Chapter 19

Construction of the Leaf Senescence Database and Functional Assessment of Senescence-Associated Genes

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Abstract

Leaf senescence is the last phase of plant development and a highly coordinated process regulated by a large number of senescence-associated genes (SAGs). By broad literature survey, we constructed a leaf senescence database (LSD) in 2011 and updated it to Version 2.0 in 2014 (http://www.eplantsenescence.org/ and http://psd.cbi.pku.edu.cn/) which contains a total of 5357 genes and 324 mutants from 44 species. These SAGs were retrieved based on genetic, genomic, proteomic, physiological, or other experimental evidence and were classified into different categories according to their functions in leaf senescence or morphological phenotype of mutants. To provide comprehensive information for SAGs, we made extensive annotation by both manual and computational approaches. In addition, we predicted putative orthologues of the SAGs in other species. LSD has a user-friendly interface to allow users to make text queries or BLAST searches and to download SAGs sequences for local analysis. Functional analyses of putative SAGs reveal that WRKY75, AZF2, NAC16, and WRKY26 are positive regulators of leaf senescence, while MKP2 and CTR1 perform negative regulation to leaf senescence. This database has been served as a valuable resource for basic research on the function of SAGs and evolution of plant leaf senescence, as well as for the exploration of genetic traits in agronomically important plants.

Key words Leaf senescence, Senescence-associated gene, Database annotation, Orthologue

1 Introduction

Senescence is the final development phase of plant leaves, in which leaf cells initiate active degenerative processes, such as the degradation of chlorophylls, proteins, and other macromolecules [1]. The released nutrients are then transferred to growing leaves, developing fruits and maturing seeds [2]. Leaf senescence can either be naturally induced during development or stimulated by environmental factors including darkness, nutritional deficiency, and various stresses [1]. Efficient senescence is essential for plants

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to accumulate nutrients which can be used in the next season or generation. However, premature senescence which is a protective mechanism when plants undergo stress leads to the decrease of crop yield and quality [3].

Leaf senescence is a highly coordinated process regulated by a large number of senescence-associated genes (SAGs), which are upregulated during senescence [1]. Many advances in the understanding of the molecular mechanisms of leaf senescence have been achieved through the identification and characterization of hundreds of SAGs and their corresponding mutants in *Arabidopsis thaliana*, *Lycopersicon esculentum*, and *Nicotiana tabacum* [4–6]. Recently, genome-scale analyses have been widely used in leaf senescence studies, and thousands of SAGs have been identified and categorized by using the ATH1 Arabidopsis GeneChip microarray or RNA-sequencing [3], providing a systematic view of transcriptional regulation in leaf senescence. Among them, more than 200 transcription factors, including WRKY, NAC, MADS, MYB, bZIP, and bHLH family members, are involved in the regulation of leaf senescence [5, 6], indicating that leaf senescence is governed by complex transcriptional regulatory networks.

Interestingly, recent findings show that, in addition to the senescence-specific regulation of gene expression by transcription factors, leaf senescence is also controlled by a higher-order regulatory epigenetic mechanism through differential alteration of chromatin structure at distinct gene loci [7]. In *Arabidopsis*, a previous study reported that expression levels of many regulatory factors of leaf senescence are affected in plants overexpression of a histone methyltransferase *SUVH2* (SUPPRESSOR of VARIEGATION HOMOLOG 2), and leaf senescence is delayed in these plants. At the beginning of leaf senescence, active markers such as H3K4me3 enriches at WRKY53 locus [8], indicating a senescence-induced gene expression. Similarly, Brusslan et al. reported that the active markers H3K4me3 or H3K9ac is significantly increased in At5g13080 that encodes WRKY75 [9], another important transcription factor regulating leaf senescence [4].

Molecular and genetic studies of leaf senescence in recent years led to the accumulation of a large volume of scattered information related to SAGs. Using bioinformatics tools along with manual curation, we constructed a leaf senescence database (LSD) in 2011 and updated it to Version 2.0 in 2014 (http://www.eplantsenescence.org/, http://psd.cbi.pku.edu.cn/). During the past 5 years, this database has been not only used extensively in our own leaf senescence research but also accessed by plant scientists working in the plant genetics, genomics, and molecular breeding.

2 Construction of the Database

2.1 Pipeline

Figure 1 shows the pipeline we used to construct the LSD and to make functional analyses of the SAGs. We started with PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) keyword search and collected SAGs as well as the phenotype information of their mutants through an extensive literature survey. General descriptions and database annotations related to leaf senescence were retrieved from literature papers and online databases and entered into the LSD through a specially designed computational tool and manually confirmed.

We obtained 324 SAGs with mutational evidence (LSD Version 2.0) through the above approach. These SAGs formed the core dataset of the database. In order to provide users with candidate SAGs for further experimental validation, we collected potential SAGs generated through microarray expression profiling of the model organism *Arabidopsis* and made it publicly available [3]. We downloaded the computationally identified SAGs of an economically important monocot species banana (*Musa acuminata*) from the Banana Genome Hub (http://banana-genome.cirad.fr/). Taken together, a total of 5357 SAGs from 44 species were identified and manually verified based on genetic, genomic, proteomic, physiological, and other experimental evidence (Table 1).

