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RNAi-mediated knocking-down of rlpk2 gene retarded soybean leaf senescence

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Abstract

Leaf senescence that occurs in the last stage of leaf development is a genetically programmed process. It is very significant to elucidate the molecular mechanisms that control the initiation and progression of leaf senescence and the way the senescence signal is transduced. In a previous study on artificially induced soybean leaf senescence, we cloned a novel gene designated *rlpk2* (Genbank Accession No. AY687391) that encodes a leucine-rich repeat (LRR) receptor like protein kinase. The expression level of *rlpk2* gene was shown to be strongly up-regulated during both the natural leaf senescence process in this report and the artificially induced primary-leaf-senescence process in our previous work. The RNA interference (RNAi)-mediated knocking-down of *rlpk2* dramatically retarded both the natural and nutrient deficiency-induced leaf senescence in transgenic soybean. The transgenic leaves showed more cell-aggregated surface structure and higher content of chlorophyll.

Keywords

RNAi, mediated, knocking, down, *rlpk2*, gene, retarded, soybean, leaf, senescence

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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RNAi-mediated knocking-down of *rlpk2* gene retarded soybean leaf senescence

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Abstract Leaf senescence that occurs in the last stage of leaf development is a genetically programmed process. It is very significant to elucidate the molecular mechanisms that control the initiation and progression of leaf senescence and the way the senescence signal is transduced. In a previous study on artificially induced soybean leaf senescence, we cloned a novel gene designated *rlpk2* (Genbank Accession No. AY687391) that encodes a leucine-rich repeat (LRR) receptor like protein kinase. The expression level of *rlpk2* gene was shown to be strongly up-regulated during both the natural leaf senescence process in this report and the artificially induced primary-leaf-senescence process in our previous work. The RNA interference (RNAi)-mediated knocking-down of *rlpk2* dramatically retarded both the natural and nutrient deficiency-induced leaf senescence in transgenic soybean. The transgenic leaves showed more cell-aggregated surface structure and higher content of chlorophyll.

Keywords: soybean, leaf senescence, RNA interference, gene knock-down.

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In higher plants the last step of leaf development is senescence. It is an active, genetically controlled programmed cell death, during which cells undergo a transition from assimilation and other anabolic reactions to a catabolic pattern that leads to cell dysfunction, structure disintegration and eventually cell death^[1]. The catabolic events in the senescing leaf include chlorophyll degradation, chloroplast breakdown, proteolysis and nucleic acid hydrolysis. Most of genes involved in the photosynthesis and carbon assimilation are down-regulated, whereas other genes, referred to as senescence-associated genes (SAGs) are up-regulated. Most of protein products of SAGs are involved in the breakdown and mobilization of macromolecules during leaf senescence. Although several components such as GTP-bound protein and AP2 transcriptional factor in *Arabidopsis thaliana* were reported to be involved in the regulation of this progress^[2], the molecular mechanisms underlying the initiation and progression of leaf senescence are far from well elucidated. The

question as to how internal and environmental signals are transduced to regulate leaf senescence remains open.

Most of the plant receptor-like protein kinases (RLKs) consist of three domains—the extracellular receptor domain, the single-pass transmembrane domain and the intracellular kinase domain. Once specific ligands bind with the receptor domain, which usually causes the intracellular domains to be autophosphorylated or transphosphorylated and eventually the RLK to be activated, the extracellular signals are transduced into the inside of cells^[3]. The unique structures imply that the RLKs play important roles in regulating plant developmental processes and transducing environmental signals. Several RLKs have recently been found to be involved in a diverse range of developmental and stress signal transduction pathways, such as *Brassica* SRK in self-incompatibility^[4], *A. thaliana* ERRECTA in determining organ shape^[5], CLAVATA1 in meristem maintenance^[6], and rice (*Oryza sativa*) Xa21 in resistance to bacterial pathogen^[7]. As in many other developmental processes, the involvement of RLKs in senescence has also been suggested. The transcription levels of *PvSARK* in bean and *AtSIK1* in *Arabidopsis* were found to increase in senescent leaves^[8,9], although their detailed function in regulating the progression of leaf senescence has not been known as yet. In our previous study on soybean leaf senescence and retarding effect of exogenous cytokinin, great changes in autophosphorylation status and catalytic activities of plasma membrane protein kinases had been found, which implied the involvement of specific protein kinases in regulating soybean leaf senescence^[10]. Four novel RLKs genes were isolated from the artificially induced senescent soybean leaves. One of them, designated *rlpk2* (GenBank Accession No. AY687391), codes for a novel RLK with 9 leucine-rich repeat (LRR) units in its receptor domain. The transcriptional level of *rlpk2* was strongly increased in the soybean primary leaves undergoing artificially senescence-inducing treatment (ST), suggesting that *rlpk2* might be involved in the regulation of soybean leaf senescence^[11]. Here we report the characterization of the organ-specific and leaf developmental expression pattern of *rlpk2*. In addition, a dramatically retarding effect was shown on soybean leaf senescence by RNA interference (RNAi)-mediated knocking-down of *rlpk2* gene expression. To our knowledge, it is the first report that demonstrates RNAi-mediated knocking-down of a specific RLK delayed leaf senescence in higher plants.

