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Identification of leaf senescence regulating genes in *Nicotiana tabacum*

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IDENTIFICATION OF LEAF SENESCENCE REGULATING GENENS IN NICOTIANA TABACUM

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Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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Résumé

Xiaoming GAO. (2022). Identification des gènes régulateurs de la sénescence foliaire chez *Nicotiana tabacum* (Thèse de doctorat en anglais). Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège.

Résumé — La sénescence des feuilles est un élément important de la sénescence des plantes et joue un rôle vital dans le développement global de la plante. La sénescence foliaire est un trait agronomique important de la culture, affectant le rendement et la qualité de la culture. Le tabac (*Nicotiana tabacum* L.) est une culture économique importante largement cultivée dans le monde et une plante modèle importante dans la recherche phytogénétique. Dans la production de tabac, les feuilles jaunies matures sont des cibles de récolte. La sénescence des feuilles est essentielle au rendement et à la qualité des produits du tabac. L'étude des gènes régulateurs de la sénescence des feuilles de tabac et l'analyse de leurs fonctions peuvent améliorer la compréhension du mécanisme de régulation de la sénescence des feuilles de tabac et fournir des nouvelles ressources génétiques pour la sélection de nouvelles variétés de tabac.

Dans cette étude, deux mutants prématurés de la sénescence foliaire du tabac, *la feuille jaune 1* (*yl1*) et *la feuille jaune 2* (*yl2*) obtenus par mutagenèse d'éthylméthane sulfonate (EMS) ont été étudiés. La sénescence foliaire dans les mutants a été initiée plus tôt que dans le type sauvage. Les mutants *yl1* et *yl2* ont été étudiés pour la caractérisation phénotypique, l'analyse génétique et la cartographie préliminaire des gènes cibles. Cette étude a posé les bases d'un clonage supplémentaire du gène régulateur de la sénescence des feuilles de tabac et d'une analyse approfondie du mécanisme de régulation de la sénescence des feuilles de tabac.

Le contenu et les résultats principaux sont les suivants:

(1) mutant *yl1*

Le mutant *yl1* de la sénescence des feuilles de tabac a été sélectionné dans la bibliothèque de mutants de mutagenèse EMS de la variété Honghuadajinyuan (HD) de tabac commun tétraploïde (*Nicotiana tabacum* L.). L'identification phénotypique a montré que *yl1* avait des caractéristiques de sénescence foliaire prématurée. La sénescence foliaire de yl1 a été initiée plus tôt au stade de développement précoce. Les feuilles inférieures de yl1 ont commencé à jaunir 50 jours après la transplantation (JAT). La chlorophylle des feuilles de *yl1* s'est dégradée plus tôt que celle du type sauvage. Dans les feuilles du même stade de développement et à la même position foliaire, l'efficacité de la photosynthèse, la teneur en protéines et le niveau d'expression du gène lié à la photosynthèse de *yl1* étaient inférieurs à ceux du type sauvage, tandis que le niveau d'expression du gène lié à la sénescence était supérieur à celui de type sauvage. Au stade de développement tardif, la teneur en chlorophylle de *yl1* a diminué plus rapidement, le taux de sénescence de *yl1* était plus rapide que celui de HD de type sauvage. Les variétés de tabac sauvage HD et Gexin3 (GX3) ont été croisées respectivement avec *yl1*. Le rapport de séparation phénotypique de la

population F2 et BC1F1 indiqué que le phénotype de *yl1* était contrôlé par un seul gène récessif. La population BC1F1 développée entre le parent GX3 et *yl1* a été utilisée pour cartographier le gène cible. Le gène cible a été cartographié à l'aide des marqueurs moléculaires SSR publiés, le résultat a indiqué que le gène cible était situé sur le groupe de liaison du tabac (LG) 11 entre les marqueurs PT60305 et PT53066, et la distance génétique était de 3,51 et 1,08 cM, respectivement. Le traitement exogènes des feuilles avec les hormones végétales a montré que *yl1* était plus sensible à l'éthylène et à l'acide jasmonique que le type sauvage.

(2) mutant *yl2*

Le mutant *yl2* de la sénescence des feuilles de tabac a été sélectionné dans la bibliothèque de mutants de mutagenèse EMS de la variété Zhongyan100 (ZY100) de tabac commun tétraploïde (*Nicotiana tabacum* L.). Les plantes *yl2* ont présenté un jaunissement précoce des feuilles. Les feuilles inférieures de *yl2* ont commencé à jaunir à 65 JAT. Les paramètres physiologiques et l'expression des gènes marqueurs ont indiqué que le phénotype *yl2* était causé par une sénescence prématurée des feuilles. La teneur en chlorophylle et en protéines de *yl2* était inférieure à celle du type sauvage ZY100 tout au long des stades de développement. La teneur en chlorophylle a rapidement diminué au stade de développement ultérieur. Les variétés de tabac sauvage Cuibi1 (CB1) et Gexin3 (GX3) ont été croisées respectivement avec *yl2*. Le rapport de séparation phénotypique de la population F2 indiqué que le phénotype de *yl2* était contrôlé par un seul gène récessif. En utilisant le “Whole Genome sequencing” (WGS), 18 SNP candidats associés au phénotype du mutant *yl2* ont été obtenus. La possibilité que 18 SNP conduisent au phénotype du mutant a été exclue par validation par PCR. La population F2 développée entre CB1, GX3 et *yl2* a été utilisée pour cartographier le gène cible. Le gène cible a été cartographié à l'aide des marqueurs moléculaires SSR publiés, le résultat indiqué que le gène cible était situé à une extrémité du groupe de liaison du tabac (LG) 24. La distance génétique entre le gène cible et le marqueur SSR polymorphe le plus proche PT60487 était de 14,2 cM.

Mots-clés: le tabac; Mutagenèse EMS; sénescence prématurée des feuilles; séquençage du génome entier; SNP; SSR; hormone végétale

Abstract

Xiaoming Gao. (2022). Identification of leaf senescence regulating genes in *Nicotiana tabacum* (PhD Dissertation in English). Gembloux Agro-Bio Tech, University of Liège, Gembloux, Belgium.

Abstract —Leaf senescence plays a vital role in the overall development of plant. And it is also an important agronomic trait of crop that affects the yield and quality of crop, such as grain size and weight. Tobacco (*Nicotiana tabacum* L.) is an important economic crop widely cultivated in the world and an important model plant in plant genetic study. In tobacco production, mature yellow leaves are harvesting targets. Leaf senescence is critical to the yield and quality of tobacco products. Investigation of tobacco leaf senescence regulating genes and analysis of their functions can enhance the understanding of the regulating mechanisms of tobacco leaf senescence, and provide new genetic resources for the breeding of new tobacco varieties.

In this study, two tobacco premature leaf senescence mutants *yellow leaf 1* (*yl1*) and *yellow leaf 2* (*yl2*) obtained by ethyl methane sulfonate (EMS) mutagenesis were studied. Leaf senescence of mutants were initiated earlier than wild type. The mutants *yl1* and *yl2* were studied for phenotypic characterization, genetic analysis and preliminary mapping of target genes. This study laid the foundation for further cloning of tobacco leaf senescence regulating gene and in-depth analysis of tobacco leaf senescence regulating mechanisms.

The main contents and results are as follows:

(1) *yl1* mutant

The tobacco leaf senescence mutant *yl1* was selected from the EMS mutagenesis mutant library of tetraploid common tobacco (*Nicotiana tabacum* L.) variety Honghuadajinyuan (HD). Phenotypic identification showed that *yl1* had premature leaf senescence characteristics. The leaf senescence of *yl1* was initiated earlier in the early developmental stage. The lower leaves of *yl1* began to turn yellow at 50 days after transplanting (DAT). The chlorophyll in *yl1* leaves degraded earlier than that in wild type. In leaves of the same developmental stage and at the same leaf position, the photosynthesis efficiency, protein content, and the expression level of photosynthesis related gene of *yl1* were lower than that of wild type, while the expression level of senescence related gene was higher than that of wild type. In the late developmental stage, the chlorophyll content of *yl1* decreased more rapidly, the senescence rate of *yl1* was faster than that of wild type HD. The wild type tobacco varieties HD and Gexin3 (GX3) were crossed with *yl1*, respectively. All the F1 plants showed a normal leaf senescence phenotype similar to the wild type. In F2 populations, mutant type plants and wild type plants showed a separation ratio of 1:3. In BC1F1 population, mutant type plants and wild type plants showed a separation ratio of 1:1. The phenotypic separation ratio of F2 and BC1F1 population indicated that the phenotype of *yl1* was controlled by a single recessive gene. The BC1F1 population developed between parent GX3 and *yl1* was used to map the target gene. The target gene was

mapped using the published SSR molecular markers, the result indicated that the target gene was located on the tobacco linkage group (LG) 11 between the markers PT60305 and PT53066, and the genetic distance was 3.51 and 1.08 cM, respectively. The treatment of exogenous plant hormones on detached leaves showed that *yl1* was more sensitive to the ethylene and jasmonic acid than wild type.

(2) *yl2* mutant

The tobacco leaf senescence mutant *yl2* was selected from the EMS mutagenesis mutant library of tetraploid common tobacco (*Nicotiana tabacum* L.) variety Zhongyan100 (ZY100). The *yl2* plants displayed early leaf yellowing. The lower leaves of *yl2* began to turn yellow at 65 DAT. Physiological parameters and marker genes expression indicated that the *yl2* phenotype was caused by premature leaf senescence. The chlorophyll content and protein content of *yl2* were lower than wild type ZY100 throughout the developmental stages. The chlorophyll content was rapidly decreased in the later developmental stage. The wild type tobacco varieties Cuibi1 (CB1) and Gexin3 (GX3) were crossed with *yl2*, respectively. All the F1 plants showed a normal leaf senescence phenotype similar to the wild type. The phenotypic separation ratio of F2 population indicated that the phenotype of *yl2* was controlled by a single recessive gene. Using the whole genome sequencing (WGS), 18 candidate SNPs associated with *yl2* mutant phenotype were obtained. The possibility of 18 SNPs leading to the mutant phenotype was ruled out by PCR validation. The F2 population developed between CB1, GX3 and *yl2* was used to map the target gene. The target gene was mapped using the published SSR molecular markers, the result indicated that the target gene was located on one end of tobacco linkage group (LG) 24. The genetic distance between target gene to the nearest polymorphic SSR marker PT60487 was 14.2 cM.

Keywords: tobacco; EMS mutagenesis; premature leaf senescence; whole genome sequencing; SNP; SSR; plant hormone

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List of Abbreviations

Abbreviate	Full Name
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AFLP	amplified fragment length polymorphism
ARF	auxin response factor
BSA	Bulk Segregant Analysis
CB1	Cuibi1
CDS	coding sequence
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
CAB	chlorophyll a/b binding protein
Chls	total chlorophyll
Chr	Chromosome
cM	centiMorgans
CTK	cytokinin
DAT	days after transplanting
DH	doubled haploid
ET	ethylene
EMS	ethyl methane sulfonate
EST	expressed sequence tags
GA	Gibberellin
GS	glutamine synthetase
GX3	Gexin3
HD	Honghuadajinyuan
HXK	hexokinase
IAA	indole acetic acid
InDel	Insertion-Deletion
JA	jasmonic acid
LG	linkage group
LOD	logarithm of odd
MAPK	mitogen-activated protein kinase

MeJA	methyl jasmonate
MES	2-(N-Morpholino) ethane sulfonic acid
MS	Murashige and Skoog
N.	*Nicotiana*
NGS	next generation sequencing
QTLs	quantitative trait loci
PSII	photosystem II
qRT-PCR	Quantitative Real-time PCR
RAPD	random amplified polymorphism
RBCS	*ribulose bisphosphate carboxylase small subunit*
RFLP	restriction fragment length polymorphism
Rubisco	ribulose bisphosphate carboxylase
SA	salicylic acid
SAG12	senescence-associated gene 12
SAGs	senescence-associated genes
SDGs	senescence down-regulated genes
SNP	single nucleotide polymorphism
SSR	simple sequence repeat
TGI	Tobacco Genome Initiative
WGS	whole genome sequencing
yl1	*yellow leaf 1*
yl2	*yellow leaf 2*
ZY100	Zhongyan100

1

General Introduction

1 Advances in plant leaf senescence

Senescence is a natural phenomenon common in the plant and has important biological significance. Plant senescence is the final stage of development and ultimately leads to an orderly decline in the function of cells, tissues, organs and organisms until death or end of the physiological processes of the life cycle (Gan and Amasino, 1997; Lim et al., 2007). This process is not passive or chaotic, but rather rigorous and orderly. It is a process of procedural death, manifested by the joint regulation of external environmental factors and related genes (Gan, 2003, 2007; Guo and Gan, 2012). This complex process involves continuous changes in cell physiology, biochemistry, and gene expression levels (Breeze et al., 2011).

Leaves are important organs of plant nutrition and energy sources. Leaf senescence is the main form of plant senescence. The most obvious phenotype of senescence is the yellowing of leaves caused by the degradation of chlorophyll in chloroplasts (Ougham et al., 2005). Other metabolic changes include the increased of oxidation and hydrolysis of macromolecular substances such as proteins, lipids and nucleic acids. These hydrolyzed molecules are used in annual plants to provide feedstock for seed development, or to transfer nutrients from old leaves to shoots, stems and roots in perennial plants for leaves and flowers growth next year (Munné-Bosch, 2008).

1.1 Physiological characteristics of plant leaf senescence

Leaf senescence is generally associated with the developmental age pattern of plants and is also induced by various environmental factors. In the process of senescence, after orderly degradation and assembly of leaf cells, metabolites such as nutrients are re-migrated into the reproductive organs. Therefore, leaf senescence is not a passive process, it is an active biological process, which is conducive to the survival and reproduction of plants themselves (Lim et al., 2007). The most striking feature of leaf senescence is the dramatic metabolic shift from anabolism to catabolism, in which the number of catabolic genes expressed in senescent leaves is almost double that of anabolic genes (Guo et al., 2004). Leaf senescence occurs in an orderly manner, first with the denaturation of chloroplasts, hydrolysis and reactivation of macromolecules, followed by degradation of mitochondria and nuclei (Guo, 2013; Solomos, 1988) (Figure 1-1).

1.1.1 The change of organelles

One of the earliest physiological manifestations of senescence is the collapse of the membrane structure causing leakage, in part due to membrane lipid de-esterification (Thompson et al., 1998). With the occurrence of senescence, the chloroplast first changed. The outer layer of the chloroplast disappeared, the chlorophyll degraded, and then the thylakoid membrane gradually disintegrated, the granule structure was destroyed, the plastid globules in the matrix formed. The number of ribosomes and endoplasmic reticulum in the cytoplasm is reduced and chromosomes are aggregated.

The destruction of the membrane structure leads to an increase in cell permeability, while the vacuole collapses, and various hydrolases are dispersed throughout the cell, promoting cell disintegration and death (Nooden, 1988b). Among many organelles, only the mitochondria and nucleus maintain their intact structure until the late stage of leaf senescence (Cao et al., 2003). This indicates that although the leaves have undergone senescence process, they still require strict genetic regulation and sufficient energy to supply plants to complete the degradation and transportation of photosynthetic products to ensure the growth and development of plants (Lim et al., 2007).