In addition to manual curation, computational approaches were also employed to annotate these SAGs. We predicted the potential miRNA targets of the SAGs using the RNAhybrid method [12]. The orthologues of each SAG in other plants were retrieved

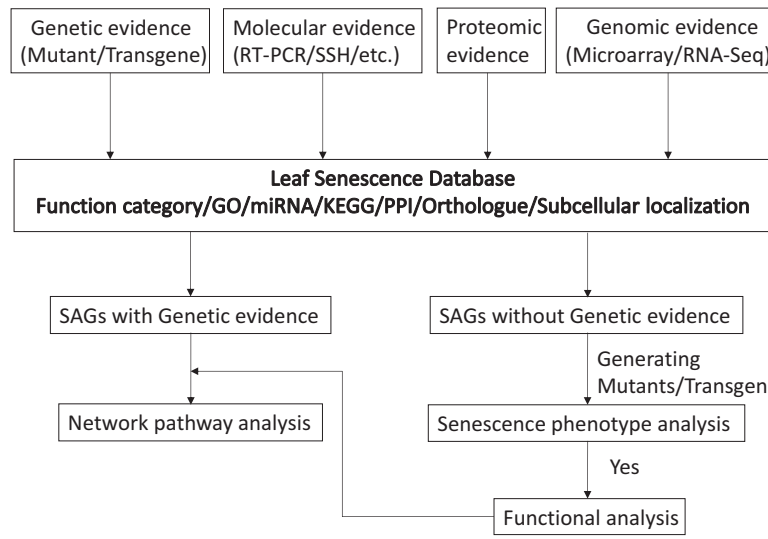


Fig. 1 The pipeline used for collecting, annotating, and functional analysis of SAGs

Table 1
Number of SAGs from 44 Species in LSD

Species	Common name	Number	Mutants	Transgenic
Arabidopsis thaliana	Thale cress	3745	191	82
Musa acuminata	Banana	882	0	0
Triticum aestivum	Wheat	256	2	0
Oryza sativa	Rice	132	12	12
Zea mays	Maize	94	0	0
Triticum turgidum	Wheat	65	0	0
Medicago truncatula	Barrel clover	31	0	0
Sorghum bicolor	*Sorghum*	26	0	0
Lycopersicon esculentum	Tomato	23	4	0
Hordeum vulgare	Barley	14	0	0
Glycine max	Soybean	12	1	2
Brassica oleracea	Broccoli	9	0	0
Nicotiana tabacum	Tobacco	9	3	3
Brassica napus	Rapeseed	8	0	1
Pisum sativum	Pea	6	1	0
Brassica rapa var. *parachinensis*	Choy sum	5	0	0
Ipomoea batatas	Sweet potato	4	3	0
Lolium perenne	Perennial ryegrass	4	0	0
Solanum tuberosum	Potato	3	0	0
Arabidopsis lyrata	Thale cress	2	0	0
Brassica campestris	Chinese cabbage	2	0	0
Medicago sativa	Alfalfa	2	1	0
Spinacia oleracea	Spinach	2	0	0
Amaranthus hypochondriacus	Grain amaranths	1	0	0
Astragalus sinicus	Chinese milk vetch	1	1	0
Brassica rapa subsp. *rapa*	Turnip	1	0	0
Camellia sinensis	Tea	1	0	0
Capsicum annuum	Pepper	1	0	0
Chenopodium rubrum	Red goosefoot	1	0	0
Crocus sativus	Saffron	1	0	0

(continued)

Table 1
(continued)

Species	Common name	Number	Mutants	Transgenic
Cucumis melo	Muskmelon	1	0	0
Daucus carota	Carrot	1	0	0
Dianthus caryophyllus	Carnation	1	0	0
Festuca arundinacea	Tall fescue	1	1	0
Festuca pratensis Huds.	Fescue	1	0	0
Fragaria x ananassa	Strawberry	1	0	0
Ipomoea nil	Japanese morning glory	1	1	0
Mangifera indica	Mango	1	0	0
Neosinocalamus affinis	Rendle	1	0	0
Nicotiana attenuata	Solanaceae	1	0	0
Petunia hybrida	Petunia	1	2	1
Platycodon grandiflorus	Balloon flower	1	0	0
Rosa hybrida	Rose	1	0	0
Vigna unguiculata	Cowpea	1	0	0
Total	44	5357	223	101

from the online database OrthoMCL-DB [13], and putative function domains of SAGs-encoding proteins were identified by InterProScan [14, 15]. Subcellular localization information of *SAG*s in *Arabidopsis* mined from literature or generated from the SUBA3 program was also added [16]. QTL information linked to the original database (http://archive.gramene.org/qtl/) was included for further studies of leaf senescence-related agronomic traits in crops such as rice, maize, and *Sorghum*. In addition, *Arabidopsis* seed information obtained from TAIR was integrated. Finally, a total of 108 images of *Arabidopsis thaliana* mutants obtained from our experimental validation for some SAGs were added into the database [6].

2.2 Data Sources and Software Tools

SAG sequences including DNA, mRNA, and protein of model organisms with completed genomes were downloaded from genome sequencing centers (Table 2) such as the *Arabidopsis* Information Resource (TAIR). For species whose genomic sequences were not available when we started to construct LSD, we downloaded the assembled transcripts from the Plant Genome Database (PlantGDB).

