



# Genetic analysis and fine-mapping of a new rice mutant, *white and lesion mimic leaf1*

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## Abstract

Rice (*Oryza sativa* L.) leaf color mutants are excellent models for studying chlorophyll biosynthesis and chloroplast development. In this study, we isolated a stable genetic *white and lesion mimic leaf1* (*wlml1*) mutant from an ethyl methanesulfonate (EMS)-mutagenized population of the *indica* cultivar TN1. Compared with wild-type TN1, the *wlml1* mutant had lower contents of chlorophyll and carotenoids, altered chloroplast ultrastructure, and altered regulation of genes associated with chlorophyll metabolism and chloroplast development. In addition, lesions formed on the leaves of *wlml1* plants grown at 20 °C and genes related to disease resistance and antioxidant functions were up-regulated; by contrast, the mutant phenotype was partially suppressed at 28 °C. These findings indicated that *WLML1* might play a role in chlorophyll metabolism and chloroplast development, as well as in biotic and abiotic stress responses. Genetic analysis showed that *WLML1* was controlled by a recessive nuclear gene, and map-based cloning delimited *WLML1* to a 159.7-kb region on chromosome 4 that includes 30 putative open reading frames. Based on these findings, the *wlml1* mutant will be a good genetic material for further studies on chlorophyll metabolism and stress responses in rice.

**Keywords** Rice · Leaf color · Chlorophyll metabolism · Chloroplast development · Stress response

## Introduction

Leaves are the main photosynthetic organs in plants, and changes in leaf color occur during growth and development, including increases in chlorophyll contents on de-etiolation and decreases in chlorophyll during senescence. The study of mutations affecting leaf color can provide insight into

these processes. Most of the existing rice mutants displaying altered leaf color are due to mutations in genes related to chloroplast development or metabolism (Zhang and Gao 2009; Yang et al. 2016, Su et al. 2017). These rice leaf color mutations result in decrease of photosynthetic efficiency and crop production (Ye et al. 2017; Ge et al. 2015), and in severe cases can even lead to plant death (Wang et al. 2018). Recently, leaf color mutants have become useful models for studying physiological and metabolic processes such as chlorophyll metabolism, leaf color breeding, disease resistance, and chloroplast structure, function and development. Leaf color mutants also provide useful visible markers for monitoring self-pollination in male-sterile lines and maintaining the purity of genetic stocks. Therefore, it is important to map and clone rice leaf color genes, and investigate how to regulate leaf color in rice. This information will provide a theoretical basis for crop breeding with high photosynthetic efficiency.

Leaf color mutants have been identified in several species, including rice (Hu et al. 1981), soybean (Palmer and Rodriguez de Cianzio 1985), maize (Lin and Yu 1995), barley (Rudoi and Shcherbakov 1998), and wheat (Marco et al. 1989). Leaf color

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mutants display a variety of leaf colors and patterns including light green, yellow, albino, purple, spots, zebra, altered shades of green, and reversible whitening (Li et al. 2018). The observed changes in leaf color reflect the changes in the type and contents of pigment within the leaves, such as chlorophyll, carotene, and anthocyanin. Chlorophyll plays important roles in determining leaf color as well as capturing and converting light energy into chemical energy (Tanaka and Tanaka 2006; Fromme et al. 2003). Most of the chlorophyll biosynthetic pathway has been determined. It begins with the formation of glutamyl-tRNA, followed by 15 enzymatic reactions involving at least 27 enzymes in higher plants (Liu et al. 2012). Seven chlorophyll biosynthesis genes have been identified in rice: magnesium-chelatase ChlD subunit (*OsCHLD*), magnesium-chelatase ChlI subunit (*OsCHLI*), magnesium-chelatase ChlH subunit (*OsCHLH*), chlorophyll synthase (*OsCHLG*), divinyl reductase gene (*OsDVR*), *protochlorophyllide oxidoreductase* (*OsPDR*), and chlorophyll a oxygenase (*OsCAO*) (Nagata et al. 2004). Mutations in these genes affect chlorophyll synthesis, resulting in changes in leaf color. For example, *dvr* mutants exhibit yellowish-green leaves and stunted plant growth (Wang et al. 2010). *Non-yellow coloring 1* (*NYC1*), which encodes a chlorophyll b reductase enzyme, results in an inhibition of chlorophyll b degradation, and hence a stay-green phenotype (Sato et al. 2009).

More than 180 color mutants were hitherto isolated and characterized in rice, and at least 80 genes (Deng et al. 2014; Sun et al. 2017). These mutations distributed in 12 chromosomes were cloned, most of which are controlled by a pair of recessive nuclear genes (Qian et al. 1996; Wang et al. 2008; Xie et al. 1995). In this study, we characterized a *white lesion mimic leaves1* (*wlm1*) mutant, which was derived from an  $M_2$  population of the *indica* rice cultivar Taichung Native 1 (TN1) after ethyl methanesulfonate (EMS) mutagenesis. This mutant displays white and lesioned leaves, and the mutant is stably inherited, segregating as a recessive mutation in a nuclear gene. We characterized the mutant phenotype, including pigment contents, chloroplast structure, and plant responses to different temperatures, and further delimited the *WLM1* locus to a 159.7-kb region on chromosome 4. The fine mapping of this gene lays the foundation for the cloning of related genes and leaf color breeding programs, as well as research on chlorophyll metabolism and mechanisms of disease resistance in plants.

## Materials and methods

### Plant materials and growth conditions

The *wlm1* mutant was derived from the  $M_2$  population of the *indica* rice cultivar TN1 after EMS mutagenesis. The *wlm1* mutant and its progeny all exhibited white and

diseased leaves, and this phenotype was stably inherited over multiple generations. The *wlm1* mutant and the *japonica* cultivar Wuyunjing 7 (W7) were used to make the segregating population for mapping. All plants were grown in the paddy fields at the China National Rice Research Institute (CNRRI) in Hangzhou (summer) and Lingshui (winter), China.