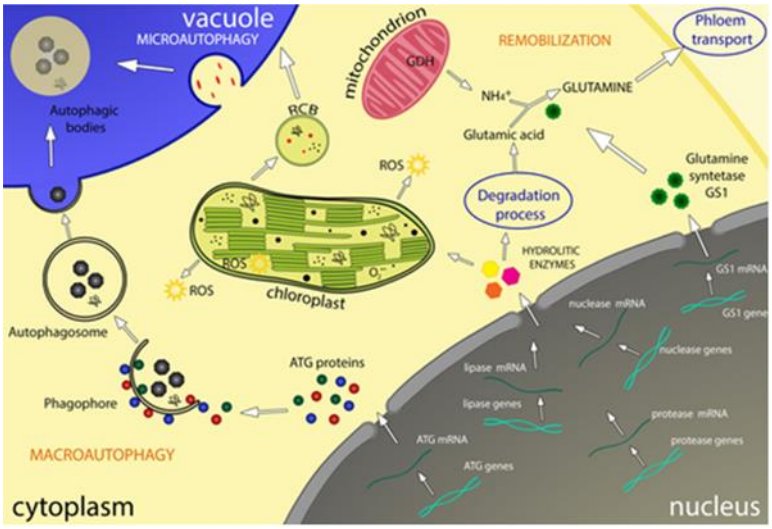

Figure 1-1 Main processes that regulate leaf senescence (Wojciechowska et al., 2018).

1.1.2 The degradation of chlorophyll

The yellowing of leaves is the first visible symptom of leaf senescence due to chlorophyll loss, so the rapid loss of chlorophyll associated with chloroplast degeneration is often used as a biomarker of senescence. Decomposition of chloroplast proteins provides a major source of nitrogen, and chlorophyll needs to be removed from chlorophyll-binding proteins to make it degradable (Thomas et al., 2001). Free chlorophyll is toxic to cells and requires proper degradation to avoid free radical production (Li et al., 2017b; Matile, 1992). Chlorophyll degradation begins in the chloroplast and involves a complex enzyme-catalyzed pathway in several subcellular compartments, which consists of multiple enzymatic reactions that produce interim and chlorophyll that are different in short-lived life, eventually accumulating in the vacuoles of senescent cells (Hortensteiner and Feller, 2002; Hortensteiner and Krautler, 2011; Kuai et al., 2018; Thomas et al., 2001). In recent years, the regulation mechanism of chlorophyll decomposition during leaf senescence has been extensively studies in various crops and plants. In *Arabidopsis*, *NYE1* and its closely homologous *NYE2* have been identified as key regulators of chlorophyll

degradation (Ren et al., 2007; Wu et al., 2016). The *I* gene controlling the Mendelian pea color and the *PaO* encoding the pheophorbide oxygenase *a* gene are one of the key enzymes involved in chlorophyll degradation, which are derived from the senescent chloroplast inner membrane (Park et al., 2007; Sato et al., 2007).

1.1.3 The degradation of lipid

Lipid degradation is activated during leaf senescence (Thompson et al., 1998). Studies on leaf developmental metabolism in *Arabidopsis* indicate that chloroplast lipids are depleted during leaf senescence (Watanabe et al., 2013). The degradation products of lipids are metabolized to sucrose, which can be transported in the phloem (Kaup et al., 2002; Thompson et al., 1998). Lipid degradation may also play a regulatory role in senescence. Studies have shown that phospholipase PLDα is involved in the regulation of plant hormone induced leaf senescence (Fan et al., 1997). The *Arabidopsis* gene *SAG101* encoding an acyl hydrolase catalyzes the release of oleic acid from glycerol trioleate in an in vitro assay and plays a regulatory role in leaf senescence (He and Gan, 2002).

1.1.4 The degradation of protein

The equilibrium system of protein synthesis and decomposition maintains the steady state of plant cell tissue, but as the leaf senescence, the protein gradually degrades, and this steady state is destroyed. Protein degradation plays a key role in nitrogen cycling during leaf senescence. Transcriptomic studies have shown that genes involved in proteolytic activity are up-regulated in large numbers (Breeze et al., 2011; Guo et al., 2004). Serine proteases and cysteine proteases are the most abundant enzymes associated with leaf senescence, while aspartic acid, threonine and metalloproteinases are also detected (Diaz-Mendoza et al., 2016; Roberts et al., 2012). Changes in plant protease activity were detected in different cell regions such as nucleus, chloroplast, cytoplasm, endoplasmic reticulum, vacuoles, mitochondria, apoplast, cell wall and specific vesicles (Diaz-Mendoza et al., 2016). The abundance of chloroplast protein decreased significantly in the late senescence. The most typical are ribulose bisphosphate carboxylase (Rubisco) and chlorophyll a/b binding protein (CAB). Rubisco is a key enzyme in plant photosynthesis, and many studies have shown that Rubisco degrades more rapidly than other proteins during leaf senescence (Wilson et al., 2002). There are also hydrolyzed proteins synthesis during leaf senescence, such as ribonuclease, lipase and cellulase. The largest source of mobile proteins in the cell is the chloroplast, α – ribulose - 1, 5 - bisphosphate carboxylase/oxygenase and light-harvesting complex of photosystem II (PSII) (LHCII) are the main source of protein (Hortensteiner and Feller, 2002). Chloroplast protein accounts for more than 80% of the total leaf nitrogen content and is the main source of mobilization of nitrogen in senescent leaves (Liu et al., 2008). During leaf senescence, chloroplast proteins can be degraded by the coordination of chloroplast proteases, senescence-associated vacuoles, and the ubiquitin/26S proteasome pathway (Diaz-Mendoza et al., 2016; Gomez et al., 2019). SAV is a specific cleavage space for chloroplast protein degradation during leaf senescence. Previous studies

have detected that a large number of chloroplast proteins are enriched in SAV (Martinez et al., 2008), and SAV may be involved in the degradation of PSI protein and related chlorophyll during leaf senescence (Gomez et al., 2019). During leaf senescence, proteins in the cytoplasm are targeted and degraded by the ubiquitin/proteasome system, and the expression of genes encoding ubiquitin-dependent proteolytic enzymes is increased (Buchanan-Wollaston et al., 2003). Studies have also shown that mutants of these enzymes have delayed leaf senescence phenotype (Woo et al., 2001).

1.1.5 The change of nucleic acid

In the late stage of leaf senescence, the intracellular nucleic acid content decreases significantly. In dark-induced senescent leaves, the total RNA content decreases faster than DNA, especially rRNA decreased most, with rRNA decreasing faster in mitochondria and chloroplasts, and cytoplasmic tRNA decaying the slowest (Buchanan-Wollaston, 1997).

1.1.6 The reuse of nitrogen

The leaf senescence process is characterized by a transition from nutrient assimilation to nutrient reuse (Masclaux et al., 2000). The availability of nitrogen generally limits the growth and reproduction of plants, and in order to survive, plants adopt a strategy of transferring nitrogen from senescent leaves into newer growing organs (Lim et al., 2007). The efficient use of nitrogen is critical to the plant's life cycle. In cereals such as wheat, rice and barely, approximately 90% of nitrogen is transferred from vegetative organs to seeds (Gregersen et al., 2008). As a result of protein degradation, a large amount of ammonium is produced in the senescent leaves, which is again assimilated into the amide group of glutamic acid, and then transferred to the the GS/GOGAT cycle by a synergistic reaction of glutamine synthetase (GS1), and the glutamine is produced with glutamate synthetase. Glutamine is the main mobile amino acid that is transported long distances to the "sink" tissues (Hortensteiner and Feller, 2002; Masclaux et al., 2000). Genes encoding enzymes involves in the GS/GOGAT cycle are up-regulated during leaf senescence (Guo et al., 2004; Masclaux et al., 2000). Recently, leaf senescence metabolomics studies on *Arabidopsis*, sunflower and tobacco have also demonstrated that glutamine accumulation during has increased (Li et al., 2017a; Moschen et al., 2016; Watanabe et al., 2013). During leaf senescence, autophagy involved in chloroplast protein degradation plays a key role in the reuse of seed nitrogen. Compared with wild type, the nitrogen transfer efficiency of autophagy mutant *atg* is greatly reduced (Guiboileau et al., 2012).

1.2 Three stages of leaf senescence

At present, many studies support the fact that leaf senescence can be divided into three stages, including the induction of leaf senescence, the degradation of intracellular substances and the ultimate death, referred to as the three stages of initiation, degeneration and termination (Noodén et al., 1997; Yoshida, 2003). (Figure

1-2)

In the early stage of development, the change of sugar metabolism plays an important role in the initiation of leaf senescence. Sugar is an important photosynthetic product of plant. Sugar signaling plays a vital role in plant leaf senescence and its development stages (Rolland et al., 2006). Usually accompanied by a decrease in photosynthesis, the concentration of sugars in the leaves is also reduced. The decrease of sugar concentration in plant leaves is a factor in inducing leaf senescence (Quirino et al., 2000). Senescence-associated genes (*SAGs*) are induced by low concentrations of sugars, and high concentrations of sugars inhibit the expression of some *SAGs* (Fujiki et al., 2000; Fujiki et al., 2001). However, some studies have found that higher sugar concentration induce leaf senescence, such as the accumulation of sugars in *Arabidopsis* and tobacco in the initial of leaf senescence (Masclaux et al., 2000; Quirino et al., 2001; Stessman et al., 2002). Hexokinase (HXK) is thought to be a receptor for sugar signaling and hexose phosphorylation. Study have found that overexpression of *AtHXK* in *Arabidopsis* can promote leaf senescence, and vice versa (Xiao et al., 2000). Overexpression of *AtHXK* in transgenic tomato can increase sugar concentration and reduce photosynthesis ability, thus accelerating leaf senescence (Dai et al., 1999).

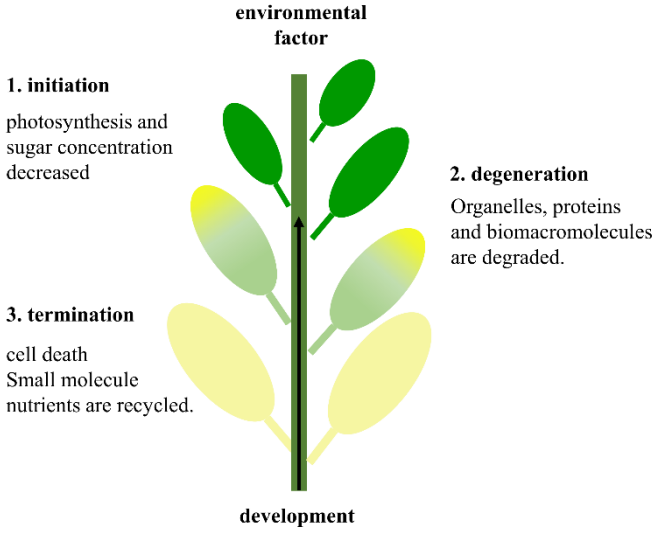

Figure 1-2 The stages of leaf senescence and their physiological characteristic

After leaf senescence is induced, the process of degeneration begins, and plant cells undergo intense physiological and biochemical reactions. *SAGs* expression and proteolytic processes are activated, and ubiquitinated protein degradation systems play an important role in this stage, up regulation of the expression of these genes lead to an increase in the activity of the corresponding enzyme coupled to it, accelerating protein breakdown and senescence (Belknapa and Garbarinob, 1996; Noodén et al., 1997). The ROS produced in the cells could not be removed in time, and the content

of various ROS scavenging enzymes decreased sharply and the activity decreased, resulting in a large accumulation of ROS in the leaves (Quirino et al., 2001). ROS can lead to the peroxidation of proteins, nucleic acids, lipid membranes and chlorophyll, causing the accumulation of malondialdehyde, hydrogen peroxide and free radicals, further accelerating the disintegration of membrane structure and aggravating the senescent process (Quirino et al., 2001; Zhang et al., 2006). Membrane degradation is one of the early events of leaf senescence (Thompson et al., 1998). Phospholipid hydrolase PLDα is thought to play an important role in membrane senescence and is involved in the process of plant hormones promoting lipid degradation and induced leaf senescence (Fan et al., 1997). *SAG101* encodes an acyl hydrolase that acts as a "accelerator" in cell membrane destruction of senescent leaf cells, which attacks membrane phospholipids. This initial attack and release of free fatty acids will disrupt the bilayer structure of the membrane, which helps other lipid degrading enzymes hydrolyze membrane lipids, thereby promoting leaf senescence (He and Gan, 2002).

The third stage enters the terminal stage of senescence and death. The appearance of the leaves gradually changes from green to completely chlorotic. At this stage, apoptosis-like characteristics such as complete lysis of vacuoles, increased membrane permeability, electrolyte extravasation, chromatin condensation, and DNA fragmentation can be detected (Yoshida, 2003). Nutrients from plant cell breakdown are migrated to developing tissues and organs that are more nutrient-demanding. Therefore, leaf senescence is an orderly event that plants form in order to adapt to the environment.

1.3 Factors affecting leaf senescence

Leaf senescence can be regulated by various internal and external factors (Figure 1-3). The internal factors are phytohormones and reproduction. The external factors include biotic and abiotic factors. The abiotic factors include light, ozone, temperature, drought, nutrient, etc., while biotic factors include pathogen infection, wounding and shading by other plants. Usually, these unfavorable factors can lead to premature leaf senescence.

1.3.1 Internal factors

The internal factors affecting leaf senescence mainly include the endogenous hormones and the development stages of plants. Under normal conditions, leaf senescence is mainly related to leaf age and it is a natural senescence. Hormones play an important role in regulating leaf senescence. Hormones may alter the phenotype of leaf senescence, while leaf senescence causes changes in various hormones in plants, and hormonal changes in turn regulate leaf senescence (Li et al., 2012). Although the role of hormone pathways in leaf senescence has not been totally determined, it is generally believed that plant endogenous hormones such as cytokinin, auxin, gibberellin, ethylene, abscisic acid, jasmonic acid and salicylic acid are closed related to senescence.

Cytokinins (CTKs) are a class of plant hormones composed of adenine derivatives, which are widely involved in plant growth and development as well as stress

resistance (Sakakibara, 2006). CTKs delays leaf senescence mainly by delaying chloroplast and protein degradation, protecting membrane integrity, delaying the degradation rate of photosynthesis related enzymes, and promoting nutrient transport (Zhang et al., 2011). In the process of leaf senescence, the endogenous CTKs content decreased, therefore, CTKs only play a regulatory role in the early stage of leaf senescence (Gan and Amasino, 1995; Smart et al., 1991). When the exogenous CTKs was applied or the endogenous CTKs content was enhanced by senescence-specific promoter, leaf senescence was significantly delayed (Gan and Amasino, 1995; McCabe et al., 2001; Ori et al., 1999). The *IPT* encoded isopentenyl transferase is a key enzyme in the CTK biosynthesis pathway. During the leaf senescence, the expression of *IPT* gene is down regulated, which reduces the endogenous CTK content and further aggravates leaf senescence (Gan and Amasino, 1995). Under drought stress conditions, endogenous CTKs levels are reduced due to decreased levels of *IPT* expression (Nishiyama et al., 2011). The enhanced activity of isopentenyl transferase not only delays leaf senescence, but also significantly enhances the drought resistance of plants (Merewitz et al., 2016; Rivero et al., 2007). However, CTKs affect the senescence process, but do not affect the onset of leaf senescence (Jibran et al., 2013). The decline in endogenous CTKs level is the main cause of leaf senescence has not been confirmed by experiments (Edlund et al., 2017).