Table 2
Sequence data source of SAGs

Species	Website
Arabidopsis thaliana (thale cress)	http://www.arabidopsis.org/
Brassica napus (rapeseed)	http://www.genoscope.cns.fr/brassicanapus/
Glycine max (soybean)	http://www.plantgdb.org/GmGDB/
Hordeum vulgare (barley)	http://plants.ensembl.org/Hordeum_vulgare/Info/Index/
Lycopersicon esculentum (tomato)	https://solgenomics.net/organism/Solanum_lycopersicum/genome
Medicago truncatula (barrel clover)	http://www.plantgdb.org/MtGDB/
Musa acuminata (banana)	http://banana-genome.cirad.fr/
Nicotiana tabacum (tobacco)	https://solgenomics.net/organism/Nicotiana_tabacum/genome
Oryza sativa (rice)	http://rice.plantbiology.msu.edu/
Solanum tuberosum (potato)	https://solgenomics.net/organism/Solanum_tuberosum/genome
Sorghum bicolor (sorghum)	http://www.plantgdb.org/SbGDB/
Triticum aestivum (wheat)	http://wheatgenome.info/
Zea mays (maize)	http://www.maizegdb.org/
Other species	http://www.plantgdb.org/

The sequence and annotation data were stored in a relational database MySQL, an open-source database management system widely used for biological database applications. Queries to the database were implemented in PHP scripts running in an Apache web server under Linux environment. Graphics are drawn using the PHP module of the GD graphics library.

To meet the general requirements of data analyses, we integrated the sequence similarity search tool BLAST [10] and the sequence analysis platform WebLab [11] into LSD (Table 3). Users can either retrieve the sequence from LSD, or upload their own sequences to search homologues against different type of sequence (DNA, mRNA, CDS, and protein) in LSD. Users may also perform further analyses for the SAG sequences using the web-based bioinformatics platform WebLab we developed.

3 Usage of the Database

3.1 User Interface

LSD provides an easy-to-use user interface with the following functionalities:

Table 3
Software tools used in the construction and annotation of LSD

Name	Website
MySQL	http://dev.mysql.com/
Blast	http://blast.ncbi.nlm.nih.gov/
WebLab	http://www.weblab.org.cn/
RNAhybrid [12]	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/
OrthoMCL-DB [13]	http://www.orthomcl.org/orthomcl/
InterProScan [14, 15]	http://www.ebi.ac.uk/interpro/scan.html
SUBA3 [16]	http://suba.plantenergy.uwa.edu.au/

1. Browse: browse SAGs (via a species table or species tree), mutant, phenotype, mutant seed, and stay-green QTL.
2. Search: Text Search and BLAST sequence similarity search.
3. Help: User Guide and FAQs.
4. Download: an FTP server with Genomic, Coding, cDNA, and protein sequences of SAGs.
5. Feedback: an online form for users to give feedback.
6. Submit: an online form for users to upload SAGs.
7. Links: links to leaf senescence-related web sites and databases.
8. About: general description of the database and the development team.

The online Help and FAQs provide instructions for the above functionalities. For example, the Text Search has the following options described in the User Guide:

(a) Search genes by locus name, alias name, or keywords.
(b) Search mutants by mutant name, mutant type, or ecotype.
(c) Search article by title, author, or keyword.
(d) Search primers by locus name, alias name.
(e) Search for interactions between miRNAs and SAGs.

3.2 Case Study

LSD collects two types of SAGs. The SAGs in the core dataset which were retrieved from literatures contain rich information obtained by both manual curation and computational annotation, while those identified through high-throughput investigation have less information. In the following section, we take two real examples to show how to search the database and what kind information can be obtained.

The first example is an *Arabidopsis* transcription factor, the ethylene-insensitive gene 2 (*EIN2*), which is a positive regulator of ethylene-induced leaf senescence. The steps to search and display the information related to this SAG is as follows:

1. Open a typical web browser such as Firefox, type in the URL of the leaf senescence database: http://psd.cbi.pku.edu.cn/.
2. Click the Text Search button in the left-side menu bar to open a text search window.
3. In the text search window, type in the locus name of the Arabidopsis EIN2 gene AT5G03280 and click the Submit button.
4. A table of search results shows the entry name of this SAG.
5. Click the link AT5G03280 to display the rich information of this SAG (Fig. 2).

Figure 2 shows the screen dump obtained as the above text search steps. The information is divided into several sections. The first section (Fig. 2a) contains general information described as follows:

1. Locus name: clicking the link AT5G03280 brings up a page in the *Arabidopsis* Information Resource.
2. Alias: *EIN2*.
3. Organism: *Arabidopsis thaliana*.
4. Taxonomic identifier: clicking this link NCBI brings up the NCBI taxonomic information page for *Arabidopsis thaliana*.
5. Functional category: Hormone response pathway.
6. Effect of senescence: promote.
7. Gene description: a brief description for this gene such as "involved in ethylene signal transduction."
8. Evidence: Genetic evidence – Mutant [1].
9. References: the literature citation related to this SAG.
10. Gene Ontology: clicking each link brings up to the Gene Ontology information database including biological process, cellular component, and molecular function.
11. Pathway: clicking REACT_15518 brings up a page in the REACTOME pathway database.
12. Protein-protein interaction: clicking 3702.AT5G03280.1 brings up a protein-protein interaction page in the STRING database.
13. Sequence: clicking Genomic, mRNA, CDS, or Protein shows DNA, RNA, or protein sequences.