### Agronomic trait measurements

Wild-type TN1 and *wlm1* plants were planted in the same paddy fields at the CNRRI in Hangzhou in the summer of 2017, and the same growth periods were maintained for all plants. The main agronomic traits (plant height, tiller, leaf length, leaf width, spike length, number of primary branches, and 1000-grain weight) were investigated once the plants reached maturity. Data from three biological replicates were compiled for each trait.

### Photosynthetic rate measurement

In the morning, a photosynthetic apparatus (Li-6800, Li-Cor, Lincoln, Nebraska, USA) was used to measure the photosynthetic rate of both the white and diseased parts of corresponding sites on leaves from mutant and wild-type plants that had reached maturity. Three biological replicates were used.

### Observing temperature sensitivity in seedlings

Mature seeds from *wlm1* and TN1 plants were soaked in sterile water in a 96-well plate in the same growth environment for 15 days. Seedlings that had grown to a similar size were transferred to a growth chamber (Panasonic, MLR-352H-PC) for 7 days under 12 h light/12 h dark at constant temperatures of either 20, 28, or 40 °C. To measure the thermo-sensitivity of the plants, phenotypes were observed, chlorophyll pigment contents were measured, and the expression levels of thermo-sensitivity related genes were quantified.

### Pigment content measurement

During the mature stage, corresponding sections were cut from the leaves of TN1 and *wlm1* plants, immersed in extraction solution (mixture of 2/3 95% acetone and 1/3 ethanol) and incubated in the dark at 26 °C for 24 h. The optical density of sample solutions was measured with an ultraviolet spectrophotometer (UV-2600) at 663, 645, and 470 nm. Three biological replicates were conducted for each plant type. The contents of chlorophyll (Chl a and Chl b) and

carotenoid (Car) in each leaf sample was calculated according to Arnon's method (Arnon 1949).

### Observation of chloroplast ultrastructure

At the four-leaf stage, leaves from wild-type and *wlm1* mutant plants were cut into small pieces, fixed with 2.5% glutaraldehyde (diluted with phosphate buffer, pH 7.2), and placed under vacuum until the specimens were immersed. For mutant plants, both green and white sections of the leaves were collected for comparison. Specimens were then fixed, dehydrated, embedded, sliced, dyed, and then examined and photographed using a transmission electron microscope (Hitachi H-7650, Tokyo, Japan) (Li et al. 2010; Wu et al. 2016).

### Genetic analysis and fine mapping

To identify the gene responsible for the *wlm1* phenotype, we first examined its segregation. To that end, we performed a reciprocal cross between *wlm1* and the *indica* cultivar TN1. The phenotypes of plants in the F<sub>1</sub> and F<sub>2</sub> populations were observed and analyzed using the  $\chi^2$  test.

For fine mapping, an F<sub>2</sub> segregating population was derived from the crosses between the *wlm1* mutant and the *japonica* cultivar W7. The parents and 2878 F<sub>2</sub> individuals were planted in the paddy field. Among the F<sub>2</sub> individuals, 645 that displayed the mutant phenotype were used to map the *WLM1* gene. 23 *wlm1*/W7 F<sub>2</sub> individuals with the mutant phenotype were used for linkage analysis. All F<sub>2</sub> plants displaying the leaf color mutant phenotype were used for fine mapping. Total genomic DNA was extracted from fresh leaves using the CTAB (cetyltrimethylammonium bromide-based) method.

Insertion/deletion (InDel) and simple sequence repeat (SSR) markers were developed based on the sequence differences between the two rice varieties, *japonica* Nipponbare (<http://rgp.dna.affrc.go.jp>) and *indica* 93-11 (<http://www.rise.genomics.org.cn>). The 232 SSR markers were uniformly distributed among 12 chromosomes and the primer sequences for the molecular markers are listed in Supplemental Table 1.

### RNA extraction and quantitative real-time PCR (qRT-PCR)

Real-time fluorogenic quantitative PCR was performed to analyze leaf color-related gene expressions in the wild type and the *wlm1* mutant. The total RNAs in the wild type and the mutant leaves at the four-leaf stage were extracted to synthesize the first-strand cDNA by reverse transcription. The volume of the PCR system was 10  $\mu$ L and contained the following: 1  $\mu$ L of cDNA template, 0.5  $\mu$ L of 10  $\mu$ M each

primer (F/R), 5  $\mu$ L of 2 $\times$ SYBR (TOYOBO), and ddH<sub>2</sub>O up to 10  $\mu$ L. The PCR conditions were: 1 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 1 min. Real-time PCR was performed with a Real-Time PCR System (ABI 7900). The expression levels were normalized against the expression of *Histone H3*. The primers used for the quantification of related genes are listed in Supplemental Table 2.

### Statistical analysis

All results are expressed as mean values ( $\pm$  standard deviation, SD) based on three biological replicates. Statistical significance was assessed using Student's unpaired *t* test. Probability values of <5% were considered to be statistically significant, single asterisk (\*) and double asterisk (\*\*) represents the significant at the level of 5 and 1%, respectively. The statistical analysis of the gene relative expression levels, pigment content, photosynthetic rate and agronomic traits in this study all follow the methods described above.