1 Materials and methods

1.1 Plant materials and transformation

Soybean (*Glycine max*) Kefeng 34, a cultivar obtained from the Academy of Tianjin Agriculture Science was grown in the experimental field. The growth and development stages of soybean had been demarcated previously^[12]. Soybean plants have five nodes with four unfolded

leaflets at V4 and seven nodes of unfolded leaflets at V6 stage. R1 and R2 describe the flowering and bearing pod stage respectively. The second trifoliate leaf at each stage was sampled.

Both *Agrobacterium*-mediated cotyledon^[13] and growing point^[14] transformation methods were used in this study with Kanamycin resistance as selection marker. The transformed seedlings were screened out and transferred to soil. PCR method was used to confirm the exogenous DNA insertion in genomes of putative transgenic soybean plants. The effect of RNA interference was characterized by determining the expression level of *rlpk2* in transgenic plants by RT-PCR method.

1.2 Construction of binary expression vector of *rlpk2*-RNA interference cassette

E. coli DH5 α and *Agrobacterium* LBA4404 were held in our laboratory. Both of the plasmid pKANNIBAL and pART27 used to construct the *rlpk2*-RNAi binary vector were gifts by Dr. Peter M. W.'s laboratory. Structural analysis of full-length *rlpk2* cDNA (GenBank Accession No. AY687391) was carried out by bio-informatic tools to predict kinase conserved domain. A 301-bp DNA fragment in non-conservative region at downstream of the last kinase subdomain was chosen as a target sequence of RNA interference (RNAi). The target fragment was amplified by RT-PCR method with a pair of primers F2: 5'-GGT ACC ATC GAT GTT GAT GAA GAG TTT TCT GGA G-3' (*Kpn* and *Cla* sites were introduced) and R2: 5'-CTC GAG GAT CCA TAC ATT TGC ACA CGT TCA AAC-3' (*Xho*I and *Bam*HI sites were introduced), and then inserted either in sense orientation between *Cla*I/*Bam*HI sites or in antisense orientation between the *Kpn*I/*Xho*I sites separated by a PDK intron in pKANNIBAL. The resultant plasmid harboring *rlpk2*-RNAi cassette was named pKR23-2. The entire *rlpk2*-RNAi cassette comprising sense and antisense fragments of *rlpk2* interspersed by the PDK intron was excised from pKR23-2 by flanking *Not*I sites and inserted into binary vector pART27 yielding the construct pART27-1^[15]. The pART27-1 plasmid with the selectable marker kanamycin resistance gene in plant and streptomycin resistance gene in bacterium was transformed into soybean plants by *Agrobacterium*-mediated transformation method as described previously. Molecular biology methods were performed as described by Sambrook et al.^[16].

1.3 Extraction of genome DNA

The genome DNA was isolated from soybean leaves as previously described by Lodhi et al.^[17]. Introduction of the transgenes was confirmed by PCR method with R2 as both forward and reverse primer, the amplification product of a 1400-bp fragment contained entire *rlpk2*-RNAi cassette (Fig. 1). 32 cycles of amplification were performed under the condition of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 100 s and at last an elongation of 72 °C for 10 min.