a

Locus name	AT5G03280	
Alias	EIN2	
Organism	*Arabidopsis thaliana*	
Taxonomic identifier	[NCBI]	
Function category	Hormone response pathway:ET	
Effect for Senescence	promote	
Gene Description	Involved in ethylene signal transduction. Acts downstream of CTR1. Positively regulates ORE1 and negatively regulates mir164A,B,C to regulate leaf senescence.	
Evidence	Genetic evidence:Mutant [Ref 1]	
References	1: Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis. Science 2009 Feb 20;323(5917):1053-7	
Gene Ontology	biological process	transport
	cellular component	membrane
	molecular function	transporter activity
Pathway	Reactome	REACT_15518
Protein-Protein Interaction	STRING	3702.AT5G03280.1-P
Sequence	AT5G03280.1 | Genomic | mRNA | CDS | Protein	

Mutant information

Mutated 1	**Mutant name**	ein2-34
	Mutant/Transgenic	mutant
	Ecotype	Col-0
	Mutagenesis type	EMS
Mutated 2	**Mutant name**	AAF-OXein2-5
	Mutant/Transgenic	transgenic
	Ecotype	Col-0
	Mutagenesis type	cross
Mutated 3	**Mutant name**	ein2-1/GVG:GmSARK
	Mutant/Transgenic	mutant
	Ecotype	Col-0
	Mutagenesis type	cross
Mutated 4	**Mutant name**	ein2-5EIL1ox
	Mutant/Transgenic	Double mutant
	Ecotype	Col-0
	Mutagenesis type	cross
Mutated 5	**Mutant name**	ore3
	Mutant/Transgenic	mutant
	Ecotype	Col-0
	Mutagenesis type	EMS

Fig. 2 A typical LSD entry: the Arabidopsis *EIN2* gene (AT5G03280). (**a**) Basic and mutant information. (**b**) miRNA interaction. (**c**) Ortholog Group, Cross Links, Mutant Image, and Subcellular Localization

b

miRNA Interaction

Details

```
target: AT5G03280.1
miRNA: ath-miR854a
mfe: -28.3 kcal/mol
p-value: 0.059681

position:  50
target 5' U   U    A        U     A 3'
           CUC CUCU UCUCUAUC CUCGU
           GAG GAGG AGGGAUAG GAGUA
miRNA  3'                        G 5'
```

```
target: AT5G03280.1
miRNA: ath-miR854b
mfe: -28.3 kcal/mol
p-value: 0.052700

position:  50
target 5' U   U    A        U     A 3'
           CUC CUCU UCUCUAUC CUCGU
           GAG GAGG AGGGAUAG GAGUA
miRNA  3'                        G 5'
```

```
target: AT5G03280.1
miRNA: ath-miR854c
mfe: -28.3 kcal/mol
p-value: 0.056671

position:  50
target 5' U   U    A        U     A 3'
           CUC CUCU UCUCUAUC CUCGU
           GAG GAGG AGGGAUAG GAGUA
miRNA  3'                        G 5'
```

```
target: AT5G03280.1
miRNA: ath-miR854d
mfe: -28.3 kcal/mol
p-value: 0.066216

position:  50
target 5' U   U    A        U     A 3'
           CUC CUCU UCUCUAUC CUCGU
           GAG GAGG AGGGAUAG GAGUA
miRNA  3'                        G 5'
```

```
target: AT5G03280.1
miRNA: ath-miR854e
mfe: -28.3 kcal/mol
p-value: 0.056286

position:  50
target 5' U   U    A        U     A 3'
           CUC CUCU UCUCUAUC CUCGU
           GAG GAGG AGGGAUAG GAGUA
miRNA  3'                        G 5'
```

Fig. 2 (continued)

c

Ortholog Group

Ortholog Groups: OG5_153355

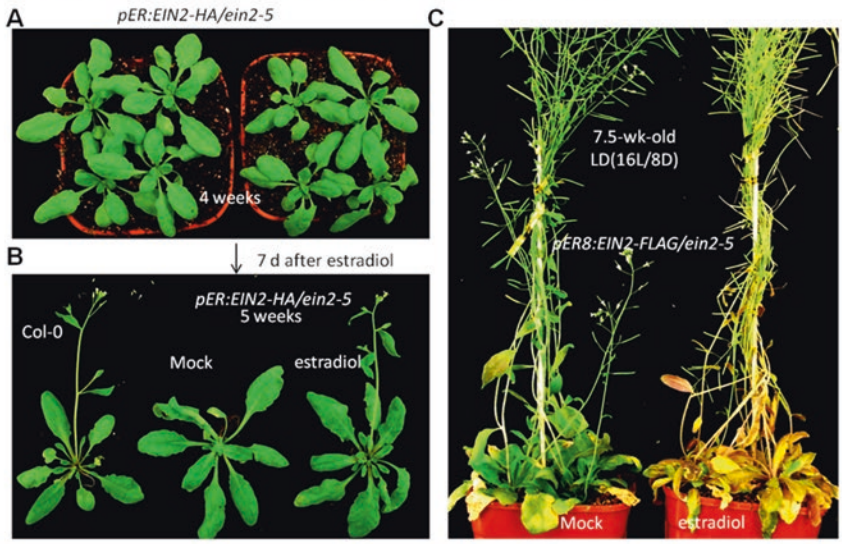
Accession	Taxon
NP_195948 (AT5G03280)	Arabidopsis thaliana
196299	Chlamydomonas reinhardtii
NP_001050996	Oryza sativa Japonica Group
NP_001058920	Oryza sativa Japonica Group
NP_001058922	Oryza sativa Japonica Group
e_gw1.197.128.1	Physcomitrella patens subsp. patens
e_gw1.54.248.1	Physcomitrella patens subsp. patens
30078.m002320	Ricinus communis
XP_002954234	Volvox carteri f. nagariensis