## Results

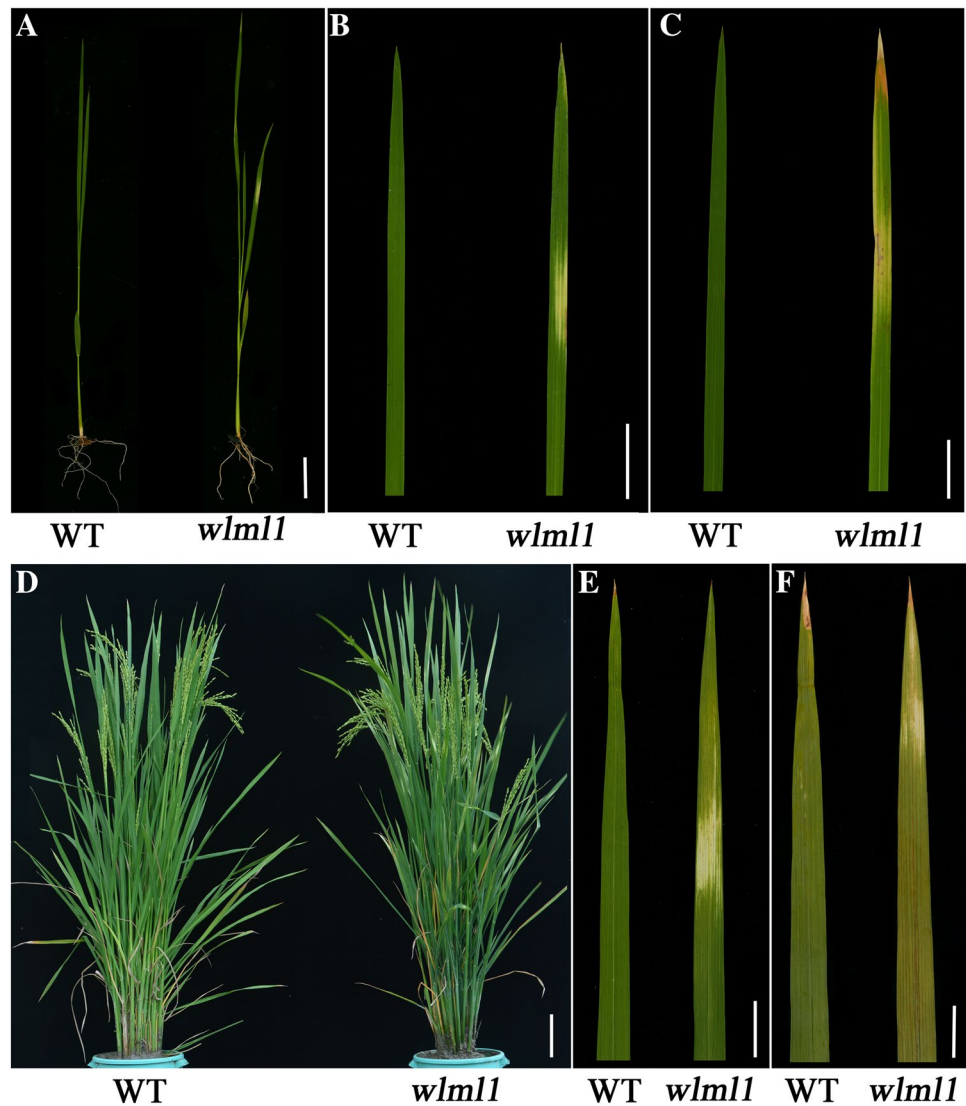
### Phenotypic and agronomic traits analysis

At the four-leaf stage, *wlm1* mutant leaves displayed yellow patches and some lesions that were not present on the leaves of wild-type (TN1) plants (Fig. 1a–c). These yellow patches changed to white, and the lesions increased in abundance on the white patches (Fig. 1c–e). At the mature growth stage, the agronomic traits (effective tiller number, panicle length, total grain number per panicle, grain length, grain width, and seed-setting rate) were not significantly different between the wild-type and mutant plants; however, plant height and grain weight were significantly reduced in the mutant (Table 1).

### Analysis of photosynthetic rate, photosynthetic pigment content, and chloroplast ultrastructure

The leaf-color phenotype observed in the same *wlm1* plant was not consistently exhibited in all leaves (variegated phenotype), so the photosynthetic rate and photosynthetic pigments were measured by sub-area. Compared to the wild-type rates (Fig. 2a), the photosynthetic rates of the white sections and the lesioned sections of mutant leaves declined by 34 and 36.3%, respectively. The content of Chl a, Chl b, and carotenoid (Car) pigments in the white leaf sections from the *wlm1* mutant declined by 56.6, 56.5, 55.6%, respectively (Fig. 2c), but the pigment contents did not differ between green leaf sections from the *wlm1* mutant and those from wild-type plants (Fig. 2b).

**Fig. 1** Phenotype comparison between wild-type TN1 and *wlm11* mutant plants at various growth stages. **a–c** Seedlings and leaves from wild-type TN1 and *wlm11* mutant plants at the four-leaf stage. **d–f** Pictures of seedlings and leaves from wild-type TN1 and *wlm11* mutant plants at the mature stage. Scale bars represent 2 cm (**a, e, f**); 1 cm (**b, c**); and 10 cm (**d**)



