-2* relative to Nip. Table S2 provides a summary of the RNA-seq analysis. We defined a gene as being differentially expressed when its expression level differed by at least two-fold between *sgd1-2* and Nip. We obtained 563 DEGs between Nip and *sgd1-2*, of which 383 genes were upregulated in *sgd1-2* and the remaining 180 genes were downregulated (with a false discovery rate [FDR] $P < 0.05$). 9 DEGs were selected for RT-qPCR validation of the RNA-seq data. The RT-qPCR results were consistent with those of the RNA-seq data (Table S3). We then performed a GO enrichment analysis with these DEGs, which revealed an enrichment for genes related to metabolism, stress, cellular processes, and transcriptional control in the mutant (Fig. 1).

3.2 Tocopherol Deficiency Induces Cell Membrane Damage

We collected 1-week-old Nip and *sgd1-2* seedlings to quantify their membrane lipid profiles and membrane integrity. We chose this earlier time point because of the many DEGs that were enriched in GO categories known to be related to lipid and fatty acid metabolism. We did not observe differences between Nip and *sgd1-2* in terms of their total fatty acid contents (Fig. 2A). Tocopherols were thought to solely affect membrane lipids produced in the ER, but not those produced in plastids, as shown in Arabidopsis. We examined the cell membrane penetrability of Nip and *sgd1-2* seedlings grown under ideal conditions. We determined that the cell membrane of the *sgd1-2* mutant was more permeable than that of Nip even under optimal growth conditions, indicative of damage to the membrane caused by the lack of tocopherols (Fig. 2B).

3.3 Tocopherol Deficiency Induces Ectopic Stress Response

Relative conductivity measures ion leakage and thus reflects damage to cell membranes caused by cold stress. We asked if *sgd1-2* displayed symptoms typically associated with a cold stress response when grown under normal growth conditions. We measured *CBF*/*DREB1* expression levels in 1-week-old Nip and *sgd1-2* seedlings. Under normal growth conditions, the six *CBF/DREB1* genes (*OsDREB1A*, *OsDREB1B*,

OsDREB1C, *OsDREB1E*, *OsDREB1F*, and *OsDREB1G*) were expressed to higher levels in *sgd1-2* relative to Nip (Fig. 3A). Moreover, the free proline content of the *sgd1-2* mutant was almost two times that of Nip (Fig. 3B). These findings suggest that *sgd1-2* evoked an ectopic stress response due to the lack of tocopherols.