Cross Link

Database	Entry ID	E-value	Start	End	InterPro ID	Description
PIRSF	PIRSF037378	0.0	1	1294	IPR017187	Ethylene-insensitive protein 2
PANTHER	PTHR11706	0.0	2	626	IPR001046	Natural resistance-associated macrophage like
PANTHER	PTHR11706:SF4	0.0	2	626	IPR017187	Ethylene-insensitive protein 2
Pfam	PF01566	1.6E-88	38	390	IPR001046	Natural resistance-associated macrophage like
PRINTS	PR00447	1.0E-17	97	123	IPR001046	Natural resistance-associated macrophage like
PRINTS	PR00447	1.0E-17	125	144	IPR001046	Natural resistance-associated macrophage like
PRINTS	PR00447	1.0E-17	199	222	IPR001046	Natural resistance-associated macrophage like
PRINTS	PR00447	1.0E-17	301	320	IPR001046	Natural resistance-associated macrophage like
PRINTS	PR00447	1.0E-17	361	380	IPR001046	Natural resistance-associated macrophage like

Mutant Image

overexpression of EIN2C leads to early flowering and senescence.



Subcellular Localization

Localization	plasma membrane
Evidence	SUBAcon
Pubmed ID	23180787

Fig. 2 (continued)

For those SAGs with one or more mutants, we retrieved the information for each mutant and made them available including mutant name and type, ecotype, and mutagenesis type. For example, the mutant name of mutant 1 of this SAG (*EIN2*) is "*ein2-34*," the ecotype is "Col-0," and the mutagenesis type is "EMS" (Fig. 2a). Users may find additional information such as chlorophyll content, leaf color marker gene expression for each mutant by clicking the name link, e.g., "*ein2-34*" to access the mutant page.

As shown in Fig. 2b, the predicted potential miRNA targets for EIN2 and the link to miRBase for the miRNAs were added. The Ortholog Group section lists orthologs of this SAG, i.e., AT5G03280, from other plants with links to the OrthoMCL database (orthomcl.org/). And the Cross Link section gives the domain and motif information of the protein sequence of the SAG with links to the original database such as PANTHER (http://www.pantherdb.org/), Pfam (http://pfam.xfam.org/), and PRINTS (http://www.bioinf.man.ac.uk/dbbrowser/PRINTS/). Subcellular localization information of *SAG*s in *Arabidopsis* mined from literature or generated by the SUBA3 program was added. Finally, mutant images for some SAGs generated from our laboratory were added into the database. For example, users may find transgenic plants overexpressing *EIN2* and exhibit early flowering and early senescence phenotype (Fig. 2c).

For those potential *Arabidopsis* and banana SAGs either identified by microarray profiling or predicted by computational tools, there are fewer annotations than that of the SAGs in the core dataset (Fig. 3). However, the general information, the ortholog groups, and the cross-links may give some evidence for users to carry on experimental validation.

4 Functional Assessment of Putative SAGs

In order to verify whether SAGs collected in LSD really affect leaf senescence process, we made functional assessment for several candidate SAGs collected through high-throughput approaches. T-DNA insertion lines were selected using the SIGnAL database (http://signal.salk.edu/) and ordered from ABRC. If multiple insertions were available in the same genes, the selection was based on the position of the insertions that disrupt the gene function as much as possible, such as those insertions located within exon regions. Some RNAi lines were generated by ourselves or obtained from other laboratories for further study if the mutant line is not available in the SALK collections [4].

4.1 Plant Materials

All of the transgenic lines and mutants were derived from the wild-type *Arabidopsis thaliana* Columbia (Col-0) ecotype and cultivated in growth chambers under long-day conditions (LDs; 16 h light/8 h dark) at 22 °C under fluorescence illumination

Basic information	
Locus name	AT1G01070
Organism	*Arabidopsis thaliana*
Taxonomic identifier	[NCBI]
Function category	Others
Effect for Senescence	unclear
Gene Description	nodulin MtN21 family protein. Gene expression is increased during leaf senescence
Evidence	Genomic evidence:microarray data [Ref 1]
References	1: Breeze E, Harrison E, McHattie S, Hughes L, Hickman R, Hill C, Kiddle S, Kim YS, Penfold CA, Jenkins D, Zhang C, Morris K, Jenner C, Jackson S, Thomas B, Tabrett A, Legaie R, Moore JD, Wild DL, Ott S, Rand D, Beynon J, Denby K, Mead A, Buchanan-Wollaston V High-resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals a distinct chronology of processes and regulation. Plant Cell 2011 Mar;23(3):873-94
Gene Ontology	cellular component — membrane
Protein-Protein Interaction	STRING — 3702.AT1G01070.1-P
Sequence	AT1G01070.1 | Genomic | mRNA | CDS | Protein AT1G01070.2 | Genomic | mRNA | CDS | Protein

Ortholog Group

Ortholog Groups: OG5_147140

Accession	Taxon
NP_001077514	Arabidopsis thaliana
NP_172612	Arabidopsis thaliana
NP_172613	Arabidopsis thaliana
NP_192052	Arabidopsis thaliana
NP_192053	Arabidopsis thaliana
NP_192054	Arabidopsis thaliana
NP_563617 (AT1G01070)	Arabidopsis thaliana
NP_849280	Arabidopsis thaliana
NP_973734	Arabidopsis thaliana
NP_974494	Arabidopsis thaliana
NP_974495	Arabidopsis thaliana