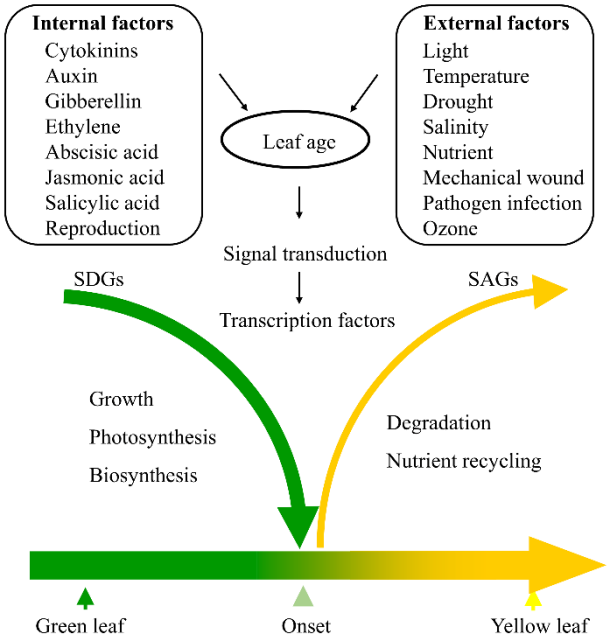

Figure 1-3 The leaf senescence regulatory network

Auxin is a hormone essential for plant growth and development, and is involved in the regulation of many developmental processes, such as apical dominance,

embryonic and postembryonic development, rhizome development, vascular differentiation, branching and flowering (Gan, 2010; Khan et al., 2014). Earlier studies have shown that auxin has a regulatory effect on abscission and senescence (Nooden, 1988a), however, more evidence is needed to demonstrate whether there is a direct or indirect relationship between leaf senescence and the metabolic pathways and signaling of endogenous auxin (Mueller-Roeber and Balazadeh, 2014; Rivero et al., 2007). Auxin is generally thought to delay plant leaf senescence. The bioactive auxins are indole acetic acid (IAA) and indole butyric acid (Kelley and Estelle, 2012). In the process of leaf senescence, the endogenous IAA content can be increased by two-fold (Quirino et al., 1999). However, exogenous application of IAA can effectively inhibit the expression of *SAG12* and delay leaf senescence (Noh and Amasino, 1999). van der Graaff et al. (2006) showed that the auxin content and the expression of IAA synthesis gene increased during leaf senescence (van der Graaff et al., 2006). *YUCCA6* encodes flavin monooxygenase, a rate limiting step that catalyzes the de novo synthesis of auxin. *YUCCA6* overexpression plants and its knockout mutants *yuc6-1D* all show higher auxin content and significant longevity, and the expression of *SAG12*, *NAC1* and *NAC6* in *yuc6-1D* was lower than that of the wild type during the whole senescent process (Kim et al., 2011). This is consistent with earlier finding that exogenous auxin can temporarily reduce *SAG12* expression (Noh and Amasino, 1999). Ellis et al. (2005) found that ARF2 (Auxin Response Factor) can promote the change of *Arabidopsis* developmental stage, which is consistent with the role of auxin in plant growth and development (Ellis et al., 2005). Therefore, it is speculated that auxin may regulate developmental controlled senescence by directly switching the developmental process and the timing of age-related changes. Delayed senescence in mutant *oresara14* encoded ARF2, which inhibits the auxin response genes in *Arabidopsis*, this finding that further demonstrates the role of auxin in leaf senescence (Lim et al., 2010).

Gibberellin (GA) is a kind of biguanide phytohormone. In the process of leaf senescence, the content of GA active ingredient is gradually reduced (van der Graaff et al., 2006). Exogenous application of GA can delay leaf senescence, while exogenous application of GA inhibitor can promote leaf senescence, so GA are considered to be a class of hormones that delay leaf senescence (Jibran et al., 2013; Schippers et al., 2007; Yu et al., 2009). However, GA do not directly act on leaf senescence, but rather delay leaf senescence by antagonizing ABA or reducing respiration (Jibran et al., 2013). Study have also shown that GA and CTKs have synergistic effects in inhibiting leaf senescence (Greenboim-Wainberg et al., 2005). The DELLA proteins, including GA INSENSITIVE (GAI), REPRESSOR OF GA1-3 (RGA), RGA-LIKE1 (RGL1), RGA-LIKE2 (RGL2), and RGA-LIKE3 (RGL3), constitute the negative regulators of the GA signaling pathway (Peng and Harberd, 1997; Silverstone et al., 1998; Yu et al., 2004). RGL1 repressed the transcription activation of WRKY45, which strongly expressed in senescent leaves, its mutation can delay leaf senescence, while its overexpression significantly promotes leaf senescence (Chen et al., 2017). RGA and GAI attenuate the transcriptional activity of WRKY6 on its downstream *SAGs*, and WRKY6 is a positive regulator of dark-

induced leaf senescence (Zhang et al., 2018a). Although basic study on GA and leaf senescence regulation has reached some conclusions, the molecular mechanism by which GA regulate leaf senescence still needs to be uncovered.

Ethylene (ET) is a gaseous hormone associated with plant maturation and senescence that promotes the senescence of leaves and flowers and accelerates fruit ripening (Abeles et al., 1988). In the process of leaf senescence, ET content increased, and the expression of ET synthase related genes increased in senescent leaves (van der Graaff et al., 2006), which fully reflects the important role of ethylene in leaf senescence. In addition, ET also affects the division, elongation and volume of plant cells, and responds to biotic and abiotic stress (Jibran et al., 2013; Zhou et al., 2009). The analysis of the mechanism of action of ET in leaf senescence is already very clear. ETHYLENE INSENSITIVE 2 (EIN2) is a core member of ET signaling pathway, and its mutant leaves exhibit a phenotype of delayed leaf senescence (Gan, 2010; Oh et al., 1997). ET regulates the expression of important leaf senescence associated transcription factors ORESARA1 (ORE1) through EIN2 signaling pathway, and synergizes with other transcription factors to control leaf senescence (Kim et al., 2009). Further studies revealed that ET can inhibit the expression of *miR164*, which can specifically degrade ORE1 (Kim et al., 2009; Li et al., 2013). In senescent leaves, the accumulation of ET activates senescence positive regulator EIN3, which acts downstream of EIN2 and directly binds to the promoter of *miR164* to inhibit its expression, indirectly promoting the expression of ORE1 (Li et al., 2013). However, ethylene induced leaf senescence is age-dependent and it does not induce leaf senescence in seedlings (Jing et al., 2005).

Abscisic acid (ABA) is a sesquiterpenoid phytohormone present in various tissues and organs of higher plants and is involved in many physiological processes such as growth and development, senescence, drought resistance and salt tolerance (Cutler et al., 2010; Fujii and Zhu, 2009; Jibran et al., 2013; Lee et al., 2011; Zhu, 2002). During leaf senescence, ABA content increased sharply, also the expression of *SAGs* and genes involved in the ABA synthesis pathway increased, exogenous ABA showed induction of *SAGs* expression (Breeze et al., 2011; van der Graaff et al., 2006; Weaver et al., 1998). ABA can promote the expression of chlorophyll degradation genes, inhibit the expression of chlorophyll synthesis genes, and lead to chlorophyll degradation (Liang et al., 2014). Exogenous application of ABA also leads to a decrease in chlorophyll content (Yang et al., 2002). All of these indicate a link between ABA signaling and leaf senescence. Although ABA is widely believed to promote leaf senescence, the underlying molecular mechanism is not clear. Previous studies shown that ABA promotes senescence by causing ethylene biosynthesis (Riov et al., 1990). However, recent studies confirmed ethylene independent ABA signaling and that PYL9 (pyrabactin resistance 1-like 9) promotes ABA-induced leaf senescence by inhibiting PP2Cs (Protein Phosphatases 2C) and activating SnRK2s (sucrose nonfermenting 1-related protein kinase 2s) (Zhao et al., 2016). At present, the clearest study of the mechanism of ABA regulation of leaf senescence is the AtNAP (*Arabidopsis thaliana* NAC-like Activated by *apetala3*/*pistillata*) pathway. AtNAP is

a member of the NAC transcription factor family, which has a promoting effect on leaf senescence (Guo and Gan, 2006). AtNAP activates the expression of *SAG113* that encoding PP2C, which negatively regulates ABA mediated stomatal closure, resulting in water loss and ultimately cell death (Zhang and Gan, 2012; Zhang et al., 2012). Studies suggested that ABA induced stomatal closure is the core stage of this crosstalk between ABA mediated plant responses to biotic and abiotic stress signals (Lee and Luan, 2012). In addition, AtNAP promotes ABA biosynthesis and chlorophyll degradation by inducing the expression of *AAO3* (*Abscisic Aldehyde Oxidase 3*), *NYC1* (*Non-Yellow Coloring 1*) and *ABI5* (*ABA Insensitive 5*) (Liang et al., 2014; Sakuraba et al., 2014; Yang et al., 2014).

The application of jasmonic acid (JA) can promote leaf senescence, so JA has always been considered as one of the important causes of leaf senescence. A volatile JA derivative isolated from *Artemisia* (*Atthemisia absinthum*) can cause rapid loss of chlorophyll in oat (*Avena sativa*), which was identified as methyl jasmonate (MeJA), and further studies clearly indicate that the application of external MeJA induces leaf senescence in many plant species (Reinbothe et al., 2009; Ueda and Kato, 1980). JA concentration in senescent leaves is four-fold higher than non-senescent leaves, JA biosynthesis and the expression of some JA synthesis related genes was up regulated during leaf senescence (He et al., 2002; Seltmann et al., 2010; van der Graaff et al., 2006). COI1 (coronatine insensitive 1), which is an F-box protein that acts as a JA receptor (Kelley and Estelle, 2012). Leaf senescence phenotype was observed in wild type when JA treatment was applied exogenously, but no senescence phenotype was observed on *coi1* mutant. JA relies on COI1 to inhibit the transcription of RAC (rubisco activase), an enzyme which allows for carbamate formation during carbon fixation, while the decrease in RAC levels leads to various typical senescence related features, including leaf yellowing, chlorophyll loss, up regulation of senescence induced genes and down regulation of senescence inhibited genes (He et al., 2002; Shan et al., 2011). JAZ (jasmonate zim domain) proteins are key transcriptional repressor of JA signaling pathway and play important role in plant defense, growth and leaf senescence (Figueroa and Browse, 2012; Jiang et al., 2014; Yan et al., 2007; Zhang et al., 2015). The senescence phenotypes of *jaz4* and *jaz8* were stronger than the wild type, while the *JZA4* and *JZA8* overexpression plants exhibited a delayed senescence phenotype under MeJA treatment. Therefore, JAZ4 and JAZ8 as inhibitors of JA signaling pathway, have a negative regulatory effect on JA-induced leaf senescence (Jiang et al., 2014). JAZ7 plays a key role in delaying dark induced leaf senescence. Compared with wild type, *jaz7* showed a more sever leaf senescence phenotype, suggesting that JAZ7 plays a negative regulatory role in dark induced leaf senescence (Yu et al., 2015). The bHLH (IIIe basic-helix-loop-helix) family transcription factors MYC2, MYC3 and MYC4 are downstream response factors of JA, and MYC2, MYC3, MYC4 redundantly bind to *SAG29* promotor and activate its expression leading to the activation of JA induced leaf senescence (Chen et al., 2011; Fernandez-Calvo et al., 2011; Qi et al., 2015). MYC2 also mediates JA induced leaf senescence by directly combining downstream ANAC019, ANAC055 and ANAC072 (Bu et al., 2008; Zhu et al., 2015). MYC5 functions redundantly with MYC2, MYC3

and MYC4 to positively regulate JA induced leaf senescence (Song et al., 2017). In addition, JA regulates leaf senescence by interacting with other hormonal signaling pathways such as SA, auxin and ET (Hu et al., 2017; Jiang et al., 2014; Li et al., 2013; Xie et al., 2014).

Salicylic acid (SA) is a phenolic phytohormone. SA is especially known to be associated with disease resistance (Vlot et al., 2009), but there are also many studies showing that it plays a role in leaf senescence. In *Arabidopsis*, SA content increases with leaf senescence (Lim et al., 2007). When chlorophyll begin to decline significantly, the SA content in the leaves increases (Breeze et al., 2011). Twelve of eighteen genes involved in SA synthesis and signaling pathways are up-regulated in senescent leaves (van der Graaff et al., 2006). SA treatment induces the expression of many senescence associated genes, such as *SEN1* (Schenk et al., 2005), *WRKY6* (Robatzek and Somssich, 2001), *WRKY53* (Miao et al., 2004), *WRKY54* and *WRKY70* (Besseau et al., 2012), *WRKY75* (Guo et al., 2017). In contrast, WRKY46, WRKY51, WRKY63 and WRKY75 increase the level of SA by activating the transcription of *SID2* (*salicylic acid induction deficient 2*) (Zhang et al., 2017). About one-fifth of the senescence associated genes in SA deficient mutant *NahG* (*naphthalene oxygenase*) are significantly inhibited (Buchanan-Wollaston et al., 2005). In the SA signal transduction mutants *pad4* (*phytoalexin deficient 4*) and *npr1* (*non-expressor of pathogenesis-related genes 1*), the expression of senescence associated genes was suppressed and the leaf senescence process was delayed (Morris et al., 2000). Study has shown that the mitogen-activated protein kinase (MAPK) signaling cascade, which play an important role in leaf senescence regulation, regulate leaf senescence by regulating SA level (Li et al., 2016). NPR1, NPR3 and NPR4 are receptors for SA (Fu et al., 2012; Wu et al., 2012b). Through NPR1, NPR3 and NPR4, SA participates in programmed cell death and senescence caused by autophagy (Wang et al., 2016; Yoshimoto et al., 2009). Recent study has shown that the level of SA can be suppressed by ANAC090 at the pre-senescent stage (Kim et al., 2018).

1.3.2 External factors

Light is not only a source of energy for photosynthesis, but it also acts as a regulator of plant growth and development by the change of photoperiod and light intensity, and has an irreplaceable important role in regulating plant senescence (Wu et al., 2012a). Shading or insufficient light can reduce photosynthesis ability and promote leaf senescence. Methods for inducing leaf senescence using dark treatment have been applied to leaf senescence studies in several plant species. Conversely, prolonged exposure to high light causes premature leaf senescence due to loss of chlorophyll and decrease of photosynthesis efficiency (Procházková and Wilhelmová, 2004). It has also been found that *Arabidopsis* under long-day conditions, has a premature leaf senescence phenotype leaded by the accumulation of hexose sugar due to strong light (Wingler et al., 2006).

Both high and low temperatures can damage the leaves and accelerate leaf senescence. Under high temperature stress, the content of active oxygen in chloroplast

was increased, the chloroplast and thylakoid membranes were damaged, the photosynthesis-related proteins were degraded, and the photosynthesis electron transport activity was changed, which affect the photosynthesis of leaves and induced leaf senescence (Ougham et al., 2008). When subjected to low temperature stress, cell membrane damage is associated with membrane lipid peroxidation and decreased or even loss of protein activity caused by free radicals and reactive oxygen, which may further lead to disturbance of photosynthesis and related signaling pathway response, accelerating plant senescence (Sharma et al., 2005).