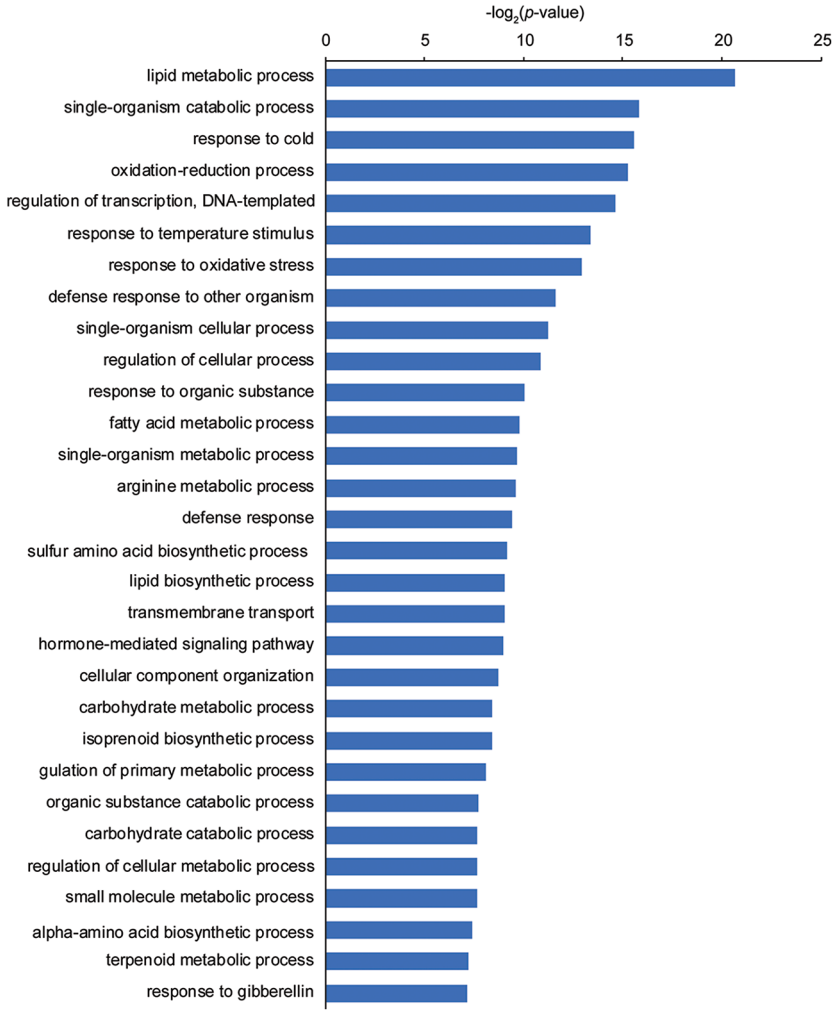

Figure 1: GO analysis of DEGs between Nip and the *sgd1-2* mutant. GO categories enriched among the DEGs between 2-week-old Nip and *sgd1-2* seedlings grown under optimal growth conditions are shown ($P < 0.05$)

3.4 Tocopherol Deficiency Induces the Accumulation of SLR1

Previous research demonstrated that *CBF/DREB1* proteins prevent Arabidopsis development by modulating the abundance of DELLA proteins [29]. We thus applied exogenous GA_3 to Nip and *sgd1-2* seedings and tested them for α-amylase activity using half-seeds without the embryo. We determined that both Nip and *sgd1-2* seeds formed plaques, reflecting α-amylase activity, but the *sgd1-2* mutant formed plaques of smaller diameter than Nip (Fig. 4A). We also treated Nip and *sgd1-2* seedlings with exogenous GA_3, finding that the mutant was less responsive to the treatment than Nip, as evidenced by the shorter stature of the mutant and the modest sheath elongation seen over time (Fig. 4B).

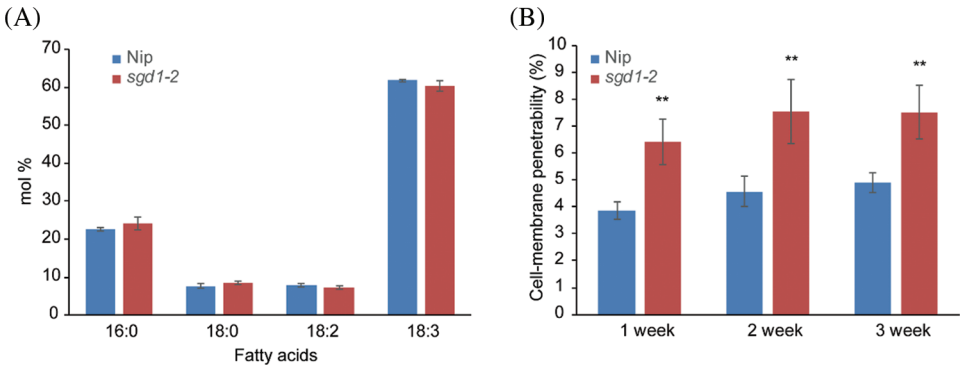

Figure 2: Tocopherol deficiency caused cell membrane damage in *sgd1-2*. (A) Total fatty acid composition in the leaves of 1-week-old Nip and *sgd1-2* seedlings. Values for each fatty acid (number of carbons: number of unsaturated bonds) are means ± standard deviation (SD, n = 3 biological replicates) and are expressed as mol %. (B) Cell membrane penetrability in leaves of Nip and *sgd1-2* seedlings under optimal growth conditions. Values are means ± SD, n = 5 biological replicates, $^{**}P < 0.01$