1.4 Extraction of total RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from tissues using the guanidine thiocyanate-phenol according to molecular cloning manual^[16]. RNase free DNaseI was used to digest the small amount genomic DNA remaining in the extracted RNA samples. A control PCR using the extracted RNA as template was run to confirm the absence of genomic DNA in the RNA sample. RT-PCR was carried out using ImProm-IITM reverse transcriptase (Promega) according to the manufacturer's instructions. Amplification of soybean ribosome 18s rRNA gene was used as an internal control to normalize for sample-to-sample variations in total RNA amounts and for reaction efficiency. Reactions were carried out in the Whatman Biometra T1 thermocycler. Internal control primers (SRNA-1: 5'-CCT TGC TTG TTG CTT TAC TAA ATA G-3' and SRNA-2: 5'-ATG CAC CTT TTC GTT TGT TTC GGA G-3') yielded an amplification product of 209 bp. The *rlpk2* gene specific primers (3R-2: 5'-CCG AAT AGC TGG GAC ATT TGG ATA T-3' and SPK285: 5'-GAT AAA GAT CTG CTA CAG AAG TAG A-3') yielded an amplification product of 491 bp. *gtr* gene specific primers (*gtr*1-1: 5'-GAT GTC CAA AGT ACA AGT GGG ATT C-3' and *gtr*1-2: 5'-AGC ATC TCC GAG AGT GGC TTG TAG A-3') yielded an amplification product of 512 bp. The semi-quantitative RT-PCR amplification was run with the program: 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C for 33 cycles followed by 10 min at 72 °C. All RT-PCR results shown in this paper were independently repeated three times and the results were identical.

1.5 Characterization of leaf surface structure with scanning electron microscope

The leaf structural difference between wild type and

Fig. 1. *rlpk2*-RNAi expression cassette. R2-reverse, sense insertion of cDNA fragment (301 bp) of *rlpk2*; R2-forward, antisense insertion of cDNA fragment (301 bp) of *rlpk2*; PDK, an intron of pyruvate orthophosphate dikinase gene.

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transgenic soybean plants was determined by a Hitachi S-3500N scanning microscope.

1.6 Measurement of chlorophyll content

The chlorophyll content was spectrophotometrically measured as described by Arnon^[18].

2 Results

2.1 Characterization of the spatial and temporal expression patterns of *rlpk2* gene

Our previous study has revealed a dramatic up-regulation of *rlpk2* expression by artificial senescence-inducing treatment in soybean primary leaves^[11]. Here we report the further investigation on organ specificity of *rlpk2* gene and changes of mRNA level of *rlpk2* during natural soybean leaf senescence. Higher expression level of *rlpk2* was detected in radicles, cotyledons, roots, young pods and flowers, whereas that of the lower ones was detected in the mature leaf (the third trifoliolate leaf at V5 stage) and young pod (Fig. 2). The second trifoliolate leaf at V2, V4, R1 and R2 stages was sampled respectively to detect the expression level of *rlpk2* during natural soybean leaf

Fig. 2. Organ-specific expression of *rlpk2*. Lane1, radicle; lane2, cotyledon; lane3, root; lane4, stem; lane5, mature leaf (the third trifoliolate leaf at V5 stage); lane6, full-opened flower; lane7, young pod; M, DNA molecular marker (DL2000, TaKaRa).

senescence by semi-quantitative RT-PCR method. During the transition from vegetative to reproductive growth the expression level of *rlpk2* increased gradually in soybean leaves, indicating an obvious correlation with leaf senescence. The mRNA level of *rlpk2* decreased at R2 stage when the sampled leaves became over senesced (Fig. 3).

Fig. 3. Effect of natural senescence on the expression level of *rlpk2* gene in the second trifoliolate leaf at V4 (lane1), V6 (lane2), R1 (lane3), and R2 (lane4) stage in soybean plants. M, Marker DL2000.

2.2 Identification of *rlpk2*-RNAi transgenic plants

Both *Agrobacterium*-mediated cotyledon^[13] and growing point^[14] transformation methods were used to transfer pART27-1 plasmid into soybean. The kanamycin resistant seedlings were screened out and transferred to soil. All the putative *rlpk2*-RNAi transgenic seedlings had grown in soil for more than 3 weeks before their genomic DNA were extracted from leaves to detect the introduction of *rlpk2*-RNAi cassette into soybean genomes. The result of PCR amplification with R2 as primer revealed six different *rlpk2*-RNAi transgenic lines, which were designated *rlpk2*-RNAi-1-6 respectively (Fig. 4(a)). Three of them (*rlpk2*-RNAi-1, -5, -6) were obtained by *Agrobacterium*-mediated cotyledon transformation, whereas the others were by growing point transformation (Fig. 5).

As described previously, *rlpk2* gene was strongly ex-

Fig. 4. Identification of transgenic plants. (a) Identification of transgenic plants with PCR method. Lane 1, positive control of PCR amplification with binary vector pART27-1 as template; lane2-9, PCR amplification with genomic DNA of putative *rlpk2*-RNAi transgenic plants as templates, six of them was confirmed; lane10, negative control of PCR amplification; M, Marker DL2000. (b) RT-PCR characterization of expression levels of *rlpk2* gene in leaves of the *rlpk2*-RNAi transgenic plants identified in (a). Lane1-6, line 6-1 of *rlpk2*-RNAi transgenic plant; lane7, wild type; M, Marker DL2000. The total RNA was extracted from the second trifoliolate leaf at V6 stage for each sample.