Drought and salinity are one of the main abiotic factors contributing to premature leaf senescence. They reduce the quantity and yield of harvestable "sink" organs by inducing premature senescence of plant "source" organs and affecting the transport and utilization of photosynthesis assimilation products (Albacete et al., 2014; Thomas, 2013). Studies have also shown that under environmental stresses such as drought, salinity and low temperature, plants can increase the expression level of ABA synthesis related genes and the content of ABA to induce leaf senescence (Guo and Gan, 2005). Drought promotes the accumulation of ethylene and abscisic acid in plant, promotes the degradation of protein and chlorophyll, leads to a decrease in photosynthesis and accelerate plant senescence (Lim et al., 2007). From the analysis of senescence gene expression in *Arabidopsis* leaves, it was observed that there is extensive overlap in gene expression data of salt induced senescence and normal senescence (Allu et al., 2014).

Mineral nutrients can also affect the speed of leaf senescence. Low levels of nutrients can lead to morphological and physiological dysplasia of plants, especially affecting leaf development and chloroplast photosynthesis, and accelerating plant leaf senescence.

Mechanical wound can promote the leaf senescence. Overexpression of wound-responsive RNA-binding proteins UBA2 can lead to an increase in the expression levels of many *SAGs*, which ultimately leads to premature senescence of the leaves (Kim et al., 2008).

Pathogen infection can also lead to an increase in the expression level of *SAGs*, thereby promoting leaf senescence (Espinoza et al., 2007). Ozone can cause significant damage to plant leaves. It reduces stomata and thus reduces carbon absorption; it directly reduces photosynthetic carbon fixation, which slows crop growth and ultimately leads to premature aging of plants (Wilkinson et al., 2012).

Overall, leaf senescence is a comprehensive reflection of internal factors and external factors. After long-term evolution, plants have evolved a series of responses to biotic and abiotic stresses. These responses are achieved through a large number of complex plant hormone related signaling pathway. Biotic and abiotic factors integrate with plant hormones and plant age to regulate plant leaf senescence.

2 Research methods of tobacco gene cloning

2.1 Characteristics of tobacco genome

Tobacco belongs to the Solanaceae genus. Tobacco (*Nicotiana tabacum* L.) is an allotetraploid (2n = 4x = 48) produced by the hybridization and natural chromosome doubling of *N. sylvestris* (2n = 24) and *N. tomentosiformis* (2n = 24) (Leitch et al., 2008). Tobacco has a very large genome, the genome size of allotetraploid tobacco is about 4.5 Gb, and it has more than 70% of repetitive sequences, while the genome size of its diploid ancestors *N. sylvestris* and *N. tomentosiformis* are about 2.7 and 2.4 Gb, respectively (Leitch et al., 2008; Renny-Byfield et al., 2011; Sierro et al., 2013a). These characteristics of the tobacco genome have brought enormous difficulties and challenges to the study of tobacco genome.

2.2 Advances in tobacco genome research

Although the tobacco genome is complex and huge, in recent years, with the rapid development of sequencing technology, the research on tobacco genome has made great progress, especially in the sequencing of tobacco genome.

In 2003, the Tobacco Genome Initiative (TGI) was launched. This is the first genome sequencing project for tobacco to characterize the open reading frame of *N. tabacum* cultivar Hicks Broadleaf. The TGI generated approximately 689 Mb of genomic sequence by Sanger sequencing and assembled into 81959 contigs with an average length of 1.2 kb and 871255 singletons with an average length of 688bp (Wang and Bennetzen, 2015). But the TGI did not get a high-resolution genetic map that could locate sequencing information on tobacco chromosomes. On this basis, in 2007, Blindler et al. generated the first linkage map of tobacco (*N. tabacum* L.) through microsatellite markers (Bindler et al., 2007). In 2011, Bindler et al. used two tobacco varieties Hicks Broadleaf and Red Russian to construct a mapping population and obtained high density tobacco genetic maps, which enabled the localization of target trait genes, the study also further enriched the microsatellite markers (Bindler et al., 2011). In 2012, Bombarely et al. used the Illumina HiSeq2000 technology to sequence the diploid *N. benthamiana* genome to assemble approximately 3 Gb of genome, containing about 141000 scaffolds and 16000 unigenes (Bombarely et al., 2012). In 2013, Sierro et al. reported the diploid *N. sylvestris* and *N. tomentosiformis* draft genomes, and constructed a physical map containing 9750 contigs of bacterial artificial chromosomes (BACs) (Sierro et al., 2013a; Sierro et al., 2013b). In 2014, Sierro et al. reported high quality draft genomes of the three main varieties (TN90, K326, Bsama Xanthi) of tetraploid tobacco *N. tabacum*, identified more than 90000 genes and combined them with previously reported tobacco genetic and physical maps (Sierro et al., 2014). In 2017, Edwards et al. used new improved assembly techniques to significantly improve the quality of the tobacco genome (Edwards et al., 2017). The release of important tobacco genome sequences and the construction of genetic maps will greatly advance tobacco study.

2.3 Homologous cloning

The use of homologous cloning is one of the effective methods for studying crop gene function before the sequence of crop genome has not been sequenced or there is no high-quality reference genome sequence. The principle is mainly based on the sequence conservation of different species of genes to design primers, amplification and cloning of the corresponding genes. Depending on the important functional gene information that has been cloned from the model plants and the Solanaceae crops, this method can be used to clone homologous genes that control important traits in tobacco, such as *NsyCMS* (Lv et al., 2015). (There are a large number of studies on homologous cloning of tobacco genes, all of which are published on Chinese journals, not list here.)

2.4 Map-based cloning

The map-based cloning technique was first proposed in 1986 by British scientist Alan Coulson to isolate the target gene based on the location on the chromosome of the target gene. The classical map-based cloning mainly includes the following steps: (1) constructing isolated populations such as F2, DH (doubled haploid) and BC (backcross) according to the phenotype; (2) developing molecular markers, such as RAPD (random amplified polymorphism), RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeat). With the release of genome sequence, we are able to develop more molecular markers, such as InDel (insertion-deletion) and SNP (single nucleotide polymorphism) markers, which improve the efficiency of molecular marker screening and gene mapping; (3) constructing a genetic linkage map, and locating the target gene in the characteristic region of the chromosome through the developed molecular markers; (4) developing and screening more molecular markers, enlarging mapping population, and performing fine mapping; (5) using chromosome walking to obtain candidate genes, and determining the target gene by gene function verification.

2.4.1 Map-based cloning of tobacco gene

The progress of tobacco map-based cloning is seriously lagging behind other model crops, such as rice. Very few genes have so far been obtained using map-based cloning, only two have been successfully associated traits with genes (Edwards et al., 2017; Michel et al., 2018). Most studies have focused on the identification of disease resistance related quantitative trait locus (QTL) and gene regions (Table 1-1). Yi et al. (1998) mapped the known *Rk* gene which conditions resistant to race1 and race 3 of root-knot nematode in tobacco by RAPD. Bao et al. (2019) used SSR molecular markers to locate the known gene *Ph* related to tobacco black shank resistance in linkage group 20 (LG20), and developed a regional linkage map. In addition, two other studies used SSR molecular markers to locate genes associated with qualitative characters to linkage groups (Vontimitta et al., 2010; Wu et al., 2014). In other studies, QTLs related to quantitative characters such as disease resistance and curing of tobacco were also identified (Vontimitta and Lewis, 2012; Tong et al., 2012a; Lan et al., 2014; Cheng et al., 2015; Gong et al., 2016; Sun et al., 2018; Zhang et al., 2018b; Cheng et al., 2019).

2.4.2 Development of tobacco molecular markers

The most critical aspect of map-based cloning is the development of molecular markers that are closely linked to the target trait. Based on the release of tobacco genome sequences and expressed sequence tags (EST), a large number of SSR markers have been developed. Bindler et al. used two different tobacco varieties Hicks Broadleaf and Red Russian in constructing a mapping population, published more than 5000 SSR markers and obtained a high-density tobacco genetic map containing 24 linkage groups (LGs) with 2363 SSR markers loci (Bindler et al., 2011; Bindler et al., 2007). Tong et al. developed a total of 4886 SSR markers (including 1365 genomic SSRs and 3521 EST-SSRs), which were functional in a set of eight tobacco varieties of four different types and were essentially non-overlapping with the set published by

Table 1-1 Map-based cloning in tobacco

Gene/QTL/Region	Function	Molecular marker type	Reference
Rk	resistance to Root-Knot Nematode	RAPD	(Yi et al., 1998)
region	leaf surface chemistry traits	SSR	(Vontimitta et al., 2010)
QTL	resistance to black shank	SSR	(Vontimitta and Lewis, 2012)
QTL	resistance to brown spot	SSR	(Tong et al., 2012a)
QTL	resistance to bacterial wilt	SSR	(Lan et al., 2014)
region	white stem	SSR	(Wu et al., 2014)
QTL	plant height	SSR	(Cheng et al., 2015)
QTL	curing	SSR, SNP	(Gong et al., 2016)
NtEGY1, NtEGY2	nitrogen utilization efficiency	SSR	(Edwards et al., 2017)
QTL	resistance to brown spot	SSR, InDel	(Sun et al., 2018)
QTL	resistance to black shank	SSR	(Zhang et al., 2018b)
NtTPN1	potato virus Y	SSR, SNP	(Michel et al., 2018)
Ph	resistance to black shank	SSR, RAPD, SCAR	(Bao et al., 2019)
QTL	resistance to *cucumber mosaic virus*	SNP	(Cheng et al., 2019)

QTL: quantitative trait locus; RAPD: random amplified polymorphism; SSR: simple sequence repeat; InDel: insertion-deletion; SNP: single nucleotide polymorphism; SCAR: sequence-characterized amplified region.

Bindler et al. (Tong et al., 2012b). These markers were widely used by scientists in subsequent studies. However, SSR markers also have the disadvantages of low throughput and low efficiency in the development and verification process.

With the rapid development of high-throughput sequencing technologies, it is no longer difficult to complete large genome sequencing. For tobacco with large genome and low genetic diversity, the development of single nucleotide polymorphism (SNP) markers is of great significance. In the past five years, four sets of tobacco SNPs have been developed in tobacco. In 2015, based on RAD-Seq (restriction-site associated DNA sequencing), Xiao et al. developed 4138 and 2162 SNP markers with a total length of 1944.74 cM and 2000.9 cM based on reference genome and non-reference genome, respectively (Xiao et al., 2015). In 2016, Gong et al. discovered 10891 SNPs using specific locus-amplified fragment sequencing (SLAF-Seq) (Gong et al., 2016). In 2018, Thimmegowda et al. reported SNPs discovery in 18 flue-cured Virginia tobacco by whole genome resequencing (Thimmegowda et al., 2018). In 2019, Cheng et al. identified a large number of SNPs using RAD-Seq (Cheng et al., 2019). All SNPs mentioned above were integrated to tobacco 24 linkage maps of previously published (Bindler et al., 2011), and significantly increased the number and density of markers of genetic linkage maps.

3 Advances in tobacco leaf senescence

Adapted from the reference: Gao X.M.; Guo Y.F. (2018). Research progress of leaf senescence related genes in tobacco. *Journal of Plant Sciences*, 6, 87-92.

Tobacco (*Nicotiana tabacum*) is an economic crop widely grown in the world. In addition, as a model plant, tobacco plays an important role in the field of plant genetics and transgenic research. Tobacco leaves are processed to make cigarettes and other tobacco products to meet the needs of people. Mature leaves are used for these tobacco products. The process of tobacco leaf maturation is actually the process of senescence and has great influence on the quality of tobacco leaves and tobacco products. In the process of tobacco cultivation, “topping” blocks the transfer of nutrients to the reproductive organs and young stem, so that photosynthetic products can accumulate more in the leaves, which is beneficial to the accumulation of dry matter in tobacco leaves. But at the same time this practice changes the normal aging pattern of tobacco leaves. Therefore, tobacco leaf senescence can be regarded as a special aging process, which has important value in the study of the molecular mechanism of plant senescence. In recent years, the molecular mechanisms of leaf senescence have been well studied in model plants as well as major food crops. But the research on tobacco leaf senescence is still limited.

3.1 Leaf senescence related genes in tobacco

NtCP1 and *NtCP2* are genes encoding cysteine proteinase in tobacco. *NtCP1* is a highly senescence specific gene, which is expressed only in natural aging tobacco

leaves, and cannot be induced by adverse environmental conditions. *NtCP2* is expressed in mature tobacco leaves and down regulated in senescence leaves. It is significantly down regulated after drought and high temperature treatment (Beyene et al., 2006). *NtCP23* and *MC* are also tobacco cysteine proteinase encoding genes. The expression of *NtCP23* is similar to *NtCP1*, except that it can be detected at the early stage of leaf growth and development. *MC* shows the highest expression at the early stage of leaf development, and decreases gradually with leaf senescence (Uzelac et al., 2016). These protease encoding genes may be involved in the degradation of proteins during leaf senescence. Nitrogen in aging leaf cells is generally converted into glutamine through the glutamic acid synthesis cycle and transported to young leaves and reproductive organs through vascular bundle in the form of glutamine to achieve the purpose of nitrogen reuse. *NtGln1-3* is related to the reutilization of nitrogen in tobacco, encoding glutamyl ammonia synthetase, transcript of which is higher in young leaves and aging leaves, but cannot be detected in mature leaves (Brugiere et al., 2000; Uzelac et al., 2016). In the process of senescence, the expression level of *NtPSA1*, which encoding non-catalytic subunits of 26S proteasome, is higher in activity growing tissues, and lower in aging leaves and flowers (Bahrami and Gray, 1999; Uzelac et al., 2016). *NtHIN1* and *NtH1N18* are the response factors of polyamine, a substance that can lead to mitochondrial dysfunction in tobacco. The expression level of *NtHIN1* and *NtH1N18* are significantly increased during leaf and flower senescence (Takahashi et al., 2004). The Ndh complex, which is encoded by the *ndhF* gene, increases the reduction of the electron transport protein and promotes the generation of reactive oxygen species, causing the dysfunction of the chloroplasts and thus promoting leaf senescence. Compared with wild type tobacco, leaf senescence of *ndhF* gene knockout tobacco line is more than 30 days delayed (Zapata et al., 2005). *CYP82E4*, a member of P450 family, encodes a protein that controls the conversion of nicotine and demethylation nicotine, and its expression is significantly up regulated with leaf senescence (Chakrabarti et al., 2008).