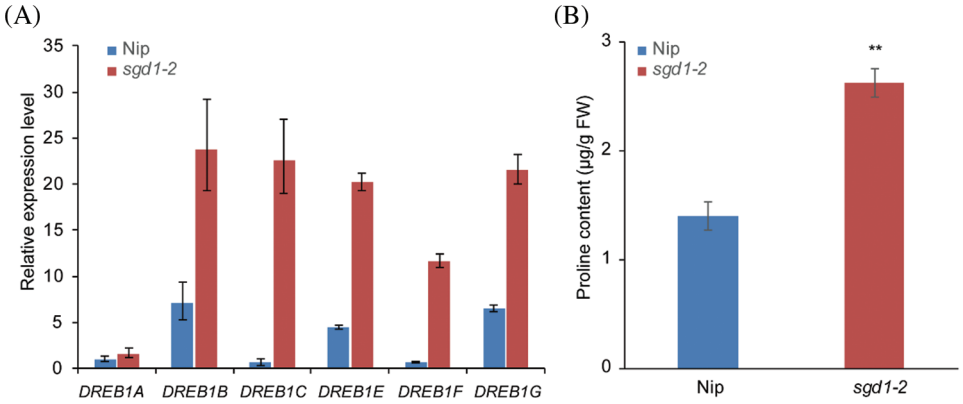

Figure 3: The *sgd1-2* mutant showed an ectopic stress response. (A) Expression levels of *CBF/DREB1* genes in 1-week-old Nip and *sgd1-2* seedlings grown under natural growth conditions. Values are means ± SD, n = 3 biological replicates. (B) Free proline contents in one-week-old Nip and *sgd1-2* seedlings grown under natural growth conditions. Values are means ± SD, n = 3 biological replicates, $^{**}P < 0.01$

We carried out an immunoblot analysis to ascertain SLR1 abundance in Nip and *slr1-2* and discovered that *sgd1-2* accumulated more SLR1 than Nip, in agreement with the lower sensitivity of this mutant to GA (Fig. 5A). Indeed, lower bioactive GA levels resulted in an accumulation of DELLAs, leading to growth inhibition [37,38]. We also measured the levels of endogenous GA_1 in 10-day-old Nip and *sgd1-2* seedlings to investigate whether the higher SLR1 abundance in the *sgd1-2* mutant was caused by altered GA metabolism. However, we observed no difference for the GA_1 levels of Nip and *sgd1-2* (Fig. 5B, Table S4). We also measured *SLR1* expression in the two genotypes and determined that *SLR1* was expressed to much higher levels in the *sgd1-2* mutant than in Nip (Fig. 5C). We conclude that the lack of tocopherols inhibits rice development via the GA signaling pathway.