Cross Link

Database	Entry ID	E-value	Start	End	InterPro ID	Description
PANTHER	PTHR31218	5.4E-110	15	290	No hit	NA
Pfam	PF00892	4.5E-5	18	110	IPR000620	Drug/metabolite transporter
SUPERFAMILY	SSF103481	4.7E-6	28	114	No hit	NA
Pfam	PF00892	1.4E-8	176	282	IPR000620	Drug/metabolite transporter
SUPERFAMILY	SSF103481	5.4E-6	211	287	No hit	NA

Fig. 3 An LSD entry (AT1G01070) without mutant information

(100–150 μE/m²/s) [4]. Seeds were sterilized and stratified in the dark at 4 °C for 3 days and germinated on Murashige and Skoog (MS) medium (pH 5.7) supplemented with 1% sucrose and 0.8% (w/v) agar. T-DNA insertion null alleles for SAGs in the Col-0 background were obtained from the randomly mutagenized

T-DNA lines (SALK collection) at the *Arabidopsis* Information Resource (TAIR). Homozygous plants were identified from segregating T3 populations by genotyping with gene-specific primers.

4.2 Experimental Conditions

To test whether experimental conditions are suitable for leaf senescence phenotype analysis, we took the mutants and transgenic plants with known senescence phenotype [4]. Plants with significant delayed or promoted senescence phenotype were used, such as *ein2-5*, *ein3-1*, *atnap*, *wrky75*, *wrky53*, and *EIN3ox*. They were grown in soil under long-day conditions (16 h light/8 h dark) along with wild-type controls and senescence phenotypes were observed every week.

4.3 Large-Scale Screening of Senescence-Associated Mutants

We utilized the same approach described above for large-scale phenotype analyses [4], mainly focused on transcriptional factors (NAC, WRKY, bZIP, and zinc finger gene families) as well as genes involving in signal transduction (e.g., protein phosphate or dephosphate). Not surprisingly, most of the mutants could not be distinguished from the wild type, probably due to functional redundancy or lack of effect on senescence. Previously, we found WRKY75 and AZF2 were positive regulators of leaf senescence, while a protein phosphatase AtMKP2 showed negative regulation to leaf senescence [4].

Recently, we found that *nac16* mutants, a T-DNA insertion line of NAC16 (SALK_001597C), showed a delayed senescence phenotype, suggesting that NAC16 was a positive regulator of leaf senescence (Fig. 4).

Compared with Col-0 wild-type plants, no difference in overall development, bolting, and flowering time could be observed in *nac16* mutants (Fig. 4a). However, if rosette leaves of 5-week-old plants were analyzed, we observed that *nac16* mutant plants showed delayed leaf senescence phenotypes compared to the wild-type plants (Fig. 4b). Senescent yellowing leaves can be observed on wild-type plants of the same age as *nac16* plants, which did not have any leaves undergoing senescence at this point (Fig. 4b). For 7-week-old plants, most of leaves in Col-0 became yellow, while leaves of *nac16* were still green (Fig. 4c). It suggests that NAC16 was a positive regulator of leaf senescence, which had been confirmed by other researchers [17]. In addition, we also found that transgenic plants overexpressing *NAC102* and *WRKY26* exhibit earlier senescence phenotype, indicating that NAC102 and WRKY26 were also positive regulators of leaf senescence (data not shown).

4.4 Reanalysis of Mutant CTR1

CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), a Raf-like Ser/Thr protein kinase, is a negative regulator of ethylene signaling. Ethylene has been known as an endogenous modulator of senescence, including fruit ripening and flower and leaf senescence.