**Table 1** Comparison of agronomic traits between wild-type and *wlm11* mutant plants

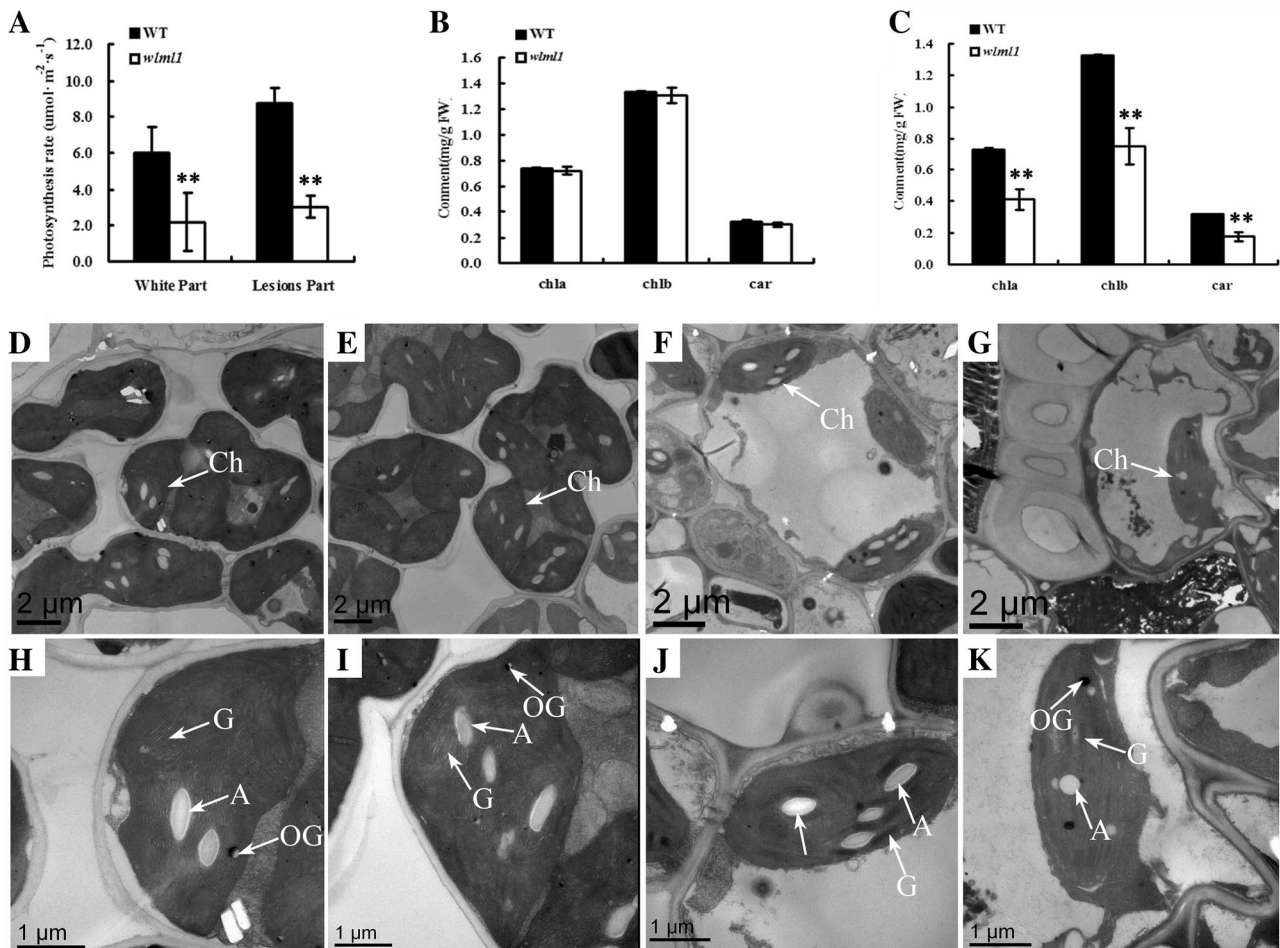
Agronomic trait	Wild type	Mutant
Plant height (cm)	109.4 ± 1.91	100.3 ± 1.21**
Tiller number	23.57 ± 4.65	26.75 ± 1.16
Panicle length (cm)	25.21 ± 0.93	24.19 ± 1.3
No. of grains per panicle	140.71 ± 13.25	136.1 ± 11.83
Grain length (cm)	7.07 ± 0.29	7.2 ± 0.32
Grain width (cm)	2.902 ± 0.12	2.91 ± 0.17
1000-grain weight (g)	24.56 ± 0.24	23.91 ± 0.46*
Seed-setting rate (%)	79.2 ± 5.06	76.1 ± 7.73

Data represent the mean ± SD of three biological replicates (Student's *t* test: \* $P < 0.05$ , \*\* $P < 0.01$ )

To explore the cause of the observed decline in photosynthetic pigments in *wlm11* plants, we observed difference of chloroplast ultrastructures in the leaves from TN1 and *wlm11* plants at the four-leaf stage by transmission electron microscopy. The chloroplasts in the green sections of leaves from both TN1 (Fig. 2c, g) and *wlm11* (Fig. 2d, h) plants were all intact. However, the white-section chloroplasts of *wlm11* leaves were severely degraded, there were few normal chloroplast structures; In addition, the number of chloroplasts decreased and an absence of the grana lamellae (Fig. 2e, i).

### Genetic analysis and fine mapping of *WLML1*

A genetic analysis was conducted on the  $F_1$  and  $F_2$  populations that were generated from reciprocal crosses between *wlm11* mutants and wild-type TN1 plants. The leaf color of all  $F_1$  plants from either cross (*wlm11* × TN1 or TN1 × *wlm11*) was consistent with that of the wild type. Out of the 656  $F_2$



**Fig. 2** Photosynthetic rate, photosynthetic pigment content, and chloroplast ultrastructure of wild-type TN1 and *wml1* mutant plants. **a** Photosynthetic rates of the white sections and lesioned sections of *wml1* mutant leaves compared with corresponding sections from wild-type TN1 leaves. **b** Content of photosynthetic pigments in green leaves from wild-type TN1 and *wml1* mutant plants. **c** Content of photosynthetic pigments in white leaves from *wml1* mutants compared with leaves from wild-type TN1 plants. **d–k** Chloroplast ultrastructure in leaves from wild-type TN1 plants (**d, h**), and green (**e, i**), white (**f, j**), and lesioned (**g, k**) sections of leaves from *wml1* mutant plants. *FW* fresh weight, *Ch* chloroplast, *N* nucleus, *G* grana stacks, *A* amyloplast, *OG* osmophilic plastoglobuli. Data represent the mean ± SD of three biological replicates (Student’s *t* test: \**P* < 0.05, \*\**P* < 0.01)

pared with leaves from wild-type TN1 plants. **d–k** Chloroplast ultrastructure in leaves from wild-type TN1 plants (**d, h**), and green (**e, i**), white (**f, j**), and lesioned (**g, k**) sections of leaves from *wml1* mutant plants. *FW* fresh weight, *Ch* chloroplast, *N* nucleus, *G* grana stacks, *A* amyloplast, *OG* osmophilic plastoglobuli. Data represent the mean ± SD of three biological replicates (Student’s *t* test: \**P* < 0.05, \*\**P* < 0.01)

plants from the *wml1* × TN1 cross, 496 displayed the wild-type phenotype and 160 displayed the mutant phenotype. Out of the 524 F<sub>2</sub> plants from the TN1 × *wml1* cross, 389 displayed the wild-type phenotype and 135 displayed the mutant phenotype. In both crosses, the segregation ratio of the wild-type to the mutant phenotype was approximately 3:1 according to a  $\chi^2$  test (Table 2). The genetic analysis showed that *WLML1* was a single recessive nuclear gene.