Fig. 5. Phenotype of *rlpk2*-RNAi transgenic seedlings. All transgenic leaves showed deeper green than those of wild type. *rlpk2*-RNAi-1, -5, -6 were obtained by *Agrobacterium*-mediated cotyledon transformation, whereas *rlpk2*-RNAi-2, -3, -4 were obtained by growing point transformation. WT1 was the wild type control of cotyledon transformation, WT2 was that of growing point transformation (Except for *agrobacterium*-inoculation and antibiotic selection, the wild type controls were processed in the same way as transgenic plants).

pressed in the second trifoliolate leaf at V6 stage during natural soybean senescence (Fig. 3). Therefore total RNA of the second trifoliolate leaf (35 d after its expansion) at V6 stage was isolated from wild type and the six transgenic plants respectively. The expression levels of *rlpk2* gene in these samples were measured by semi-quantitative RT-PCR. The results showed that expression levels of *rlpk2* were greatly decreased in the 35 d leaves of transgenic soybean plants obtained by either cotyledon or growing point transformation method. Furthermore, the mRNA of *rlpk2* could hardly be detected in *rlpk2*-RNAi-1, -3 lines whereas very high level was detected in wild type leaves (Fig. 4(b)). These results strongly suggested the successful knocking-down of *rlpk2* gene expression by RNA interference in transgenic plants.

2.3 Knocking-down of *rlpk2* gene delayed leaf senescence of transgenic plants

All six transgenic soybean seedlings showed deeper green leaves than wild type plants (Fig. 5). The deeper green color of leaf was observed along with the growth of *rlpk2*-RNAi plants, from the juvenile leaves just fully expanded 3 d to the senescent ones at R1 stage (Fig. 6(a) (c), (e)). Senescence of mature leaves was greatly retarded in transgenic plants (Fig. 6(b)). Glutamyl-tRNA reductase (GTR) is a key enzyme for chlorophyll synthesis^[19]. RT-PCR analysis showed that the expression level of *gtr* gene decreased with leaf age. However, the expression level of *gtr* in all detected *rlpk2*-RNAi leaves was substantially higher than those in wild type plants (Fig. 7(a)).

Fig. 6. Knocking-down of the *rlpk2* gene expression delayed leaf senescence of transgenic plants. (a) Juvenile leaves (the topside trifoliolate leaf at V5 stage, 3 d after fully expanding); (b) mature leaves (the fifth trifoliolate leaf at R1 stage, 40 d after expanding while onset of leaf senescence had been initiated in the corresponding wild type leaf); (c) senescent leaves; (d) nutrient deficiency-induced leaf senescence (9 d after fully expanding); (e) mid single leaflets from the second trifoliolate leaf at V2 stage; (f) graphs of surface scanning of (e) by scanning electron microscope (200×); (g) graphs of surface scanning of the 4th trifoliolate leaf at R1 stage (1000×). WT, wild type plants; RNAi, *rlpk2*-RNAi transgenic plants.

It was also found that knocking-down of *rlpk2* gene greatly increased the chlorophyll content in 40 d expanded transgenic leaves (Fig. 7(b)). To investigate the effect of knocking-down of *rlpk2* on the onset of nutrient deficiency-induced leaf senescence, both 30 d seedlings of wild type and six transgenic lines were transplanted from soil to vermiculite and irrigated with pure water for 10 d. Leaf fading caused by nutrient deficiency was observed initiating from the tip of all young leaves, which represents the developmentally oldest region of a piece of leaf, but the transgenic leaves showed significantly less senescence than wild type leaves (Fig. 6(d)).

Surface scanning of leaves by the scanning electron

microscope revealed more cell-aggregated structures and more rigid trichomes on the surface of young *rlpk2*-RNAi transgenic leaves than those of wild type leaves (Fig. 6(e) (g)). In addition, knocking-down of *rlpk2* gene caused abnormal development of ovary and stigma in all six transgenic plants, hence sterile flower, whereas the empty pART27 vector-transformed soybean plants developed normally under the same conditions. Further study on the relationship of *rlpk2* knocking-down and reproductive development of soybean is in progress.