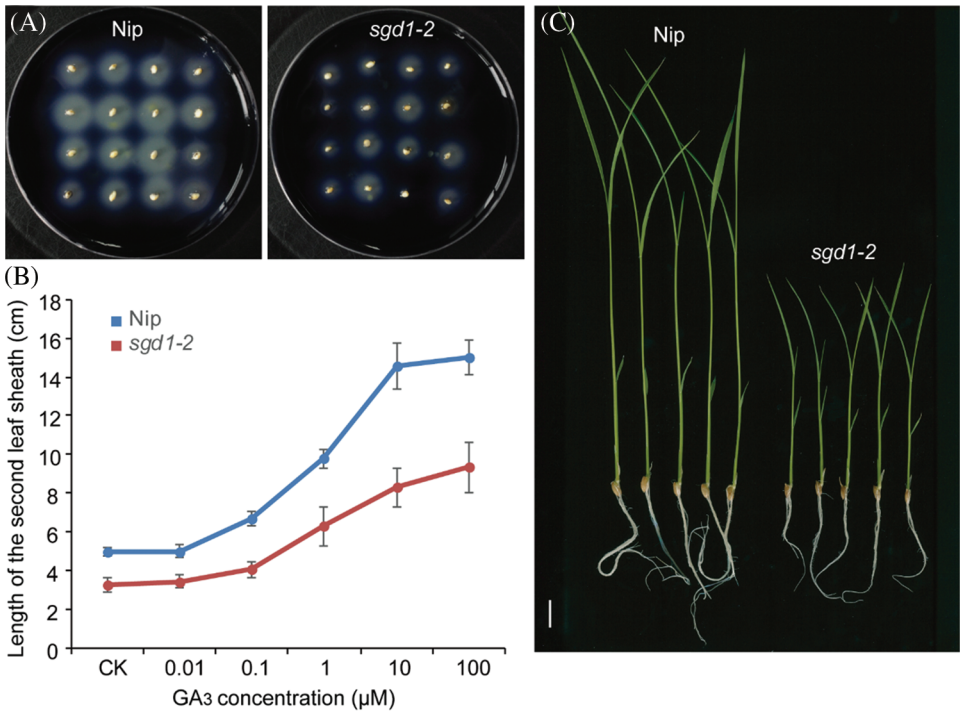

Figure 4: The *sgd1-2* mutant had reduced sensitivity to GA. (A) α-Amylase production from half-seeds of Nip and *sgd1-2* without the embryo. (B) Length of the second leaf sheath in response to GA_3 treatment in 7 days old Nip and *sgd1-2* seedlings. Values are means ± SD, n = 15. (C) Image of Nip and *sgd1-2* seedlings treated with 100 μM GA_3. Scale bar, 2 cm

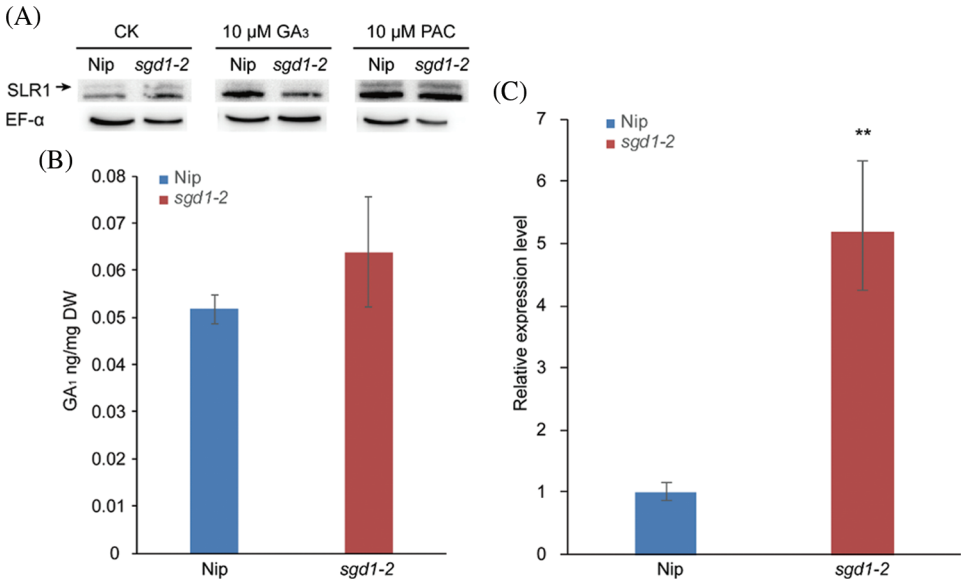

Figure 5: SLR1 abundance and *SLR1* expression levels in *sgd1-2*. (A) Immunoblot analysis of SLR1 in 10-day-old Nip and *sgd1-2* seedlings. The anti-SLR1 antibody detected two bands, with the upper band being degraded upon GA_3 treatment but increasing in intensity following PAC treatment, indicating that the upper band (black arrow) was SLR1. (B) Endogenous GA_1 contents in 10-day-old Nip and *sgd1-2* seedlings. Values are means ± SD, n = 3 biological repeats. DW, dry weight. (C) Relative *SLR1* expression levels in 10-day-old Nip and *sgd1-2* seedlings. Values are means ± SD, n = 3 biological repeats. $^{**}P < 0.01$

4 Discussion

Tocopherols have been shown to be crucial for rice growth and development. The *sgd1-2* mutant showed substantial growth retardation throughout its life cycle. Although tocopherols are considered to have antioxidant properties [39], we did not detect evidence for oxidative damage in the *sgd1-2* mutant, indicating that the growth retardation phenotype of this mutant was not a result of oxidative damage. The *sgd1-2* mutant of rice therefore underscores the fundamental differences in the functions of tocopherols in rice and Arabidopsis. The maize (*Zea mays*) *sucrose export defective1* (*sed1*) mutant exhibits growth retardation reminiscent of the rice *sgd1-2* mutant under ideal growth conditions [13]. Tocopherols may therefore be crucial for the growth of monocots but not dicots.