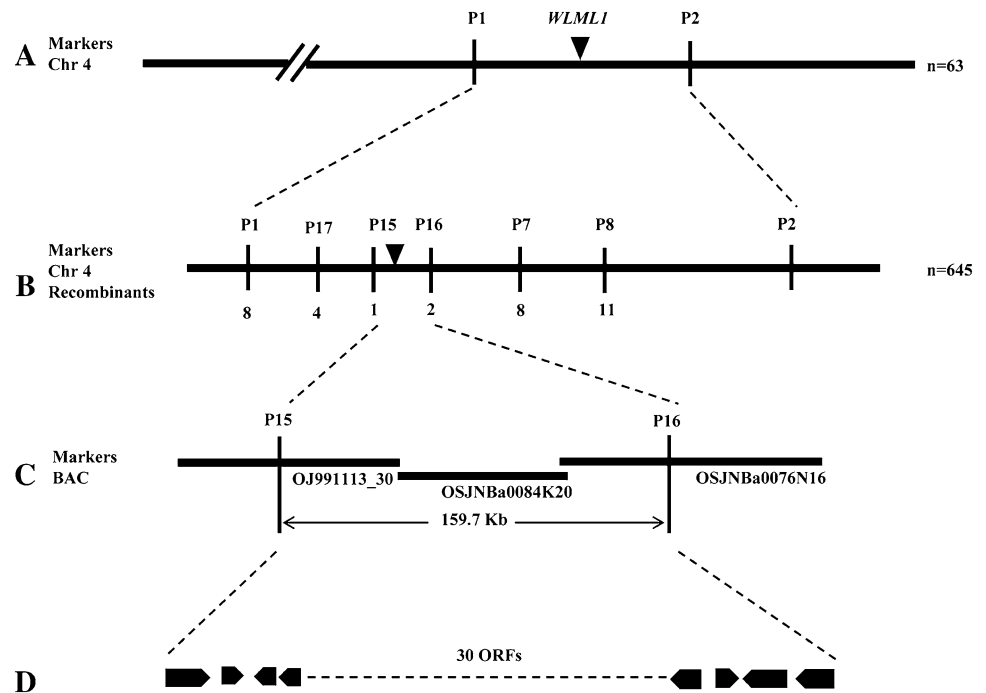
**Table 2** Segregation analysis of *wml1* crosses

Segregation population (F <sub>2</sub> )	Wild type	Mutant type	$\chi^2$ (3:1)	<i>P</i> -value
<i>wml1</i> × W7	496	160	0.1301	0.7183
W7 × <i>wml1</i>	389	135	0.1628	0.6865

The F<sub>2</sub> population generated from the *wml1* × W7 cross was used for fine mapping of *WLML1*. *WLML1* was initially mapped between markers P1 and P2 on chromosome 4 according to 23 individuals displaying the *wml1* phenotype and 232 pairs of SSR markers (Fig. 3a). To further define *WLML1*, the mapping population was expanded to 645 plants and new SSR and InDel markers were designed between P1 and P2 according to sequence differences

between the rice varieties *japonica* Nipponbare (<http://rgp.dna.affrc.go.jp>) and *indica* 93-11 (<http://www.rise.genomics.org.cn>). Finally, the locus was delimited to a 159.7-kb region between markers P15 and P16. According to the genomic annotation database RAP-DB (<http://rapdb.dna.affrc.go.jp/>), this region contains 30 predicted Open Reading Frames (ORFs) (Fig. 3b–d).

**Fig. 3** Fine mapping of *WLML1*. **a** *WLML1* was initially mapped to a region on chromosome 4 between markers P1 and P2. **b** Fine mapping of *WLML1* using 645  $F_2$  plants from the *wlml1* × W7 cross. The numerals indicate the number of recombinants. **c** *WLML1* was located between markers P15 and P16 within a 159.7-kb genomic DNA region. **d** 30 ORFs are candidates for *WLML1*



### Relative expression of genes related to leaf color in leaves from wild-type and mutant plants

The leaves from *wlml1* mutant plants display white patches and lesions. Changes in leaf color are associated with chlorophyll metabolism and chloroplast development. Therefore, we examined the transcript abundance of genes associated with chlorophyll synthesis, photosynthesis, chlorophyll degradation, and chloroplast development in *wlml1* mutant and wild-type seedlings. We found that genes associated with chlorophyll synthesis (*DVR*, *CHLH*, *OsPORA*, *OsPORB*, and *CAOI*) were significantly down-regulated, in *wlml1* mutants compared with wild-type plants, while most chlorophyll degradation genes (*SGR* and *OsPAO*) were significantly up-regulated, except for *OsPCCR* (Fig. 4a). Some genes related to chloroplast development were down-regulated (*PsbA*, *PsaB*, *V2*, *V3*, *LhcP2* and *Rps15*), while others were up-regulated (*V1*, *RpoC1*, and *RpoC2*) (Fig. 4b).

The formation of lesions in leaves is also related to disease resistance and production of reactive oxygen species (ROS). All tested disease resistance-related genes (*PBZ1*, *PR1A*, *PR1B*, and *PR10*) and antioxidant related genes (*OsCATB*, *OsAPX2*, and *OsPOD1*) were significantly up-regulated (Fig. 4c).

