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Research article

Characterization and fine-mapping of a novel premature leaf senescence mutant *yellow leaf and dwarf 1* in rice

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SA
Salicylic acid
SNP
Single nucleotide polymorphism
WT
Wild type
YLD
Yellow leaf and dwarf
ROS
Reactive oxygen species

ABSTRACT

Leaves are the main organs in which photosynthates are produced. Leaf senescence facilitates the translocation of photosynthates and nutrients from source to sink, which is important for plant development and especially for crop yield. However, the molecular mechanism of leaf senescence is unknown. Here, we identified a mutant, *yellow leaf and dwarf 1* (*yld1*), which exhibited decreased plant height and premature leaf senescence. Nitroblue tetrazolium and diamiobenzidine staining analyses revealed that the concentrations of reactive oxygen species were higher in *yld1* leaves than in wild type leaves. The photosynthetic pigment contents were significantly decreased in *yld1*. The *yld1* chloroplasts had collapsed and were filled with abnormal starch granules. Combining bulk segregant and MutMap gene mapping approaches, the mutation responsible for the *yld1* phenotype was mapped to a 7.3 Mb centromeric region, and three non-synonymous single nucleotide polymorphisms located in three novel genes were identified in this region. The expression patterns of the three candidate genes indicated that *LOC_Os06g29380* had the most potential for functional verification. Plant hormone measurements showed that salicylic acid was highly accumulated in *yld1* leaves when compared with wild type leaves, and *yld1* was more sensitive to salicylic acid than wild type. This work lays the foundation for understanding the molecular regulatory mechanism of leaf senescence, and may reveal new connections among the molecular pathways related to leaf senescence, starch metabolism and salicylic acid signaling.

1. Introduction

Leaves are important organs that produce photosynthates for plant development. Leaf senescence is a natural process and facilitates the transport of photosynthates and nutrients from source to sink, which is important for plant development and especially for crop yield (Yang et al., 2011). Leaf senescence is characterized by the collapse of chlorophyll, the hydrolysis of lipids, proteins and nucleic acids, and cell death (Lim et al., 2007). Leaf senescence is controlled by a fine-tuned network and any misregulated senescence will lead to premature senescence or delayed senescence, which normally causes a loss of crop yield. Therefore, the progress of leaf senescence needs to be under strict molecular regulation during plant development (Schippers, 2015).

The molecular mechanisms involved in leaf senescence have

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been well studied in terms of hormone and environmental signaling pathways. In the hormone signaling pathway, abscisic acid (ABA), ethylene, jasmonic acid (JA) and salicylic acid (SA) are accumulated in senescent leaves, and can accelerate leaf senescence (Zhang and Zhou, 2013; Khan et al., 2014; Schippers, 2015). A number of genes involved in hormone signaling pathways participate in leaf senescence, such as *ETHYLENE-INSENSITIVE 3*, a positive regulator, which induces senescence through a partial dependence on the action of *ORESARA 1* (Kim et al., 2009; Li et al., 2013). The overexpression of *Oryza sativa* NAC-like, activated by apetala3/pistillata, induced by ABA accelerates leaf senescence by directly targeting genes related to chlorophyll degradation and genes associated with senescence (Chen et al., 2014; Liang et al., 2014). *WRKY53*, a SA-inducible transcription factor, promotes leaf senescence by activating the expression of *ORESARA 9*, *SENESCENCE-ASSOCIATED GENE 12* and *CATALASE1/2/3* (Miao et al., 2004).

Several environmental factors are involved in the leaf senescence process, such as shading and drought. So far, the molecular networks involved in the leaf senescence regulated by environmental factors have been extensively studied. For example, *WRKY22* transcription is promoted by dark-induced senescence (Zhou et al., 2011). During drought stress, *NAC with transmembrane motif 1-like 4* positively regulates leaf senescence by activating the expression of *Respiratory burst oxidase homolog C* and *Respiratory burst oxidase homolog E*, resulting in an increase in the level of reactive oxygen species in the leaf (Lee et al., 2012). However, a number of questions remain to be addressed regarding leaf senescence.

In this work, we identified a premature senescence mutant, *yellow leaf and dwarf 1* (*yld1*), which mainly exhibited decreased plant height and premature leaf senescence. Higher concentrations of senescence-related substances, such as superoxide radicals and hydrogen peroxide, accumulated in *yld1* leaves. The *yld1* chloroplasts had collapsed and were filled with numerous abnormal starch granules. The mutation responsible for the *yld1* phenotype was narrowed down to three non-synonymous single nucleotide polymorphisms (SNPs) located in three genes. Only one gene, which had not been previously reported, encoding a phospholipid-transporting ATPase, was proposed to be the candidate. Moreover, the *yld1* leaves accumulated more SA and were more sensitive to SA than wild type (WT) leaves. Our work provides a novel component involved in the leaf senescence regulatory network, and provides a possible connection between the SA signaling pathway and leaf senescence.