3.2 Regulation of tobacco leaf senescence

Isopentenyl transferase plays a key role in cytokinin biosynthesis. The *IPT* gene, that encoding isopentenyl-transferase was cloned from *Agrobacterium tumefaciens* (Barry et al., 1984). Compared with wild type tobacco, transgenic tobacco harbored P_{SAG12}-*IPT* showed the phenotype of delayed leaf senescence, the number of flowers increased by 83.7%, the biomass increased by 40.3%, the number of seeds increased by 52.4%, but no significant difference in leaf number and plant height was observed (Iqbal et al., 2017). A deeper study of P_{SAG12}-*IPT* transgenic tobacco showed that under nutrient deficient conditions, necrotic lesions were detected in old, but otherwise green leaves of unfertilized P_{SAG12}-*IPT* transgenic tobacco. The leaves of the same leaf position of wild type tobacco were yellow at the same growth stage, but no necrotic lesions appeared. The necrotic lesions are caused by the over reduction of the electron transport chain, resulting in an imbalance between light trapping and energy consumption (Wingler et al., 2005). In addition, the expression of pathogenesis-

related genes *PR-1b* and *PR-Q* are significantly higher in old P_{SAG12}-*IPT* transgenic tobacco leaves with necrotic lesions (Wingler et al., 2005). Maria Pilarska et al. (2017) used the P_{SAG12}-*IPT* transgenic tobacco to prove that lipid peroxidation is not related to leaf senescence (Pilarska et al., 2017). Brutting et al. (2017) used *Nicotiana attenuata* expressing *IPT4* from *Arabidopsis*, driven by the promoter of *SAG12*, proved that cytokinins in leaves are sufficient to alter ontogenic patterns of defense metabolites (Brutting et al., 2017). The maize homeobox gene *knotted1* (*kn1*) and its homologs are expressed in shoot meristems and are essential for meristem maintenance and initiation (Ori et al., 1999). Many of the phenotypes of P_{kn1}-*IPT* transgenic tobacco are similar to those observed in P_{SAG12}-*IPT* transgenic tobacco, reveal that except inhibiting differentiation; *kn1* has effect on leaf senescence regulation (Ori et al., 1999). Luo et al. (2006) manipulated the expression of *KN1* through a wound-inducible promoter *Win3.12*, and $P_{Win3.12}$- *kn1* transgenic tobacco also showed delayed leaf senescence (Luo et al., 2006). *BiP* gene encodes an endoplasmic reticulum binding protein. *BiP* over expressing transgenic tobaccos have higher tolerance to drought stress (Alvim et al., 2001). The *CKX* gene encodes cytokinin dehydrogenase, and active CKX initially detected in tobacco tissues crude extracts. Over expression of *AtCKX* in tobacco, the antioxidant capacity of transgenic tobacco increased significantly, even if the cytokinin level decreased significantly, the transgenic tobacco also showed obvious delayed leaf senescence (Mytinova et al., 2011; Paces et al., 1971).

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2

Objective and Thesis Structure

Senescence is the terminal phase of plant development that is typically associated with decline in cells, tissues, organs and whole plant, and eventually leads to the death of the whole plant. Plant senescence is mainly characterized by leaf senescence, which is a programmed cell death regulated by genes and affected by internal and external factors. In the process of leaf senescence, the intracellular macromolecular substances in the senescent leaves are degraded and converted into nutrients, and transferred to young tissues and reproductive organs to complete the life cycle. Leaf senescence affects many agronomic traits such as the quantity and quality of the seeds, the ripening of the fruit, and the storage time during post-harvest. Regulation and intervention of leaf senescence can improve the yield and quality of crops. Therefore, study of leaf senescence has great theoretical and practical significance.

Impressive progress has been made in the characterization and understanding of leaf senescence regulation, especially in model species such as *Arabidopsis* and rice. However, at present, little is known about the molecular regulation mechanism of tobacco leaf senescence. Tobacco (*Nicotiana tabacum* L.) is an important agricultural crop widely grown in the world, of which mature leaves are the raw materials for cigarette production. It also plays an important role in plant genetics and transgenic research as a model plant. The process of tobacco leaf maturation is actually senescence.

At present, the research on the molecular mechanism of tobacco leaf senescence regulation is still blank. Studies related to tobacco leaf senescence mostly use tobacco as a tool for expression of exogenous genes, thereby analyzing the function of exogenous genes in leaf senescence regulation. In addition, the dynamic changes of metabolites and transcripts in the process of tobacco leaf senescence have also been reported. But none of these studies identified specific leaf senescence regulating genes.

Mapping functional genes using ethyl methane sulfonate (EMS) mutants combined with map-based cloning technology to clone functional genes is a classical approach of forward genetics. Meanwhile, with the continuous development of high-throughput sequencing technology, using mutant combined with sequencing technology can overcome the time-consuming and labor-intensive defects of traditional methods and achieve rapid localization of functional genes, so it is increasingly becoming the main approach to clone functional genes.

A new tobacco mutant library induced by EMS mutagenesis was developed by Tobacco Research Institute of Chinese Academy of Agricultural Sciences.

Therefore, based on the above background, the objective of this study is identification of tobacco leaf senescence regulating genes.

According to the leaf yellowing phenotype, we identified mutants from M2 lines and further verified the phenotype in M3 and M4 generations. The tobacco mutants with early leaf yellowing phenotype were used as biological materials for future research activities. Establish genetic population and analyze the inheritance of premature leaf senescence phenotype. Base on the mapping populations and published polymorphic molecular markers of tobacco, the position of genes causing premature

leaf senescence phenotype in the genome will be preliminary located. In addition to the traditional map-based cloning technology mentioned above, the whole genome sequencing (WGS) technology will be used to sequence the tobacco mutants with premature leaf senescence phenotype. Compared with the reference genome, the mutant sites that may lead to the phenotype will be screened, and the functional genes that lead to premature leaf senescence phenotype will be identified.

This document started with a general introduction on the context of research progress in plant leaf senescence, research progress in tobacco genome, tobacco gene cloning, and research progress in tobacco leaf senescence.

In Chapter 3, a tobacco premature leaf senescence mutant controlled by a single recessive gene in the Honghuadajinyuan (HD) variety background was identified. And using the classical map-based cloning method to carry out gene mapping.

In Chapter 4, a tobacco premature leaf senescence mutant controlled by a single recessive gene with Zhongyan100 (ZY100) variety background was identified. We first screened candidate SNPs associated with mutant phenotype using the MutMap method and WGS. However, the results of the verification proved that the candidate SNPs were not point mutations that lead to the phenotype. Therefore, the target gene was mapped using the mapping population and SSR molecular markers.

Finally, in Chapter 5, a conclusion and discussion for the results above are proposed.

3

Characterization and Mapping of a Novel Premature Leaf Senescence Mutant *yellow leaf 1* in Common Tobacco (*Nicotiana tabacum* L.)

Characterization and Mapping of a Novel Premature Leaf Senescence Mutant *yellow leaf 1* in Common Tobacco (*Nicotiana tabacum* L.)

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Abstract: As the last stage of plant development, leaf senescence has a great impact on plant's life cycle. Genetic manipulation of leaf senescence has been used as an efficient approach in improving the yield and quality of crop plants. Here we describe an ethyl methane sulfonate (EMS) mutagenesis induced premature leaf senescence mutant *yellow leaf 1* (*yl1*) in common tobacco (*Nicotiana tabacum* L.). The *yl1* plants displayed early leaf yellowing. Physiological parameters and marker genes expression indicated that the *yl1* phenotype was caused by premature leaf senescence. Genetic analyses indicated that the *yl1* phenotype was controlled by a single recessive gene that was subsequently mapped to a specific interval of tobacco linkage group 11 using simple sequence repeat (SSR) markers. Exogenous plant hormone treatments of leaves showed that the *yl1* mutant was more sensitive to ethylene and jasmonic acid than the wild type. No similar tobacco premature leaf senescence mutants have been reported. This study laid a foundation for finding the gene controlling the mutation phenotype and revealing the molecular regulation mechanism of tobacco leaf senescence in the next stage.

Keywords: tobacco; EMS mutagenesis; premature leaf senescence; SSR markers; plant hormone

1. Introduction

Tobacco (*Nicotiana tabacum* L.) is an important agricultural crop widely grown in the world. It plays an important role in plant genetics and transgenic research as a model plant (Edwards et al., 2010; Masclaux et al., 2000). Also, it can be used as a bioreactor for vaccine production (Arntzen, 2008; Budzianowski, 2015; Monreal-Escalante et al., 2017). In addition, due to the huge biomass of tobacco, it has become a potential bioenergy plant (Andrianov et al., 2010; Fuchs et al., 2013; Vanhercke et al., 2014). In tobacco production, mature/ripe leaves are harvested, so leaf senescence is critical for both the yield and quality of tobacco products. Therefore, studying tobacco leaf senescence has important theoretical significance and a practical application value. Although plant leaf senescence regulation has been extensively studied in model systems such as *Arabidopsis* and rice, limited information is available about tobacco leaf senescence regulation at present. Several studies focused on transcriptome and metabolome of tobacco leaf senescence (Li et al., 2016; Li et al., 2017; Zhao et al., 2018a; Zhao et al., 2018b). These studies have provided global and potential gene dynamic expression changes and signaling pathways for leaf senescence regulation in tobacco, but few genes involved in regulation of tobacco leaf senescence have been identified.

Senescence is the terminal phase of plant development that typically displays as a decline at the cell, tissue, organ, and whole plant levels (Lim et al., 2007). Leaf senescence is the main part of plant senescence, and is strictly controlled at the molecular level during plant development (Schippers, 2015). In a controlled environment with sufficient nutrition and favorable growth conditions, leaf senescence depends on the leaf age and development stage of the plant (Gan and Amasino, 1997; Quirino et al., 2000). Leaf senescence is affected by both endogenous and exogenous factors, which regulate the expression of genes involved in the execution process of senescence, and eventually lead to the death of the whole plant (Ali et al., 2018). Furthermore, leaf senescence is regulated by a complex regulatory network composed of transcription factors, hormones, environmental factors, sugar metabolism, epigenetic regulation, and miRNA mediated regulation (Ali et al., 2018; Guo et al., 2004; Jibran et al., 2013; Wingler et al., 2009; Yolcu et al., 2018). Leaf senescence is not a completely passive and adverse biological process. During leaf senescence, macromolecules such as proteins, lipids, and nucleic acids are broken down. The nutrients released from the catabolism of these macromolecules, as well as other nutrients, are recycled to new buds, young leaves, developing fruits, and seeds, which influence the yield of crops (Gan and Amasino, 1997). Premature leaf senescence generally causes significant yield loss while delayed leaf senescence leads to increased grain production in many crop species (Gregersen et al., 2013; Guo and Gan, 2014).

Chlorophyll breakdown is one of the most important catabolic processes during leaf senescence in higher plants (Hortensteiner and Krautler, 2011). The yellowing of

leaves caused by chlorophyll loss is the major phenotype of plant senescence. Total chlorophyll content is the most commonly used parameter for characterizing senescence progression. Drastic changes in gene expression are associated with leaf senescence. *Arabidopsis* leaf senescence transcriptome data revealed that many transcription factor families are overrepresented in the transcriptome of leaf senescence (Breeze et al., 2011; Guo et al., 2004). In addition to transcription factors, many genes associated with metabolism and signal transduction were identified to be associated with leaf senescence. Based on their different expression patterns, genes with differential expression were classified as senescence down-regulated genes (SDGs), such as *ribulose bisphosphate carboxylase small subunit* (*RBCS*) encoding a member of the rubisco small subunit, a key enzyme in photosynthesis, and senescence up-regulated genes, which are also called senescence-associated genes (SAGs) (Thomas and Stoddart, 1980). While many of the *SAGs* could be induced by senescence as well as other senescence-inducing factors, such as environmental stresses, *SAG12* was found to be strictly induced by natural senescence (Noh and Amasino, 1999), and thus, often used as a leaf senescence marker gene. Plant hormones are important endogenous factors involved in the regulation of leaf senescence. Some of the senescence regulating plant hormones are also involved in the plant's response to environmental stresses and stress-induced senescence (Jan et al., 2019; Jibran et al., 2013; Khan et al., 2014). Thus, leaf senescence often involves complex cross talks between signaling pathways associated with multiple hormones and stress signals.

Molecular markers are powerful tools for genetic research and plant breeding that have been widely used in many types of biological research. Genetic linkage mapping based on molecular markers can clarify the structure and organization of the genome. In tobacco, a large number of simple sequence repeat (SSR) markers have been developed based on the released data of tobacco genome sequences and expressed sequence tags. Bindler et al. used two different tobacco varieties, Hicks Broadleaf and Red Russian, in constructing a mapping population, and they published more than 5000 SSR markers and obtained a high-density tobacco genetic map containing 24 linkage groups (LGs) with 2363 SSR markers loci (Bindler et al., 2011; Bindler et al., 2007). Tong et al. developed a total of 4886 SSR markers (including 1365 genomic SSRs and 3521 EST-SSRs), which were functional in a set of eight tobacco varieties of four different types and were essentially non-overlapping with the set published by Bindler et al. (Bindler et al., 2011; Bindler et al., 2007; Tong et al., 2012b). These SSR markers have been wildly used for the identification of quantitative trait loci (QTLs) associated with tobacco disease resistance (Lan et al., 2014; Sun et al., 2018; Tong et al., 2012a; Vontimitta and Lewis, 2012) and tobacco traits (Cheng et al., 2015), and they have also been used for genetic diversity analysis of tobacco varieties (Kalivas et al., 2016; Tong et al., 2018).

In this study, we identified a tobacco mutant *yellow leaf 1* (*yl1*), which exhibited a premature leaf senescence phenotype. We characterized the premature leaf senescence phenotype of *yl1*, determined the physiological parameters related to leaf senescence

and the expression of senescence marker genes, analyzed its phenotypic inheritance, and mapped the target gene.

2. Materials and Methods

2.1 Plant Materials and Genetic Populations

The *yl1* mutant was identified from the chemical mutagen EMS induced library derived from the common tobacco variety Honghuadajinyuan (HD) (Wang et al., 2017). The *yl1* mutant was identified from one of the M2 lines, and further verified in the M3 and M4 generation. HD and another wild type tobacco variety GX3 (Gexin 3) were crossed with *yl1*, respectively, to produce genetic populations. The F1 (HD × *yl1*; GX3 × *yl1*) plants were self-pollinated to generate F2 segregating populations. BC1F1 segregating populations ((HD × *yl1*) × *yl1*; (GX3 × *yl1*) × *yl1*) were generated by crossing F1 with *yl1*. All the plants were grown in a field in Zhucheng City, Shandong Province, China. Leaf senescence phenotypes were evaluated by visual observation. Segregation patterns were evaluated for the "goodness of fit" to the expected ratios using the chi-squared test.

2.2 Sampling for Measurement of Leaf Senescence Related Physiological Parameters and RNA Extraction

Plants of HD and *yl1* were grown in the field in Zhucheng City, Shandong Province, China during the 2018 growing season. Middle position leaves of each plant were collected at different developmental stages. The collection of the leaf samples was done at four developmental stages during the growing season: 35, 50, 75, and 95 days after transplanting (DAT). For each sample, one leaf from the middle position was collected from three plants randomly. Each leaf was divided into two parts along the main vein. One half was wrapped in aluminum foil, immediately immersed into liquid nitrogen, and stored at -80 °C until it was used for extracting the RNA and soluble protein, while the other half was put in sample bag and stored in the ice box for chlorophyll content measurements.