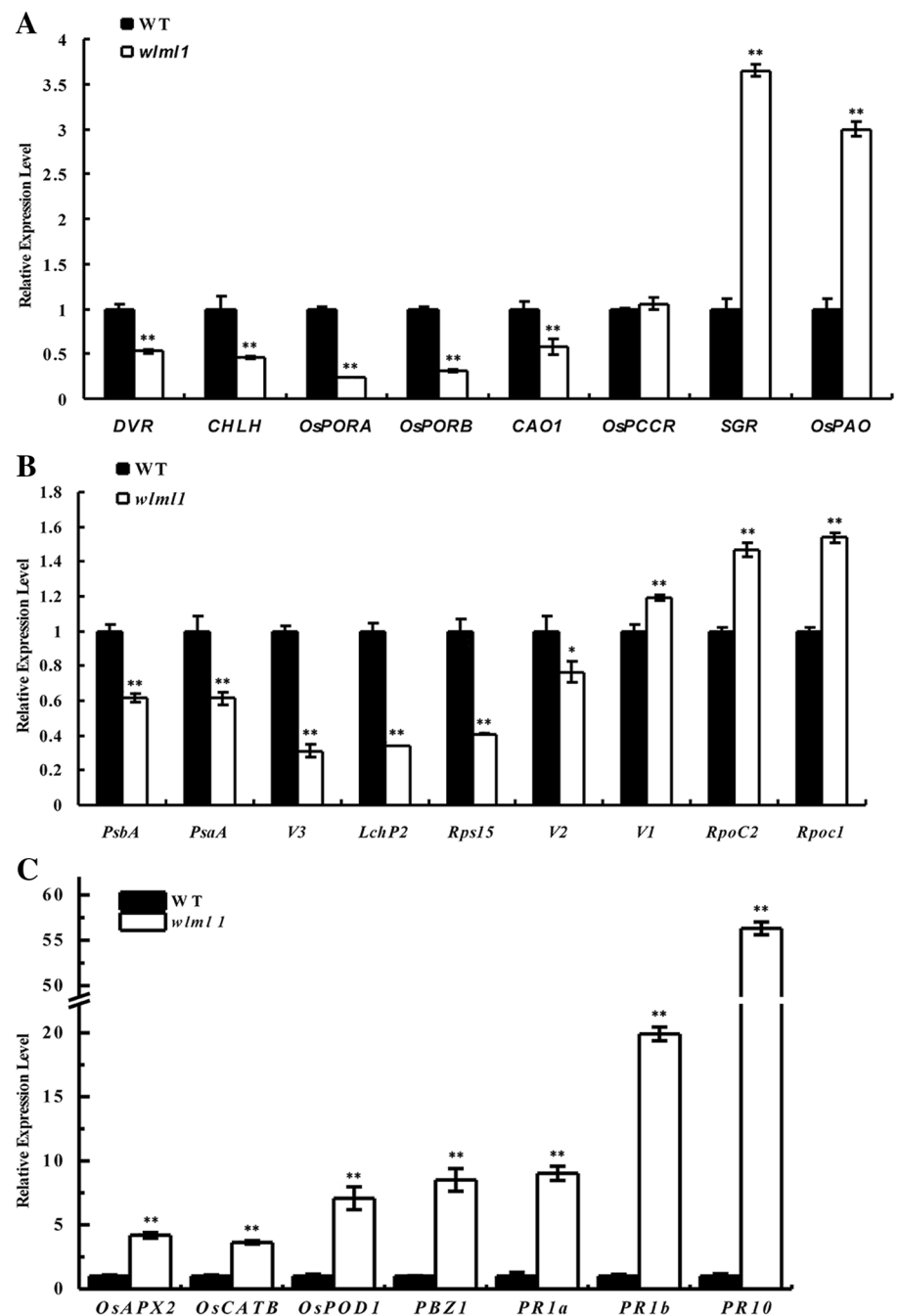
### The *wlml1* mutant is temperature-sensitive

To identify whether the phenotype in the *wlml1* mutant was affected by temperature at the seedling stage, we transplanted 15-day-old *wlml1* and TN1 seedlings into growth chambers

at different temperatures (20, 28 and 40 °C) for 7 days. There were no obvious differences in leaves color between TN1 and *wlml1* plants at 40 °C, although green withered leaf tips were observed (Fig. 5a, d), and Chl a and Chl b contents were increased in *wlml1*, compared with TN1 plants (Fig. 5g). At 28 °C, leaves were slightly yellow (Fig. 5b, e), and Chl a and Car contents were significantly reduced in *wlml1* plants, compared to wild type plants (Fig. 5h). At 20 °C, the leaves of mutant plants were also yellow and displayed a decrease in pigment content compared to the wild-type plants, but this difference was more obvious than that at 28 °C (Fig. 5c, i). Furthermore, the lesions appeared in leaves from the *wlml1* mutant plants at 20 °C but not evident in wild-type plants (Fig. 5f).

As the temperature decreased, genes associated with chlorophyll degradation (*NYC3*, *NOL*, *OsPCCR*, *OsPAO*, and *SGR*), chloroplast development (*V1*, *V2*, and *Cab1*) and oxidation resistance (*OsCATA*, *OsCATB*, and *OsPOD1*) were significant up-regulated in *wlml1* compared with TN1 (Fig. 6). For example, the ratio of expression of these genes *NYC3*, *OsPCCR*, *V1*, *V2*, *OsCATA*, and *OsPOD1* between *wlml1* and TN1 were 0.88, 0.93, 0.73, 1.09, 0.98, and 0.98 at 40 °C; and 0.96, 1.2, 0.96, 1.1, 1.09, and 1.42 at 28 °C; and 2.6, 3.15, 4.48, 3.09, 3.1, and 3.53 at 20 °C, respectively.

**Fig. 4** Relative expression of genes related to leaf color in leaves from wild-type TN1 and *wlm1* mutant plants. **a** Relative expression of genes associated with chlorophyll synthesis and degradation between wild-type TN1 and *wlm1* mutant plants. **b** Relative expression of genes associated with chloroplast development between wild-type TN1 and *wlm1* mutant plants. **c** Relative expression of genes associated with disease resistance and ROS between wild-type TN1 and *wlm1* mutant plants