3 Discussion

Senescence is a genetically programmed, indispensable

development process of higher plants. Although a number of SAGs involved in leaf senescence have been identified, the detailed molecular mechanisms underlying the progression of leaf senescence are unknown^[20,21]. Although membrane-associated receptor-like protein kinases have been found to be involved in many developmental and stress signal transduction pathways, few of them were reported to directly initiate and/or regulate leaf senescence^[22].

Soybean is an important crop on the earth. The monocarpic senescence is a typical characterization of soybean^[23]. This process has shown to be closely related to cytokinins activity^[23]. It has been reported that the exogenous application of cytokinins effectively retarded vegetable senescence and improved soybean quality and yields^[24,25]. We have cloned *rlpk2* gene from artificially induced senescent soybean leaf. It codes for a novel soybean LRR receptor-like protein kinase. The gene expression of *rlpk2* was up-regulated in both of the previously described artificial senescence-inducing system^[11] and the natural monocarpic senescence process after florescence (Fig. 3). While the expression of *rlpk2* was knocked down by RNAi method, all transgenic soybean plants showed deeper green leaves and higher chlorophyll content than wild type plants (Figs. 5, 7(b)). Both natural senescing process and nutrient deficiency-induced senescence of transgenic leaves were greatly delayed (Fig. 6). The expression level of *gtr* gene, a key factor in chlorophyll synthesis, was increased remarkably (Fig. 7(a)). All these results together implied the involvement of RLPK2 in regulating leaf senescence progression of soybean.

Fig. 7. Effects of knocking-down of *rlpk2* gene on *gtr* expression. (a) and chlorophyll content (b) in transgenic leaves. (a) Lane1–4, the ninth, seventh, sixth and fourth trifoliolate leaf of wild type at R1 stage, respectively; lane5–8, the ninth, seventh, sixth and fourth trifoliolate leaf of transgenic plant at R1 stage, respectively. (b) Leaves 40 d after fully expanding from three typical *rlpk2*-RNAi transgenic lines (R-2, R-4 and R-6) were sampled for chlorophyll content assay. Data were shown as mean values $\pm$ SE of three independent repeats.

As in many other developmental processes, senescence is initiated and controlled by internal and environmental senescence signals through specific transduction pathways. However, the questions of how the programmed process is initiated and how the senescence signals are transduced remain to be answered. Diverse LRR motifs are present in the extracellular regions of LRR-RLKs. Through protein-to-protein interactions the LRR motifs are potentially responsible for perceiving different environmental and development signals, thus triggering the protein phosphorylation-mediated signal transduction cascade, and leading to expression of a series of downstream genes followed by events of biochemical, physiological, and morphological changes^[26]. *rlpk2* gene codes for a typical LRR receptor-like protein kinase; its expression was up-regulated during natural leaf senescence (Fig. 3), and *rlpk2*-RNAi transgenic plants showed a great delay in leaf senescence progression (Figs. 5, 6, 7(b)). All the observation implied that RLPK2 is one of upstream components of soybean senescence signal transduction pathway(s). Higher expression level of *gtr* gene was observed in all detected *rlpk2*-RNAi transgenic leaves, including the juvenile to senescent ones (Fig. 7(a)). It is reasonable to postulate that RNA interference of *rlpk2* gene expression blocked the effective transduction of senescence signals, thus either oppressing the expression of genes involved in breakdown of chlorophyll and macromolecules, or maintaining / increasing the level of their synthesis. All these resulted in the retardation of soybean leaf senescence.

Study on organic expression pattern of *rlpk2* revealed higher transcript levels in radicles, cotyledons, roots, stems and flowers (Fig. 2), suggesting multiple functions of RLPK2 besides its regulation of leaf senescence. Furthermore, the observation of stronger expression of *rlpk2* in wild type flowers, especially the abnormal flower development and sterility of *rlpk2*-RNAi transgenic plants, suggested the involvement of RLPK2 in regulating the switch of vegetative growth to sexual reproductive growth of soybean.

Another interpretation for delay in the leaf senescence of *rlpk2*-RNAi transgenic plants may be that knocking-down of *rlpk2* blocks the transition of vegetative growth to reproductive growth. However, this presumption is far from sufficient to explain the observation of more resistance of *rlpk2*-RNAi transgenic plants to nutrient deficiency-induced leaf senescence (Fig. 6(d)). We may postulate that RLPK2 is an upstream component of soybean senescence signaling pathway(s), and also plays important role in controlling the transition of vegetative growth to sexual reproduction of soybean development. Further experimental study is required to verify these hypotheses.

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entation of the basic vectors pKANNIBAL and pART27 for *rlpk2*-RNAi construction.

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