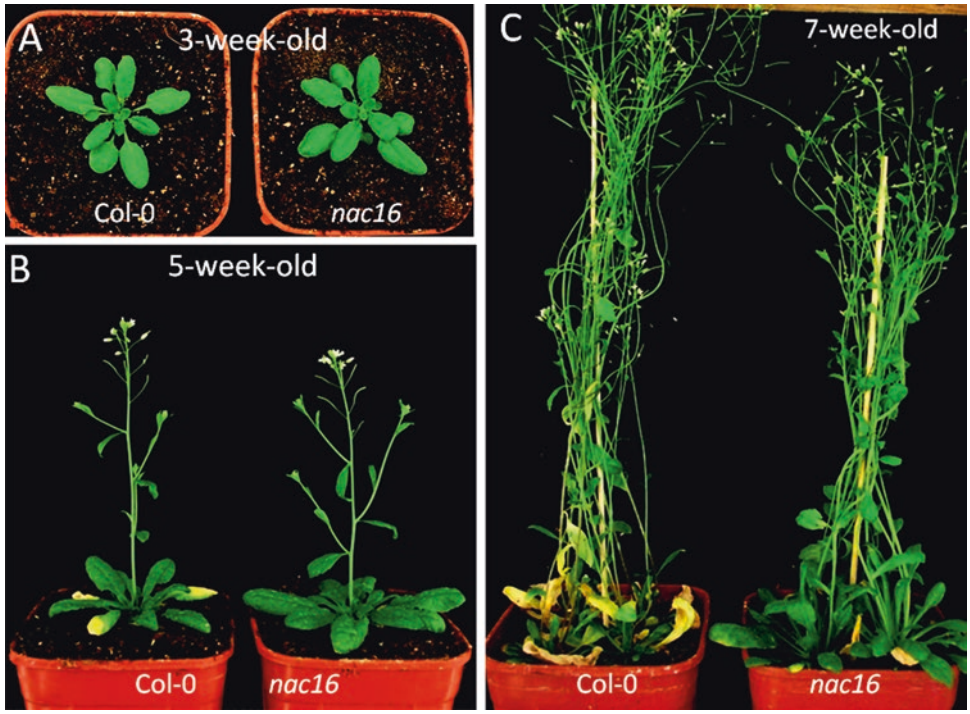


Fig. 4 T-DNA insertion line *nac16* exhibits an age-dependent delay senescence phenotype. (**a**) T-DNA insertion mutant *nac16* grown in soil under long-day (16 h light/8 h dark) conditions alongside wild-type controls (Col-0) and 3-week-old plants. (**b**) Senescence-related phenotype of 5-week-old *nac16* and wild-type Col-0 plants. (**c**) Senescence-related phenotype of 7-week-old *nac16* and wild-type Col-0 plants

However, previous studies suggested that *ctr1-1* mutant has a wild-type timing of senescence under standard growth conditions [18]. Here, we reanalyzed the senescence phenotypes of *ctr1-1* under our experimental conditions (Fig. 5).

In fact, it is difficult to find the difference between the *ctr1-1* mutant and wild-type Col-0 (Fig. 5a) based on the observation of the whole plants only. However, when all the rosette leaves were detached and arranged according to their ages, it is easy to find that most of *ctr1-1* leaves died (leaf 1–13). By contrast, only five leaves of 40-day-old Col-0 (Leaf 1–5) including cotyledon leaves died, and one leaf became yellowing (Leaf 6) (Fig. 5b). Interestingly, many rosette-like leaves which masked our observation were found in the stem of *ctr1-1* plants (Fig. 5c, d). Furthermore, *ctr1-1* mutant leaves showed significant chlorosis when excised and placed in the dark in air for several days (data not shown), to a level approaching that observed in wild-type leaves treated with ethylene. Since chlorosis is a yellowing of leaf tissue due to a lack of chlorophyll, we conclude that loss of function of the CTR1 promotes senescence process upon darkness treatment. Together, these results demonstrated that CTR1 functions as a negative regulator of leaf senescence.

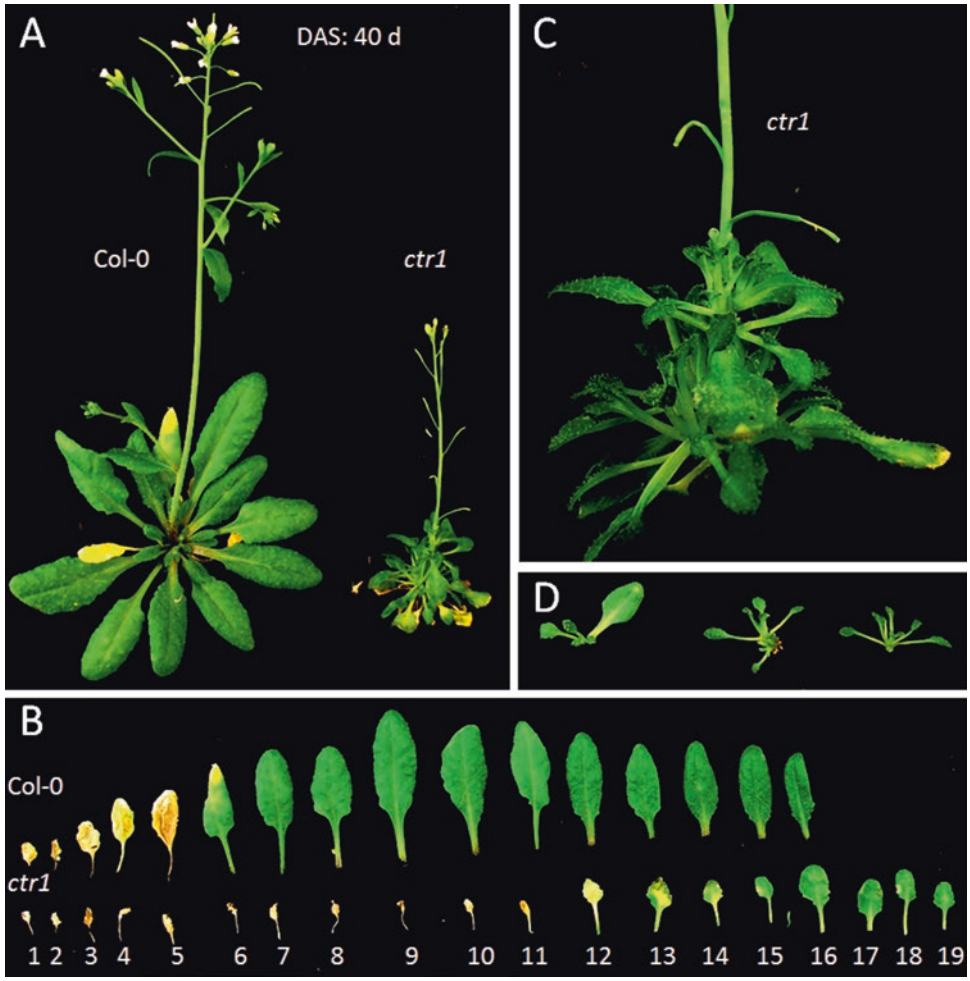


Fig. 5 Loss-of-function CTR1 accelerates leaf senescence. (**a**) Senescence phenotype in 40-day-old wild-type Col-0 and *ctrl-1* mutant. *DAS* days after soil. (**b**) Leaves in the plants (**a**) were cut and arranged according to their ages. (**c**). Loss-of-function CTR1 stimulates secondary growth shoots which were cut as shown in (**d**)