2.3 Measurement of Leaf Senescence Related Physiological Parameters

Chlorophyll was extracted and quantified as described previously (He and Gan, 2002). Briefly, 0.5 g of the leaf sample was immersed in 30 mL ethanol overnight in darkness until the leaves became completely pale. The absorbance at 649 nm and 665 nm of the extraction solution was quantified using a UV-Vis spectrophotometer (TECAN, Infinite M200, Salzburg, Austria).

Fluorescence of living leaves was measured using a portable modulated chlorophyll fluorometer according to the manufacturer's instructions (Opti-Sciences, OS1p, Hudson, USA).

Extraction of the soluble protein in each sample was according to the manufacturer's instruction (CWBIO, CW0885M, Beijing, China). Briefly, about 0.2 g of the frozen leaves' material was homogenized in 1 mL extraction reagent containing a protease inhibitor cocktail. The number of soluble proteins in each sample was measured using the BCA protein assay kit (CWBIO, CW0014S, Beijing, China) according to the manufacturer's instruction.

2.4. RNA Extraction and qRT-PCR

The total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, USA). First-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA, RR047, Dalian, China) according to the manufacturer's instruction. qRT-PCR was carried out on a Thermal Cycler Block 7500 (ABI, Waltham, USA) according to the manufacture of TB Green™ Premix Ex TaqTM II (Tli RNaseH Plus) (TAKARA, RR820, Dalian, China). The means of three biological and technical replicates were analyzed. The *Actin* was used as a reference gene for internal control, while *SAG12* and *RBCS* were used as senescence markers. The primer sequences of the genes for qRT-PCR are shown in Table 3-1.

Table 3-1 The primer sequences of genes for qRT-PCR

Gene Name	Accession Number [1]	Forward Primer Sequence [1]	Reverse Primer Sequence [1]
Actin	Nitab4.5_0009320g0010.1	CAAGGAAATCACGGCTTTGG	AAGGGATGCGAGGATGGA
SAG12	Nitab4.5_0001608g0070.1	ATTTTCAGCGGTGGCAGCT	GTAAGAAGTCGTAGGCTCG
RBCS	Nitab4.5_0006249g0010.1	CCTGCTAAGGATACAATTAG	CTCAAATTTCTTGTTGTCA

[1] The accession number and gene sequences were obtained from the N. tabacum CDS database (https://solgenomics.net) (Edwards et al., 2017).

2.5. DNA Extraction and SSR Analysis

Genomic DNA was extracted using the Plant Genomic DNA Kit (TIANGEN, DP305, Beijing, China). The DNA concentration was measured using a Nano Drop 2000 spectrophotometer (Thermo Scientific, Waltham, USA) and adjusted to 50 ng/μL. Tobacco simple sequence repeat (SSR) markers were used to map *YL1* based on the high-density genetic map of tobacco (Bindler et al., 2011). PCR was performed in a Veriti 96-Well Thermal Cycler (ABI, Waltham, USA) following the previously described method (Wu et al., 2014). Briefly, each reaction contained 7.5 μL of 2 x Taq PCR Master Mix (TIANGEN, KT211, Beijing, China), 50 ng template DNA, and 1 μL of 5 μM forward and reverse primers in a volume of 15 μL. The PCR program was as follows: pre-denaturation at 95°C for 3 min, followed by 30 cycles of 15 s at 95°C, 30 s at 50–60°C (the annealing temperature of different primer pairs), and 30 s at 72°C, and then a final extension at 72°C for 5 min. After that, 6 μL PCR products

were separated on 6% nondenaturing polyacrylamide gel and visualized with silver staining as previously described (Bassam et al., 1991).

2.6. Linkage Map and Genetic Distance

The BC1F1 population ((GX3 × *yl1*) × *yl1*) was used to obtain the markers linked to *YL1*. The polymorphic SSR markers between GX3 and *yl1* were partially selected and used to screen a small number of recessive individuals of the BC1F1 population with the premature leaf senescence phenotype, as well as their parents. After confirming the linkage between one marker and *YL1*, all adjacent polymorphic SSR markers were run across the whole recessive individuals of the population. The linkage map based on the segregations between *YL1* and the markers was constructed by QTL Ici Mapping software V4.0 (Li et al., 2008), and the threshold value of the logarithm of odd (LOD) was set at 3.0. Genetic distances were calculated and presented in Kosambi centiMorgans (cM).

2.7. Plant Hormone Treatments

Leaf discs from middle leaves of plants grown in greenhouse 50 DAT were used for plant hormone treatments. Detached leaves were cut into small discs (~1.5 cm in diameter) and floated on an incubation buffer (1/2 MS, 3 mM MES, and pH 5.8) with and without hormones in Petri dishes with filter paper. The samples were incubated at 28°C under 24 h light in a growth chamber. The plant hormone was diluted to working concentrations using the incubation buffer. The working concentrations of the plant hormones are 100 μM for 1 – aminocyclopropane - 1 - carboxylic acid (ACC) and 50 μM for methyl jasmonate (MeJA) (Zhang and Guo, 2018).

3 Results

3.1. The yl1 Plants Exhibit Premature Leaf Senescence

Compared with Honghuadajinyuan (HD), *yl1* plants showed a premature leaf senescence phenotype from about 50 days after transplanting (DAT), and throughout the subsequent developmental stage (Figure 3-1). No significant difference of leaf color was observed between HD and *yl1* 35 DAT or at earlier development stages (Figure 3-1 A). The yellowing of the bottom leaves appeared in the *yl1* plants around 50 DAT, and the leaf color was lighter than HD (Figure 3-1 B). At the flowering stage, about 75 DAT, significant difference in leaf color of the bottom and middle leaves were observed between the *yl1* and HD plants (Figure 3-1 C, E) and this difference in leaf yellowing was more profound at 95 DAT when the upper leaves of the *yl1* plants became completely yellow, whereas the majority of leaves in the HD plants still retained their green color (Figure 3-1). These results suggest that the *yl1* plants seemed to display a phenotype of premature leaf senescence.

3.2. Alterations of Leaf Senescence Related Parameters

In order to further characterize the phenotype of *yl1*, we determined the chlorophyll content, *F*v/*F*m ratio, soluble protein content, and the expression of senescence marker genes in *yl1* in comparison with HD (Figure 3-2). Consistent with the premature leaf yellowing phenotype, Chlorophyll *a* (Chl *a*), Chlorophyll *b* (Chl *b*), and the total chlorophyll (Chls) of the middle leaves in *yl1* were lower than in counterpart leaves of the HD plants at the same developmental stages. The contents of Chl *a*, Chl *b*, and Chls decreased rapidly in *yl1* 50 DAT, whereas the HD plants did not start losing chlorophyll until 75 DAT (Figure 3-2 A, B, and C). In tobacco, senescence of leaves progresses from the bottom to the top of a plant. At 75 DAT, although no significant difference in chlorophyll contents was observed in upper leaves, chlorophyll contents in the middle and lower leaves were significantly lower in *yl1* than in the HD plants (Figure 3-2 D, E, and F). At 75 DAT, the *F*v/*F*m ration and soluble protein in upper, middle, and lower leaves of *yl1* were significantly lower than in the HD plants (Figure 3-2 G, H). Furthermore, the expression of the marker genes *SAG12* and *RBCS* of the individual samples was determined. As shown in Figure 3-2 I and J, in *yl1*, expression of the senescence marker gene *SAG12* was first detected at 50 DAT and the photosynthetic gene *RBCS* became undetectable at 75 DAT. For HD plants, *SAG12* expression was first detected at 75 DAT and *RBCS* expression became undetectable at 95 DAT. Significantly lower *SAG12* and higher *RBCS* expression were detected at different leaf positions of 75 DAT plants (Figure 3-2 K, L). The premature leaf senescence phenotype of *yl1* was well reflected in the expression patterns of *SAG12* and *RBCS*.

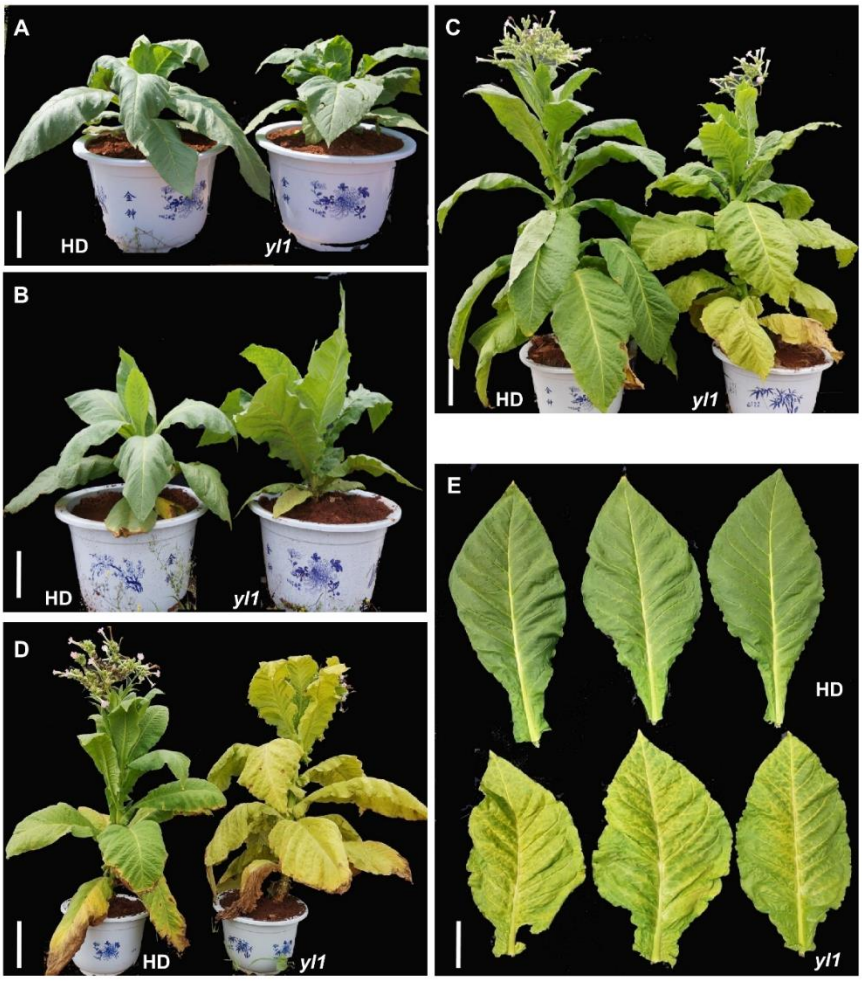

Figure 3-1 Phenotypic comparison between Honghuadajinyuan (HD) and *yl1*. The phenotype of HD and *yl1* plants grown in the field. (A) 35 days after transplanting (DAT), bar 20 cm; (B) 50 DAT, bar 20 cm; (C) 75 DAT, bar 30 cm; and (D) 95 DAT, bar 35 cm. (E) The middle leaves of HD and *yl1* 75 DAT, bar 10 cm.

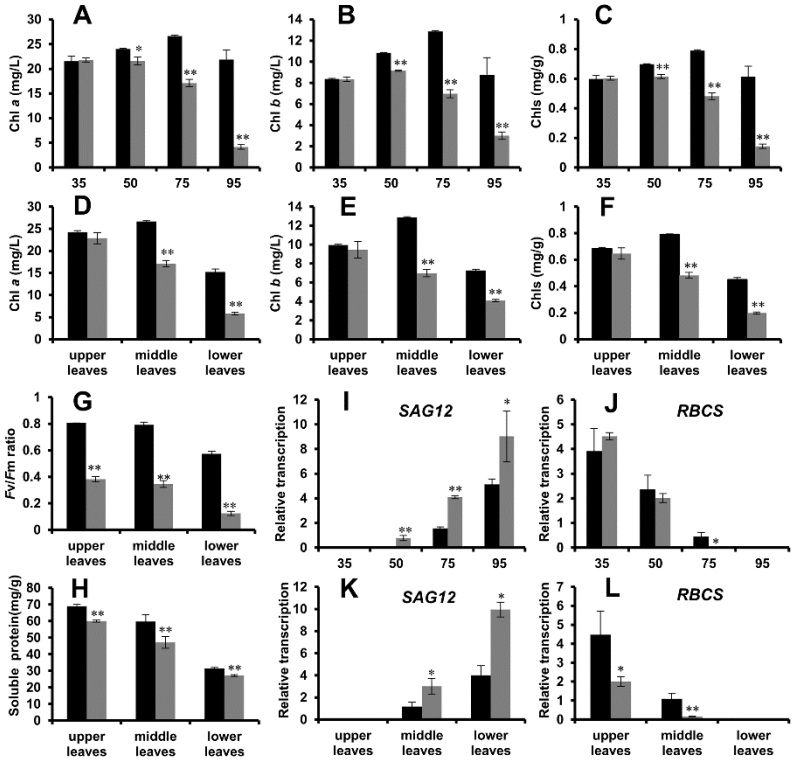

Figure 3-2 Leaf senescence related parameters of HD and *yl1*. Black and grey columns indicate HD and *yl1*, respectively. (A) Chlorophyll *a* (Chl *a*), (B) Chlorophyll *b* (Chl *b*), and (C) total Chlorophyll (Chls) in middle leaves of the same leaf position of HD and *yl1* at 35, 50, 75, and 95 DAT. (D) Chl *a*, (E) Chl *b*, (F) Chls, (G) *F*v/*F*m ratio, and (H) soluble protein in upper, middle, and lower leaves of the same leaf position of HD and *yl1* 75 DAT, respectively. Relative expression of (I) *SAG12* and (J) *RBCS* in middle leaves of the same leaf position of HD and *yl1* at 35, 50, 75, and 95 DAT. Relative expression of (K) *SAG12* and (L) *RBCS* in upper, middle, and lower leaves of the same leaf position of HD and *yl1* at 75 DAT. Values are mean ± SD of three individual replicates. * and ** represent significant difference determined by the Student's t test at $p \leq 0.05$ and $p \leq 0.01$, respectively.

3.3. The Premature Leaf Senescence of yl1 was Controlled by a Single Recessive Gene

To analyze the inheritance of the premature leaf senescence phenotype of *yl1*, two wild type tobacco varieties, HD and Gexin 3 (GX3), were crossed with *yl1*. All the F1 plants showed a normal green phenotype similar to the wild type. A total of 43 out of 154 (HD × *yl1*) and 40 out of 155 (GX3 × *yl1*) F2 plants, respectively, displayed the premature leaf senescence phenotype, showing a segregation ratio of 3:1 ($\chi^2 < \chi^2_{0.05}$ =

3.841; Table 3-2). These results suggested that the premature leaf senescence phenotype of *yl1* was controlled by a single recessive gene. To further verify this observation, BC1F1 populations were generated by crossing the F1 plants of HD × *yl1* and GX3 × *yl1* with *yl1* separately. The phenotypic segregations of the BC1F1 populations correlated to the expected ratio of 1:1 with similar numbers of wild type and mutant type plants ($\chi^2 < \chi^2_{0.05} = 3.841$; Table 3-2).