2. Materials and methods

2.1. Plant materials and growth conditions

The *yld1* mutant was identified from the ethyl methanesulfonate (EMS)-induced mutational library for the *indica* cultivar 'Shuhui527', which was treated with 0.5% (w/v) EMS for 12 h. The premature senescence and dwarf phenotype was genetically stable for more than four generations under greenhouse and field conditions. Therefore, the F1 and F2 populations derived from crosses between *yld1* and WT or 9311 were used for the genetic analysis and mapping of *yld1*. All of the plants were grown in the paddy fields at Wenjiang, Sichuan Prefecture, or at Lingshui, Hainan Prefecture, China. Agronomic traits, including plant height, number of tillers per plant, panicle length, grain numbers per panicle, seed-setting rate and 1000-grain weight, were measured three times during the evaluation.

2.2. Histological and histochemical analyses

Stems of WT and *yld1* plants at the heading stage were processed as previously reported (Xue et al., 2008). Stems prefixed in 4% paraformaldehyde were dehydrated through an ethanol series, and then embedded in Technovit 7100 resin (Heraeus Kulzer, Germany). The blocks were trimmed and sectioned at 2 μm with a disposable knife, and then stained with 1% (w/v) toluidine blue. Semi-thin sections were observed and photographed with a light microscope (Carl Zeiss), and cells were measured using a digital image processing software (ImageJ 64 version 1.45). The quantification was performed on three serial sections of three samples.

Superoxide radicals and hydrogen peroxide were detected using nitroblue tetrazolium (NBT) and diamiobenzidine (DAB), respectively, according to Rao et al. (2015). Leaves from the WT and the *yld1* mutant 10 days after the flowering stage were incubated in 0.05% (w/v) NBT or 0.1% (w/v) DAB (Sigma) at room temperature in the dark with gentle shaking for 12 h. Then, the chlorophyll was cleared by treating with 90% (v/v) ethanol at 90 °C.

2.3. Measurement of the photosynthetic pigment contents

The photosynthetic pigment contents were measured for the upper four leaves at the eight-leaf stage and the flag leaves after the flowering stage as described by Huang et al. (2015). Leaves were cut into ~2 mm pieces and soaked in 80% (v/v) acetone in the dark for 48 h at 4 °C. The concentrations of chlorophyll *a*, chlorophyll *b* and total carotenoids were determined by measuring the absorption of light with a Beckman spectrophotometer at 665 nm, 649 nm and 470 nm, respectively, and then simultaneous equations were established as in Wellburn (1994). The means from the three measurements were used for analysis.

2.4. Transmission electron microscopy (TEM) and semi-thin sectioning

Ultra-thin sectioning and TEM were performed using flag leaves of WT and *yld1* selected 10 days after the flowering stage, following the method described by Qin et al. (2013). Briefly, the leaves were prefixed with a mixed solution of 3% glutaraldehyde, and then the samples were post-fixed in 1% osmium tetroxide and dehydrated in an acetone series. Following Epox 812 infiltration, samples were embedded in acrylic resin (London Resin Company). The ultrathin sections were cut with a diamond knife, and then stained with uranyl acetate and lead citrate. Sections were examined with a TEM (HITACHI, H−600IV, Japan).

2.5. Fine mapping of the mutation

The mutation responsible for the *yld1* phenotype was primarily mapped using the bulk segregant gene mapping approach (Michelmore et al., 1991). The F2 population was developed by crossing *yld1* and 9311. A total of 300 SSR markers on the 12 chromosomes were used to analyze polymorphisms between *yld1* and 9311. The 144 SSR markers that contained polymorphisms were further used to analyze two DNA pools, each contained 10 F2 plants, with or without yellowing leaves.

The fine-mapping was performed using the MutMap approach described in Abe et al. (2012), with some modifications. Briefly, the DNA of 30 F2 individuals with yellow leaves derived from a cross between *yld1* and WT were combined in equal ratios into a bulked sample, then subjected to whole-genome sequencing using a Hiseq 2000 sequencer (PE101). In total, 87 million and 101 million sequence reads for *yld1* and WT, respectively, were obtained, corresponding to >6 Gb of total read length with >20 × coverage of the