5 Future Plan

LSD is a product of collaboration between wet-lab experimental biologists and dry-lab bioinformatics developers. The original aim of this work is to efficiently use the freely available information distributed in the online databases and literature papers for our own leaf senescence-related research. SAGs with genetic evidence were used for network and pathway analysis. Mutants and transgenic plants were generated for SAGs without genetic evidence and used for screening altered senescence phenotype mutants and functional analysis as well as gene network analysis. It turns out, however, that the dataset including SAGs and mutants and the

annotations embedded in the database are also useful for the worldwide leaf senescence research community. Currently, LSD 2.0 contains 5357 genes and 324 mutants from 44 species, with information including expression profile, primer sequence, subcellular localization, miRNA target, orthologous gene, *Arabidopsis* seed, images of *Arabidopsis* mutants, and Quantitative Trait Loci (QTL).

In 2008, the 1001 Genomes Project was launched to discover the whole-genome sequence variation in 1001 strains (accessions) of the reference plant *Arabidopsis thaliana* (http://1001genomes.org/) [19]. The resulting information is paving the way for a new era of genetics that identifies alleles underpinning phenotypic diversity across the entire genome and the entire species. More and more researchers study plant development processes, for example, flowering and senescence, and the underlying molecular regulatory mechanisms by using different ecotype plants. Currently, senescence phenotypes of more than 200 ecotypes of *Arabidopsis* plants have been collected in our laboratory. Figure 6 shows five ecotypes (Sen-0, Con-0, Dja-1, Tnz-1, and Neo-6) grown in soil under long-day (16 h light/8 h dark) condition for 7 weeks.

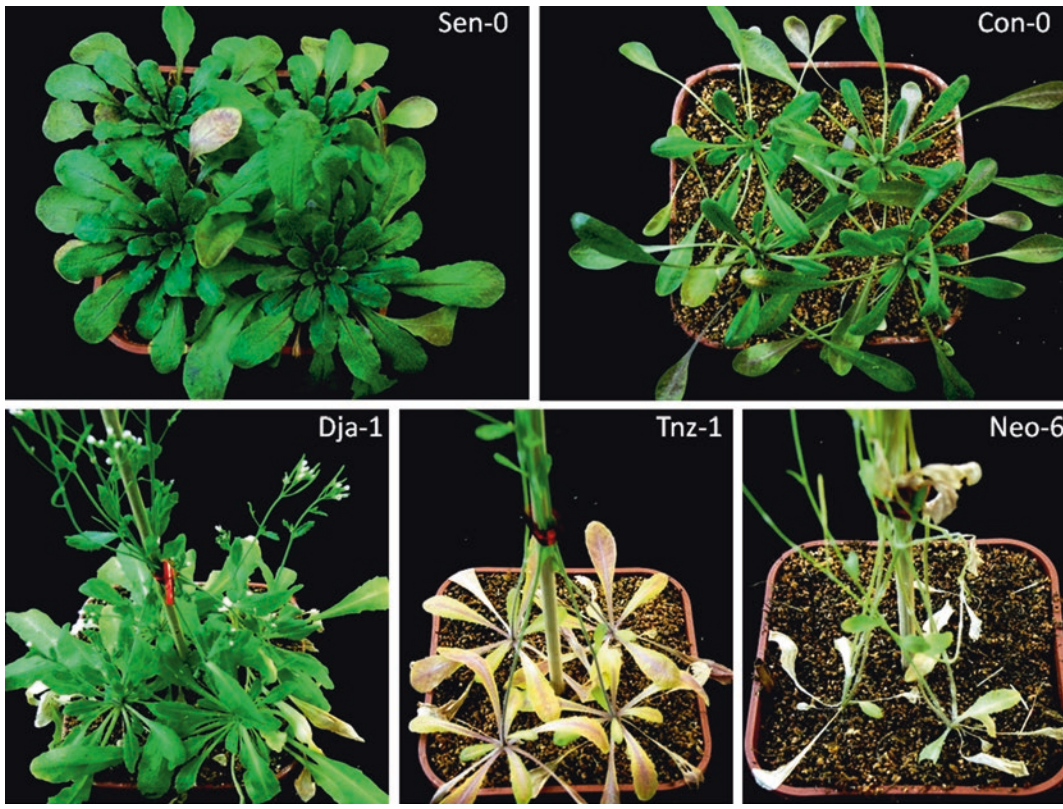


Fig. 6 Senescence phenotypes of different ecotypes under long-day conditions. Seven-week-old plants of five ecotypes (Sen-0, Con-0, Dja-1, Tnz-1, and Neo-6) grown in soil under long-day (16 h light/8 h dark) condition

Next, more than 2000 T-DNA homozygous lines of *SAG*s in *Arabidopsis* are available from our senescence-related research projects, and senescence phenotypic information will be collected and added into the database. In addition, we are constructing transgenic lines overexpressing *SAG*s in *Arabidopsis* and will add phenotype information of these mutants in the updated LSD in the future.

We will update the database with more leaf senescence-related data available and predict putative SAGs from completely sequenced plant genomes in the future. We will improve the user interface according to the suggestions and comments from the user community. We hope that the rich information of SAGs in LSD may provide a useful resource and a good starting point for the further study of the molecular mechanism of leaf senescence [5].

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