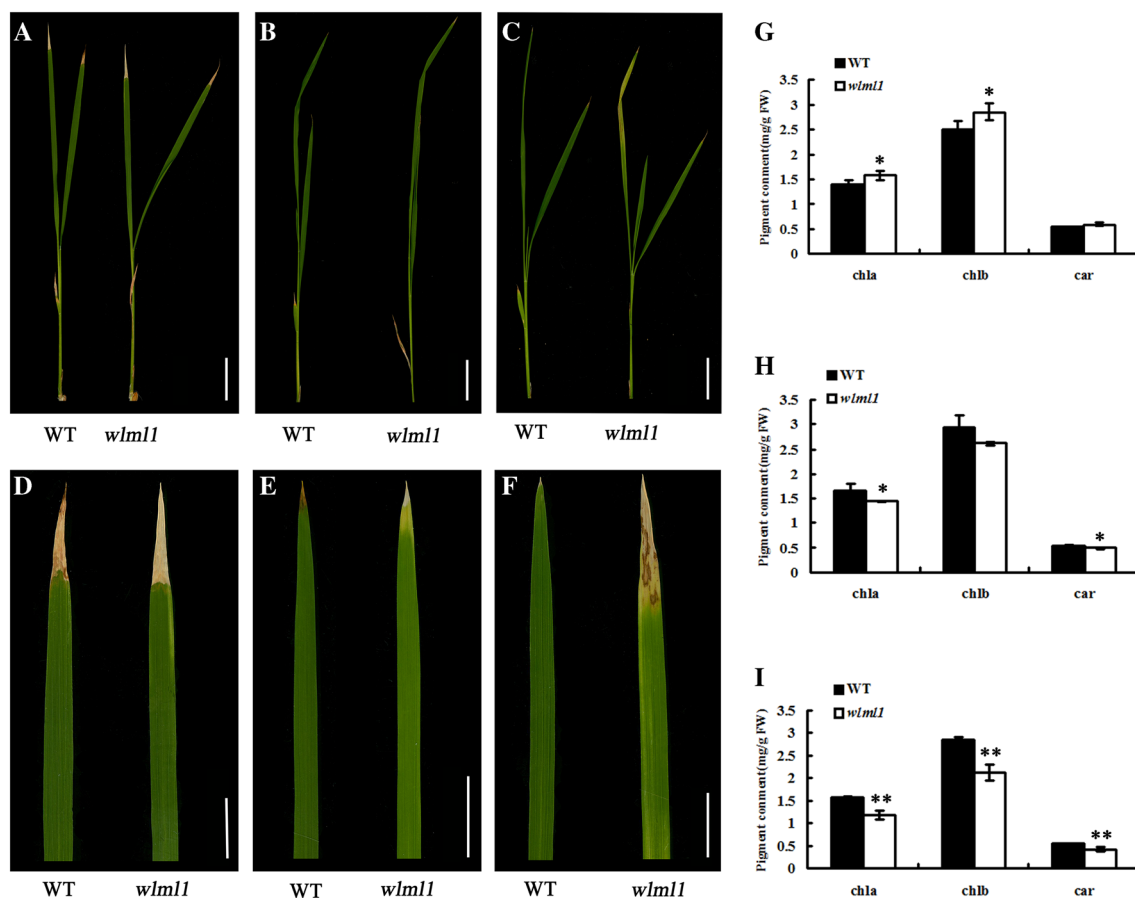


## Discussion

Leaf color markers are widely used in maintaining the genetic purity of seeds and as a marker of self-pollination to monitor male sterile lines (Deng et al. 2014; Lv et al. 2013). The only phenotypic difference between wild type and the *wlm1* mutant is a slight difference in plant height and 1000-grain weight (Table 1). Therefore, *wlm1* can be used as a visible marker in rice breeding.

There are seven genes related to leaf color on chromosome 4 that have been cloned in rice: *Rice Lesion Initiation*

1 (*OsRLIN1*), *Mitochondrial PPR25* (*OsMPR25*), *Albino Midrib 1* (*OsAM1*), *ENT-Kaurene Synthase-Like 2* (*OsKS2*), *Young Seedling Stripe1* (*OsYSS1*), *Giant Chloroplast* (*OsGIC*), and *Wax Crystal-sparse Leaf 1* (*OsWSL1*). *RLIN1* encodes a putative coproporphyrinogen III oxidase from the tetrapyrrole biosynthesis pathway, and the *rlin1* mutant showed a lesion mimic phenotype (Wang et al. 2015; Sun et al. 2011). *MPR25* encodes a pentatricopeptide repeat protein that belongs to the E subgroup of the pentatricopeptide repeat protein family. The *mpr25* mutant exhibits growth retardation and pale-green leaves with



**Fig. 5** Leaf phenotypes and photosynthetic pigment contents of wild-type and *wlm1* seedlings grown at 40 °C (a, d, g), 28 °C (b, e, h), and 20 °C (c, f, i). Data represent the mean  $\pm$ SD of three biological

replicates (Student's *t* test: \* $P < 0.05$ , \*\* $P < 0.01$ ). FW fresh weight. Scale bars are 2 cm (a–c); and 1 cm (d–f)

reduced chlorophyll content during the early stages of plant development (Toda et al. 2012). *AMI* encodes a putative potassium efflux antiporter, and the *am1* mutant exhibited a green/white variegation phenotype in the first few leaves, followed by an albino midrib phenotype in older tissues (Sheng et al. 2014; Nonomura et al. 2011). *OsKSS2* encodes a functional ent-beyerene synthase that is responsible for the biosynthesis of various diterpenoids (Tezuka et al. 2015), and the mutant has short, dark green leaves (Ji et al. 2014). *YSSI* encodes a chloroplast nucleoid-associated protein that is essential for chloroplast development in rice seedlings. The *yss1* mutant develops striated leaves at the seedling stage, but no abnormal phenotype in leaf 5 and onwards (Zhou et al. 2017). *GIC* encodes a PARC6 homolog, and the *gic* mutant has fewer and enlarged chloroplasts (Kamau et al. 2015). *WSL1* encodes a multiple organellar RNA editing factor, and *wsp1* mutants exhibit green and white sectors starting from the three-leaf stage and not entirely green panicles at the heading stage (Zhang et al. 2017). In this study, the *WLML1* locus was mapped to a 159.7-kb DNA region on chromosome 4. Both the