Table 3-2 Genetic analysis of *yl1*

Population type	Cross	Total	Wild Type	Mutant Type	Segregation ratio	χ^2 [1]
F2	HD × *yl1*	154	111	43	2.581	0.701
F2	GX3 × *yl1*	155	115	40	2.875	0.054
BC1F1	(HD × *yl1*) × *yl1*	155	83	72	1.153	0.781
BC1F1	(GX3 × *yl1*) × *yl1*	163	77	86	0.895	0.497

HD: Honghuadajinyuan, tobacco variety; GX3: Gexin 3, tobacco variety; [1] Value for significant at 0.05 and *df* = 1 is 3.841

3.4. Preliminary Mapping of YL1

The BC1F1 population developed between parent GX3 and *yl1* was used to map *YL1*. A total of 265 simple sequence repeat (SSR) markers showing polymorphism between GX3 and *yl1* were identified from 1376 pairs of SSR markers by Bindler et al. (Bindler et al., 2011; Bindler et al., 2007) and Tong et al. (Tong et al., 2012b), from which 96 markers were evenly distributed on 24 linkage groups (LGs) of tobacco and were selected to screen 19 recessive individuals from the BC1F1 population of GX3 and *yl1*. The results showed that the marker PT53066 located on LG11 was associated with the *yl1* phenotype (Figure 3-3 A). In order to obtain more linked markers, we used more polymorphic SSR markers on the flanks of PT53066 to screen the 19 mutant plants and identified three more linked markers: PT60305, PT60998, and PT60975 (Figure 3-3 B). In order to obtain the exact location of the *YL1* gene on LG11, we enlarged the BC1F1 population to get 46 premature leaf senescence individuals and screened them with the above four linked SSR markers. The isolation of each SSR marker and *YL1* was converted into genetic distance by the QTL Ici Mapping software (V4.0). The results showed that *YL1* was located between the markers PT53066 and PT60305, with the genetic distance of 1.08 and 3.51 cM, respectively (Figure 3-3 B).

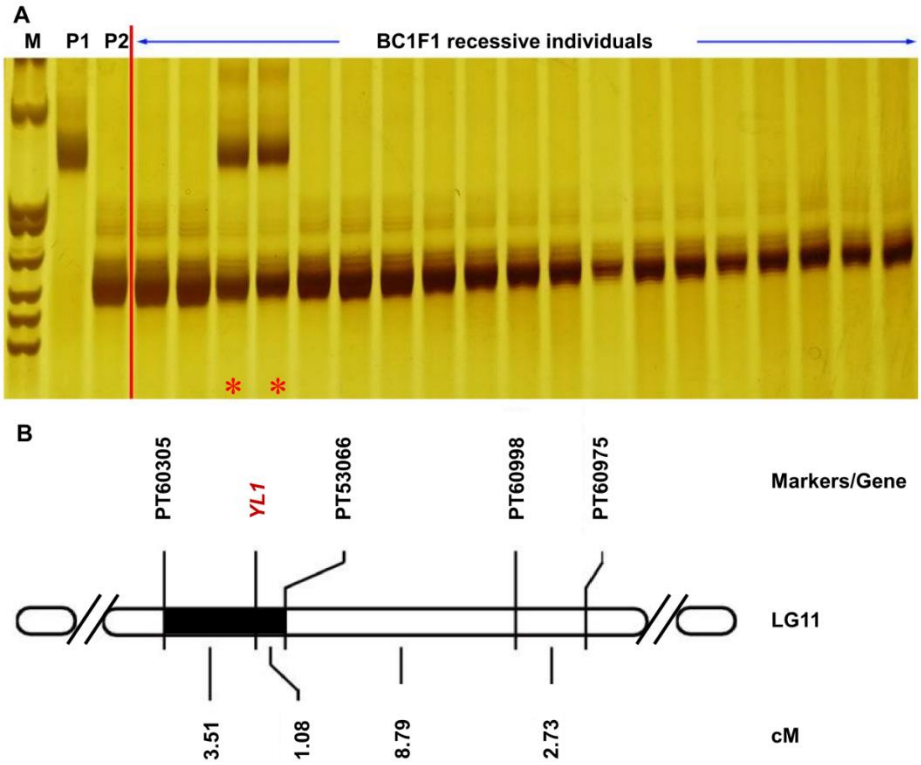

Figure 3-3 Preliminary mapping of *YL1*. (A) Linkage relationships between tobacco simple sequence repeat (SSR) markers PT53066 and *YL1* in 19 recessive individuals from BC1F1 (GX3 × *yl1*) × *yl1* population. M indicates the DNA marker pBR322/MspI; P1 indicates GX3; P2 indicates *yl1*; and * indicates the recombinant individuals. (B) Genetic map of *YL1* and four SSR markers on tobacco linkage group 11 (LG11). The black bar indicates the smallest interval of *YL1* and markers; cM indicates centimorgan.

3.5. Plant Hormone Treatments

In order to find out whether *YL1* is involved in hormone signaling pathways that regulate leaf senescence, leaf discs from the middle leaves of 50 DAT plants were used in plant hormone treatments (Figure 3-4). Without plant hormone treatment, the yellowing of the *yl1* leaves was faster than that of HD (Figure 3-4 Control). As senescence-promoting hormones, treatments of both 1 – aminocyclopropane - 1 - carboxylic acid (ACC) and methyl jasmonate (MeJA) accelerated yellowing of leaf discs with a significantly more profound effect on the *yl1* leaves, suggesting that *yl1* was more sensitive to ACC and MeJA than the HD plants (Figure 3-4).

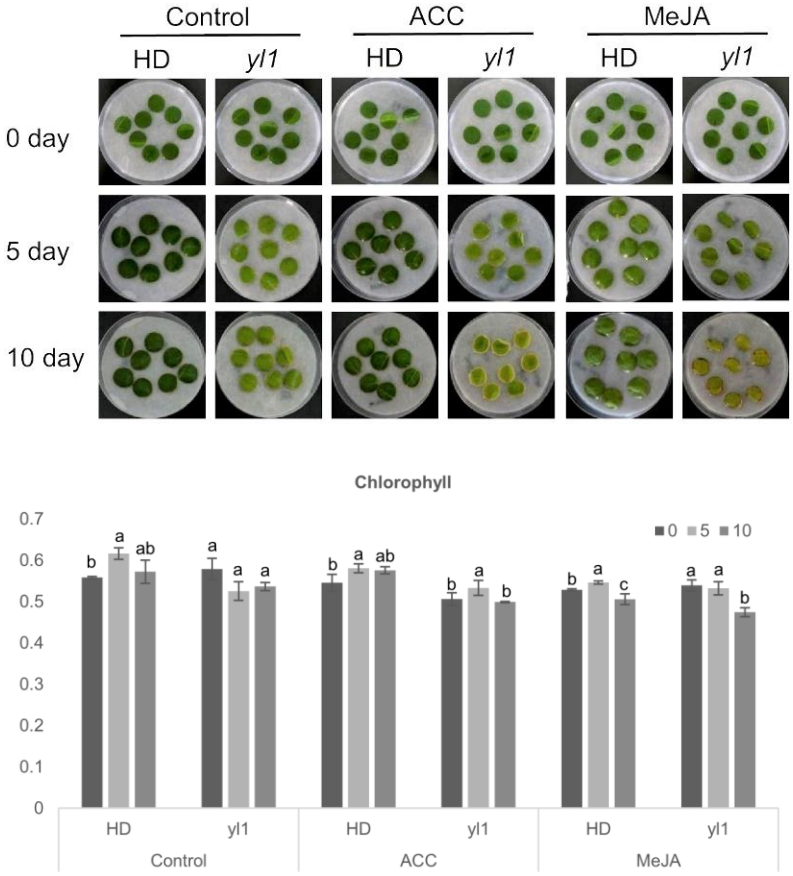

Figure 3-4 Plant hormone treatments of HD and *yl1* leaf discs. Controls were treated with the incubation buffer without hormone. ACC: 1 - aminocyclopropane - 1 - carboxylic acid; MeJA: methyl jasmonate.

4. Discussion

The *yl1* mutant has been continuously planted in Zhucheng City, Shandong Province, China, for four years. A reliable and stable early yellowing phenotype has been consistently observed in *yl1* plants over the years. The external performance of leaf senescence is that the leaf color turns yellow or even dead, while the internal performances include the chloroplast structure changing significantly or even completely disintegrating, and the decrease of the photosynthesis and protein (Gan and Amasino, 1997). One of the early physiological manifestations of leaf senescence is the collapse of the membrane structure due to membrane lipid de-esterification

(Thompson et al., 1998). With the occurrence of senescence, the outer layer of the chloroplast disappears, and the chlorophyll degrades. Chlorophyll *a* (Chl *a*) and Chlorophyll *b* (Chl *b*) are the two prevailing forms of chlorophyll, which are involved in different light harvesting. Chl *a* is associated with the energy processing centers of the photosystems, while Chl *b* is considered to be an accessory pigment that transfers light energy to Chl *a* (Tanaka and Tanaka, 2006). The chlorophyll breakdown process is initiated by conversion from Chl *b* to Chl *a* by a Chl *b* reductase (Hortensteiner and Krautler, 2011). The content of Chl *a*, Chl *b*, and total chlorophyll (Chls) in *yl1* was lower than that in HD during the whole development process, and the difference was more significant at the middle and late stages of development. Moreover, chlorophyll degradation was initiated earlier in *yl1* than in HD at an early development stage of than 50 DAT (Figure 3-2 A–C). Therefore, we believe the low chlorophyll content at late stage is due to early chlorophyll degradation, a typical premature senescence phenotype. The phenotype of the *yl1* mutant was further confirmed by the difference in *F*v/*F*m ratio, soluble protein content, and expression levels of the senescence-associated marker genes (Figure 3-2 G–L).

Mapping functional genes using EMS mutants combined with map-based cloning has been applied in many crops; however, in tobacco, it has lagged behind when compared with those of other plants such as rice. The common tobacco (*Nicotiana tabacum* L.) is an allotetraploid (2n = 48) species produced by the hybridization and chromosome doubling of *N. sylvestris* (2n = 24) and *N. tomentosiformis* (2n = 24) (Leitch et al., 2008), with 4.5 Gb genome and more than 70% repetitive sequences (Renny-Byfield et al., 2011). The difficulty of mapping tobacco genes by map-based cloning is partially due to the large genome of tobacco with lots of repetitive sequences. Wu et al. (2014) used SSR markers by Bindler et al. and Tong et al. to perform a preliminary mapping of a light color mutant *ws1* controlled by two recessive nuclear genes. The *ws1a* locus was mapped between PT54006 and PT51778 with a genetic distance of 8.04 and 3.96 cM, respectively, and the *ws1b* locus was mapped between PT53716 and TM11187, each with a genetic distance of 8.56 cM from *ws1b* (Wu et al., 2014). Michel et al. (2018) located *NtTPN1*, a gene required for potato virus Y-induced veinal necrosis in tobacco, between PT60530 and PT61143 with a distance of 0.5 and 2.9 cM, respectively (Michel et al., 2018). A tobacco black shank resistance gene *Ph* was located on the top of LG20, but there were no more PT SSR markers available for subsequent localization (Bao et al., 2019). The gene responsible for the premature senescence phenotype of *yl1* was mapped between the markers PT53066 and PT60305, and the genetic distance was 1.08 and 3.51 cM, respectively. There was one additional SSR marker, PT30027, within the mapping interval of the *yl1*. However, the SSR primer pair has no polymorphism among the mapping population. Similarly, we were not able to further narrow down the interval using the PT/TM SSR markers. Moreover, the forward and reverse sequences of PT53066, PT60305 and PT30027 primers were used in BLAST search against all the currently available genome sequences of *Nicotiana* species, including *N. tabacum* cultivars K326 (Edwards et al., 2017), TN90 and Bsama Xanthi (Sierro et al., 2014), *N. benthamiana* (Bombarely et al., 2012), and even the diploid ancestor *N. sylvestris*

(Sierro et al., 2013). We found that PT53066 and PT60305 primers were physically located on two separated genomic fragments/scaffolds, and PT30027 primers were not identified with BLAST search.

Comparative genomics is a powerful tool to isolate homologous genes. According to the COSII genetic map of two diploid *Nicotiana* species (Wu et al., 2010), gene order and gene content were well preserved between the short arm of LG11 (pseudo-chromosome) of tobacco where the *yl1* was located, and that of the chromosome 11 of tomato. Comparative genomics analyses indicated that the mapping interval of *yl1* corresponds to a 422 kb syntenic region of tomato chromosome 11, harboring 54 predicted genes (Solyc11g008760~Solyc11g010270) (Tomato Genome, 2012), and a 513 kb syntenic region of potato chromosome 11, harboring 52 predicted genes (PGSC0003DMG400016148~PGSC0003DMG400016173) (Potato Genome Sequencing, 2011), respectively. Most of the predicted genes are highly conserved between tomato and potato. However, none of these genes was predicted to be involved in plant senescence or the ethylene and jasmonic acid pathways.

Recently, a total of 1,224,048 non-redundant *Nicotiana* SSR markers were discovered and characterized in silico in seven *Nicotiana* genomic sequences, of which 99.98% are novel (Wang et al., 2018). In addition to SSR markers, single nucleotide polymorphisms (SNPs) are also commonly used molecular markers (Davey et al., 2011). With the rapid advance in next generation sequencing (NGS) technologies, it has become possible to complete genome sequencing of large crop species. Especially for tobacco, which is a crop with huge genome and low genetic diversity, it is necessary to develop SNPs from the genomic region due to their wide distribution in the genome. In the past five years, four sets of tobacco SNPs have been developed in tobacco through different varieties, sequencing populations, and sequencing methods; they have been integrated into the 24 LGs developed by Bindler et al., and new genetic linkage maps have been drawn (Cheng et al., 2019; Gong et al., 2016; Thimmegowda et al., 2018; Xiao et al., 2015). These will be good tools for the fine mapping of *YL1* in future.

In view of the important role of plant hormones in leaf senescence, we attempted to explore the relationship between the target gene and hormone pathways by the hormone treatment of detached leaves, and to provide potential methods for screening candidate genes in the future. Ethylene is one of the most important plant hormones that induce leaf senescence. Some ethylene receptors, such as EIN2, a core member of ethylene signaling pathway, play a role in other hormone signaling and act as a node in the interaction between plant hormone signals (Jibran et al., 2013). Jasmonic acid induces the expression of key enzymes involved in chlorophyll degradation, so it has long been thought to accelerate leaf senescence (Reinbothe et al., 2009). In addition, jasmonic acid regulates leaf senescence by interacting with other hormonal signaling pathways such as salicylic acid, auxin, and ethylene (Hu et al., 2017; Jiang et al., 2014; Xie et al., 2014). When HD and *yl1* were treated with ACC and MeJA, both of them had accelerated leaf senescence. It is clearly that *yl1* is more sensitive to

ACC and MeJA. However, we did not find that *yl1* showed more interesting responses to ethylene and jasmonic acid.

In summary, we have identified a novel tobacco EMS mutagenesis induced premature leaf senescence mutant controlled by a single recessive gene and have preliminarily mapped the target gene to an interval between PT53066 and PT60305 on the tobacco LG11, with the genetic distance of 1.08 and 3.51 cM, respectively. This provides good genetic material. Further cloning and characterization of the target gene will lead to a better understanding of the molecular mechanisms underlining leaf senescence in tobacco and provide useful information in the genetic manipulation of tobacco leaf senescence.

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4

Characterization and Mapping of a Novel Premature Leaf Senescence Mutant *yellow leaf 2* in Common Tobacco (*Nicotiana tabacum* L.)