Many of the DEGs were enriched in GO categories known to be involved in lipid and fatty acid metabolisms, suggesting that tocopherol deficiency may influence lipid composition in rice. However, the total fatty acid composition was identical in Nip and *sgd1-2*. Previous studies have indicated that tocopherols can change membrane lipid composition by modulating lipid biosynthesis in Arabidopsis. Lipidomic analyses using electrospray ionization triple quadrupole mass spectrometry demonstrated that tocopherols specifically affect ER fatty acid desaturation, but not that taking place in plastids [18,19]. Tocopherols may directly interact with ER-resident enzymes, influencing how the composition of membranes is determined [20]. Further investigation is needed to uncover whether and how tocopherols affect ER fatty acid desaturation in rice. Although membrane lipid composition did not show clear changes in *sgd1-2*, cell membrane penetrability was significantly higher in *sgd1-2* than in Nip, suggesting that tocopherol deficiency induced cell membrane damage in rice. Previous studies have reported how changes in tocopherol levels can affect the stability and fluidity of membranes [5,6]. Due to the comparable geometries of tocopherol and asymmetric phospholipids, research in model phospholipid membranes has suggested that tocopherol stabilizes membranes [40,41]. In rice, tocopherol is also necessary for the regular operation of cell membranes. More research is needed to determine the molecular mechanism underlying the association between the lack of tocopherol and cell membrane penetrability in rice.

The CBF/DREB1 cold response pathway is crucial for plant adaptation to cold [42]. The expression of *CBF/DREB1* genes is quickly induced in response to cold stress and causes the transcription of genes (the CBF regulon) that encode a variety of proteins to shield plants from cold stress [43]. We observed that the expression levels of *CBF/DREB1* genes were markedly higher in *sgd1-2* relative to Nip when grown under normal growth conditions. Free proline also accumulated in *sgd1-2*. In addition, many of the DEGs in *sgd1-2* were enriched in GO categories associated with stress responses, suggesting that *sgd1-2* seedlings underwent an ectopic stress response caused by tocopherol deficiency. The cell membrane is the primary site of perception for some stresses in plants [44]. Notably, we detected evidence of cell membrane damage in *sgd1-2*; together with the notion that tocopherols can change membrane lipid composition in Arabidopsis, we propose that the lack of tocopherols in the mutant may modulate the function of the cell membrane and result in the induction of a stress response even in the absence of a stimulus. Both rice and Arabidopsis plants overexpressing *CBF/DREB1* genes display substantial growth retardation even under ideal growth conditions, much like the *sgd1-2* mutant, in which *CBF/DREB1* genes were highly expressed, offering one possible explanation for the dwarf phenotype of the mutant.

CBF1 overexpression inhibits growth in Arabidopsis via the GA signaling pathway. Based on the results of the α-amylase activity assay and leaf sheath elongation following GA_3 treatment, we concluded that *sgd1-2* responded less favorably to GA_3, indicating that tocopherol deficit may also inhibit development in rice via the GA signaling pathway. In Arabidopsis, the accumulation of CBF/DREB1s in response to cold induces the expression of *RGL3*, stabilizes DELLAs, and decreases bioactive GA levels, thus leading to growth inhibition [29]. The contents of bioactive GA_1 were similar in Nip and *sgd1-2*, but we detected higher expression levels for the DELLA-encoding gene *SLR1*, as well as greater SLR1 abundance. That

bioactive GA levels were not affected may be another difference between rice and Arabidopsis. We propose that rice growth is restricted by tocopherol deficiency via the transcriptional regulation of *SLR1* expression levels, directly leading to the accumulation of SLR1 without invoking a posttranslational mechanism. These results provide fresh perspectives on how tocopherols operate during rice growth and development.

Authorship: Di Wang, Hao Gao and Xi Liu designed and conceived the research. Di Wang, Hao Gao, Jian Wang, Baoshan Cheng, Gang Li and Weijun Xu performed the experiments. Di Wang and Xi Liu wrote and revised the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix:

Supplementary Table S1: Primers used in this study

Supplementary Table S2: DEGs in *sgd1-2* relative to Nip seedlings grown under optimal growth conditions

Supplementary Table S3: Validation of RNA-seq data by RT-qPCR analysis

Supplementary Table S4: Summary of endogenous GA contents