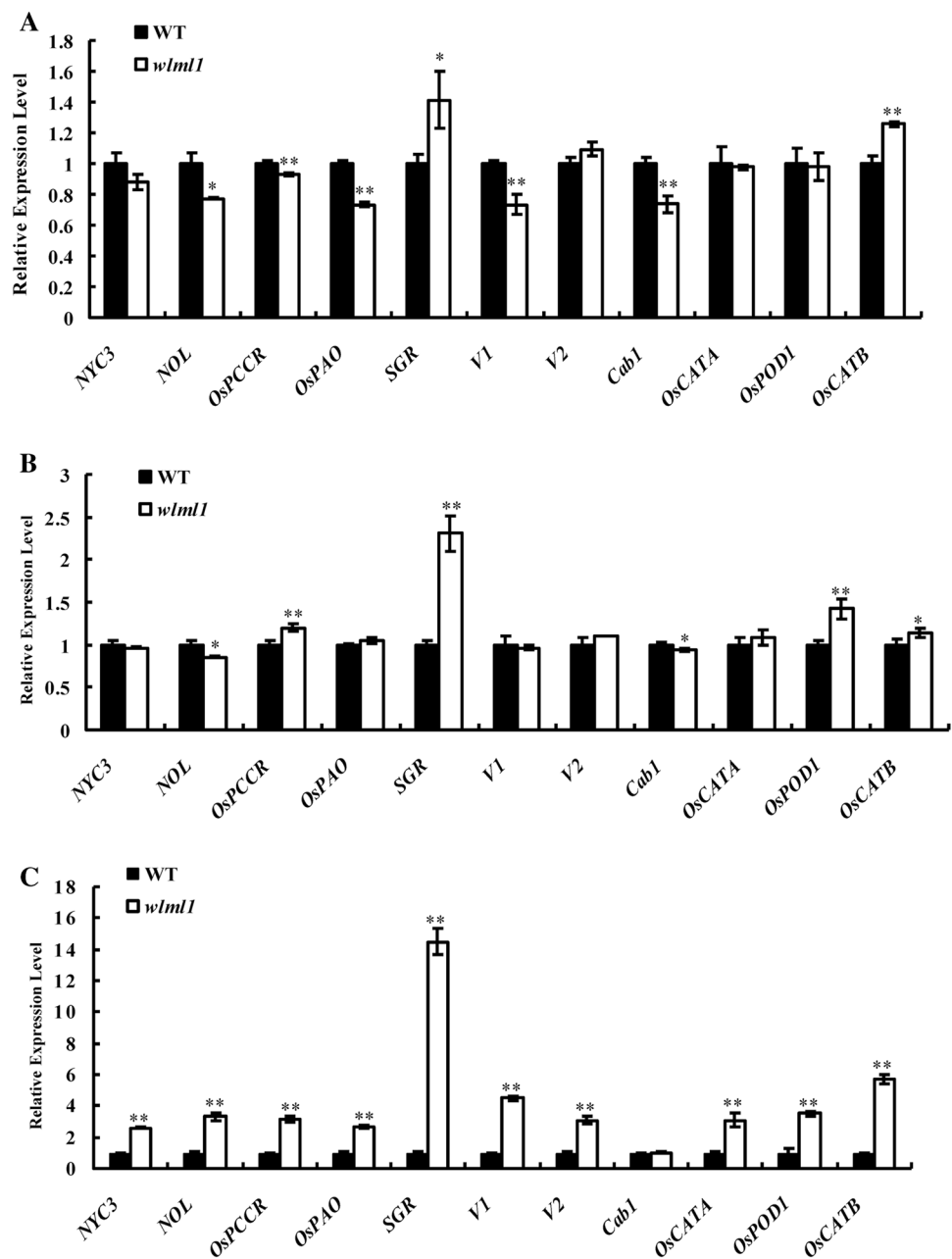
location of the *WLML1* and the mutant phenotype are different from the known leaf color mutants, so we speculate that *WLML1* is a new gene.

Together, the characteristics of the *wlm1* mutant phenotype of fewer chloroplasts, severe chloroplast degradation, and decreased pigment content (Fig. 2), as well as the up- or down-regulation of genes associated with chlorophyll metabolism and chloroplast development suggest that chloroplast dysfunction causes the leaf color change in the *wlm1* mutant (Fig. 4a, b).

ROS function as signals for the activation of defense genes. ROS also drive the cross-linking of cell wall structural proteins, function as a local trigger of programmed death in pathogen-challenged cells, and as a diffusible signal for the induction of genes encoding cellular protectants in adjacent cells (Bradley et al. 1992; Lamb and Dixon 1997; Montillet et al. 2005; Levine et al. 1994). The appearance of a lesion mimic phenotype (Fig. 1), and the up-regulation of disease resistance and ROS-related genes (Fig. 4c) in *wlm1* compared with wild-type plants suggests that the *wlm1* phenotype may be related to an imbalance of ROS.



**Fig. 6** Relative expression of genes related to chlorophyll degradation, chloroplast development and oxidation resistance under different temperatures in *wlml1* and wild-type seedlings. **a** Relative expression of genes related to chlorophyll degradation, chloroplast development, and oxidation resistance at 40 °C (**a**), 28 °C (**b**) and 20 °C (**c**). Data represent the mean  $\pm$  SD of three biological replicates (Student's *t* test: \* $P < 0.05$ , \*\* $P < 0.01$ )



The observed changes in pigment contents of the *wlml1* mutant when grown at various temperatures (Fig. 5h–j) implies that *WLML1* function may not be completely lost in the *wlml1* mutant, and was regulated by temperature. At 20 °C, the ratio of expression of genes associated with chlorophyll degradation and chloroplast development are up-regulated between *wlml1* and TN1 compared to at 28 and 40 °C. This suggests that *WLML1* regulates leaf color by accelerating chlorophyll degradation, and chloroplast development-associated genes, possibly by feedback regulation. Furthermore, the formation of lesions and higher expression of antioxidant-related genes in *wlml1* mutants grown at 20 °C compared to 28 and 40 °C suggests that *WLML1*

may participate in regulating biotic and abiotic stress in rice (Fig. 6).

## Conclusion

In this study, we identified the *wlml1* mutant, which has white and lesioned leaves and can be used as a marker in breeding to maintain the purity of varieties due to the minimal impact of the mutation on important agronomic traits in rice. We mapped *WLML1* to a 159.7-kb region on chromosome 4, which laid the foundation for subsequent map-based cloning. Since the *wlml1* mutation negatively affected

chlorophyll metabolism and chloroplast development, this mutant will be useful for in-depth studies of chlorophyll metabolism and chloroplast development. Since the mutant phenotype was influenced by temperature, this mutant can also be used to study regulation of chloroplast development at different temperatures. In addition, the *wlml1* mutant has a lesion mimic phenotype that is affected by temperature. Therefore, we speculate that the gene encoded at *WLML1* could be related to resistance to disease and stress, which we plan to investigate in future experiments. If this speculation is verified, *wlml1* will be useful for research on plant disease and stress resistance.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no potential competing interests.

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