Characterization and Mapping of a Novel Premature Leaf Senescence Mutant *yellow leaf 2* in Common Tobacco (*Nicotiana tabacum* L.)

Abstract: Leaf senescence is the final stage of plant growth and development, and has an important impact on crop yield and quality. In this study, we characterized a tobacco (*Nicotiana tabacum* L.) premature leaf senescence mutant *yellow leaf 2* (*yl2*) obtained by mutagenesis of ethyl methane sulfonate (EMS). Genetic analysis indicated that the phenotype of *yl2* is controlled by a single recessive gene. The F1 population plants crossed with tobacco mutants *yl1* and *yl2* showed the same phenotype as the wild type, indicating that *YL1* and *YL2* were two different genes. Using MutMap combined with whole genome sequencing (WGS), we identified 18 candidate single nucleotide polymorphisms (SNPs). However, the validation results of the candidate SNPs indicated that the 18 SNPs were independent of the *yl2* phenotype. Using simple sequence repeat (SSR) markers, the target gene was mapped to one end of tobacco linkage group 24 (LG24). The genetic distance between target gene to the polymorphic SSR marker PT60487 was 14.2 cM. The treatments of exogenous plant hormone on detached leaves showed that *yl2* was more sensitive to ABA and JA than the wild type. These results laid the foundation for further cloning of target gene and study its function. It also helps to reveal the molecular regulation mechanism of tobacco leaf senescence.

Key words: tobacco; EMS mutagenesis; premature leaf senescence; whole genome sequencing; SNP; SSR; plant hormone

1. Introduction

Leaves are important organs of plants for photosynthesis and synthesis of organic matter. Leaf senescence is the last stage of the leaf's life course. It is an important part of the plant senescence process and is of vital importance to the overall development of plants. Leaf senescence is a programmed cell death. The main function of leaf senescence is to degrade intracellular substances in senescent leaves and transfer the products as nutrients to young and reproductive organs to complete the plant's life cycle (Gan and Amasino, 1997). Many important agronomic traits, such as seed quantity and quality, fruit ripening, and post-harvest storage time, are affected by the senescence process (Miao and Zentgraf, 2010). Premature leaf senescence can lead to a reduction in crop yield, and a proper delay in leaf senescence can increase crop yields (Guo and Gan, 2014). A series of physiological and biochemical reactions such as leaf yellowing caused by chlorophyll degradation, photosynthesis decline and biomacromolecules degradation are the main manifestations of leaf senescence. At the molecular level, leaf senescence is mainly manifested by a decrease in total RNA content in leaves, and a number of senescence-associated genes (SAGs) were up-regulated during leaf senescence (Guo and Gan, 2005; Lim et al., 2007). Leaf senescence is closely related to plant age, plant reproductive growth and plant hormone levels, and also can be induced and regulated by a series of external environmental factors such as drought, extreme temperature, and pathogen infection (Gan and Amasino, 1997). Leaf senescence has important biological significance, but the current understanding of its mechanism is still limited. Studying the molecular mechanism of leaf senescence not only helps to further elucidate the regulation mechanism of senescence during plant growth and development, but also provides important theoretical basis for improving agronomic traits such as crop quality and yield.

Tobacco (*Nicotiana tabacum* L.) is a widely grown crop in the world. Due to the ease of operation of tobacco transgenes, it has also become an important model plant in the field of plant genetics research (Masclaux et al., 2000). In tobacco production, yellowed leaves were harvested for processing tobacco products. The yellowing of the leaves or the maturity of the tobacco leaves have a decisive influence on the formation of the main quality factors such as appearance quality, physical properties, chemical composition, smoke characteristics and cigarette safety of the tobacco products by affecting the curing process after harvesting. For example, the degradation of chlorophyll affects the color of tobacco leaves, and the degree of collapse of cell wall structure affects the looseness of tobacco leaf structure. The process of nutrient remobilization not only affects the main chemical components of tobacco leaves, but also changes the metabolism of harmful components of tobacco leaves, thus affecting the safety of flue-cured tobacco. Therefore, tobacco leaf senescence is critical to the yield and quality of tobacco products. However, compared to other model plants, such as *Arabidopsis* and rice, the study on the molecular mechanism of tobacco leaf senescence is almost blank. Most of tobacco senescence-related studies have used tobacco as a place for expression of exogenous genes, thus validating the role of

exogenous genes in leaf senescence regulation (See Chapter 1, 2.3). Recent studies have revealed the dynamic changes in metabolites during tobacco leaf senescence (Li et al., 2016; Li et al., 2017; Zhao et al., 2018a). Studies have also been conducted on transcriptome analysis of tobacco leaves from different senescence periods, and some differentially expressed genes have been obtained (Zhao et al., 2018b). But few genes involved in regulation of tobacco leaf senescence have been identified.

Using EMS mutants and map-based cloning technology to locate and clone functional genes is a classic approach to forward genetics. In the past decade, simple sequence repeat (SSR) molecular markers have been widely used in crop genetic map construction and map-based cloning. However, due to the huge tobacco genome and more than 70% repetitive sequences (Renny-Byfield et al., 2011), the extremely narrow genetic diversity severely limits the use of SSR in tobacco. Moreover, the traditional map-based cloning requires a large-scale mapping population to overcome the low polymorphism of mapping parents and absence of variants. For tobacco, such a large annual plant, it also has the disadvantages of high land cost, long time consumption and low efficiency. The development of high-throughput sequencing technologies has made it possible to quickly find target genes associated with mutant traits.

In this study, we identified a tobacco mutant *yellow leaf 2* (*yl2*) with a premature yellowing phenotype of the leaves. Measurement of the senescence related parameters confirmed that it was a premature leaf senescence mutant. Genetic analysis indicated that the phenotype was controlled by a single recessive gene. The F1 population of *yl1* crossed with *yl2* showed the same phenotype with the wild type, indicated that *YL1* and *YL2* were different genes. The BC2F2 sequencing population was created according to the MutMap (Abe et al., 2012). Whole genome sequencing of mutants and wild type were performed using Illumina platform, and 18 candidate SNPs that may lead to the mutant phenotype were predicted. However, the verification results ruled out the possibility of 18 SNPs. The location of the target gene was preliminary located on one end of tobacco linkage group 24 (LG24) using SSR molecular markers.

2. Materials and Methods

2.1. Plant Materials

The *yl2* was identified from the chemical mutagen EMS induced library for the common tobacco variety Zhongyan100 (ZY100) (Wang et al., 2017), which was treated following the described method. Briefly, seeds of ZY100 were soaked in 0.6% EMS solution for 16 h at room temperature under gentle shaking, rinsed thoroughly and sown M1 seeds in greenhouse. After two months, M1 plants were transplanted into the field to harvest M2 seeds separately. The *yl2* was identified from one of the M2 lines and further verified in M3 and M4 generation. All the materials were grown in the field in Zhucheng City, Shandong Province, China.

2.2. Sampling for Measurement of Leaf Senescence Related Physiological Parameters and RNA Extraction

Plants of ZY100 and *yl2* were grown in the field in Zhucheng City, Shandong Province, China, during the 2018 growing season. Middle position leaves of each plant were collected at different developmental stages. The collection of the leaf samples was done at five developmental stages during the growing season: 35, 50, 65, 80 and 95 days after transplanting (DAT). For each sample, one leaf from the middle position was collected from three plants randomly. Each leaf was divided into two parts along the main vein. One half was wrapped in aluminum foil, immediately immersed into liquid nitrogen, and stored at -80 °C until it was used for extracting the RNA and soluble protein, while the other half was put in sample bag and stored in the ice box for chlorophyll content measurements.

2.3. Measurement of Leaf Senescence Related Physiological Parameters

Same with Chapter 3, 2.3.

2.4. RNA Extraction and qRT-PCR

Same with Chapter 3, 2.4.

2.5. Populations for Genetic Analysis and Map-based Cloning

Two wild type tobacco varieties, Cuibi1 (CB1) and Gexin3 (GX3) were crossed with *yl2* to produce genetic populations. The F1 (CB1 × *yl2*; GX3 × *yl2*) plants were self-pollinated to produce F2 segregating populations separately. Leaf senescence phenotypes were investigated by visual observation, and the segregations were evaluated for the goodness of fit to the expected ratios using chi-squared test.

2.6. Genetic Populations for Whole Genome Sequencing

The construction of populations for whole genome sequencing (WGS) was shown in Figure 4-1 A. *yl2* was crossed with its wild type parent ZY100 to produce F1 (*yl2* × ZY100). The F1 were backcross with ZY100 twice to produce BC2F1. The BC2F1 plants were self-pollinated to produce BC2F2 populations separately. The BC2F2 population with character segregation was selected for WGS.

2.7. The Construction of DNA Library, Whole Genome Sequencing, and Data Assembly

In the BC2F2 population with character separation, 20 plants with leaf premature senescence phenotype were selected and their leaves were sampled along with leaves from the wild type parent ZY100. Genomic DNA were separately extracted using DNeasy plant mini Kit (Qiagen, 69104, CA, USA). A DNA pool (hereafter, Sample 20) were constructed by mixing an equal amount of DNA from 20 BC2F2 recessive individuals. Sample 20 and ZY100 DNA were randomly sheared, 500 bp DNA were isolated, adapters were ligated to the end to track the samples, and sequenced using

the Illumina Hiseq2000. Taking tobacco variety Honghuadajinyuan (HD) genome in unpublished China tobacco genome database as a reference genome (Figure 4-1 B). The sequenced reads were aligned with the reference genome sequence using SOAP2 software (Li et al., 2009). Based on the alignment results, the sequencing depth and coverage relative to the reference genome were calculated.

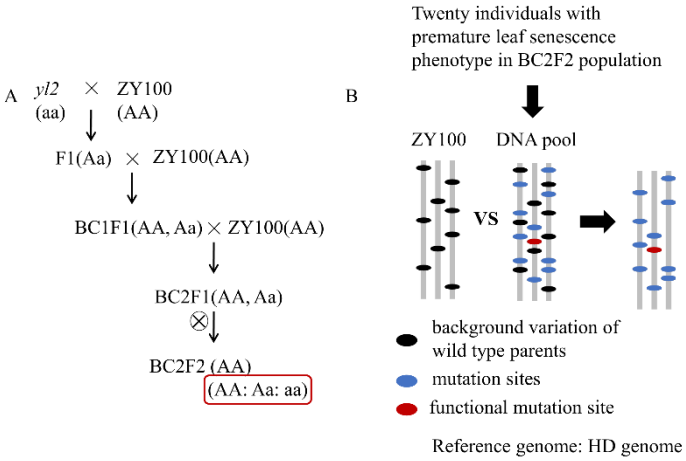

Figure 4-1 Schematic diagram of population construction and SNP screening

2.8. Bioinformatics analysis

The sequenced reads were compared with the reference genome sequence by soap2 software. According to the comparison results, the sequencing depth and coverage relative to the reference genome were calculated. The SNPs and indels of the measured genome were detected by SOAPsnp and SOAPindel software respectively.

2.9. Detection of SNPs

The raw data of Sample 20 and ZY100 sequencing were mapped to the HD pseudo chromosome using the Burrows-Wheeler Alignment (BWA) algorithm to ensure the single nucleotide polymorphisms (SNPs) between Sample 20 and ZY100 were not due to misassembled contigs. The SNPs between Sample 20 and ZY100 were detected by SOAPsnp software. SNPs were filtered by the quality value greater than 20, index greater than 0.9 given by SOAPsnp. Unique SNPs with read depth greater than 10 were considered reliable. SNPs with homozygous parent genotype and the non-synonymous changes in CDS regions were chosen for further analysis.

2.10. SNPs validation using PCR and Sanger sequencing

To validate the accuracy of the predicted SNPs, specific primers for each SNPs were designed. PCR amplification were carried out using genomic DNA of *yl2*, and genomic DNA of ZY100 and 20 BC2F2 recessive individuals used for WGS as

templates, respectively. PCR was performed in a Veriti 96-Well Thermal Cycler (ABI, Waltham, USA). Each reaction contained 25 μL of 2 x PrimeSTAR Max Premix (TAKARA, R045, Dalian, China), 50 ng template DNA, and 1 μL of 5 μM forward and reverse primers in a volume of 50 μL. The PCR program was as follows: pre-denaturation at 95°C for 5 min, followed by 30 cycles of 30 s at 95°C, 30 s at 50-60°C (the annealing temperature of different primer pairs), and 1 min at 72°C, and then a final extension at 72°C for 5 min. The specific PCR products were sequenced and then analyzed by DNAMAN 5.0.

2.11. DNA Extraction and SSR Analysis

See Chapter 3, 2.5.

2.12. Linkage Map and Genetic Distance

The F2 population (CB1 × *yl2*; GX3 × *yl2*) was used to obtain the SSR markers linked to *YL2*. The polymorphic SSR markers between CB1, GX3 and *yl2* were selected and used to screen 46 recessive individuals of the F2 population with premature leaf senescence phenotype, as well as their parents. The linkage map based on the segregations between *YL2* and the markers was constructed by QTL Ici Mapping software V4.0 (Li et al., 2008), and the threshold value of the logarithm of odd (LOD) was set at 3.0. Genetic distances were calculated and presented in Kosambi centiMorgans (cM).

2.13. Plant Hormone Treatments

The middle leaves of plants grown in greenhouse 50 DAT were used for plant hormone treatments. Detached leaves were cut into small pieces ~1.5 cm in diameter and floated on incubation buffer (1/2MS, 3 mM MES, pH5.8) with and without hormone in Petri dishes with filter paper. The samples were incubated at 28 ℃ under 24 h light in a plant chamber for ten days. Plant hormones were diluted to working concentration using incubation buffer. The working concentrations of plant hormones were as follows: 100 μM for abscisic acid (ABA), 100 μM for 1-aminocyclopropanel-1-carboxylic acid (ACC), 50 μM for methyl jasmonate (MeJA), and 100 μM for salicylic acid (SA) (Zhang and Guo, 2018).

3 Results and Discussion

3.1. The yl2 Plants Exhibit Premature Leaf Senescence

Compared with ZY100, *yl2* plants showed a significant premature leaf senescence phenotype from about 65 days after transplanting (DAT), and throughout the subsequent developmental stage (Figure 4-2). No significant difference of leaf color was observed between ZY100 and *yl2* 50 DAT or at an earlier development stage (Figure 4-2 A, B). The yellowing of the bottom leaves appeared in the *yl2* plants around 50 DAT, and the leaf color was lighter than ZY100, but the difference was not significant (Figure 4-2 B). At 65 DAT, the plants are still in a period of vigorous

vegetative growth, and flower buds have not yet formed. The lower leaves of *yl2* have shown obvious yellow, while the ZY100 plants remain green (Figure 4-2 C). At the flowering stage, about 80 DAT, the middle leaves of *yl2* have completely turned yellow, while the lower leaves of ZY100 remain green partially (Figure 4-2 D). The difference in leaf yellowing was more profound at 95 DAT. The *yl2* plants became completely yellow, whereas the majority of leaves in the ZY100 plants still retained their green color (Figure 4-2 E). These results indicated that the initiation of leaf senescence of *yl2* was earlier than ZY100. The *yl2* exhibited a phenotype of premature leaf senescence.

3.2. Alterations of Leaf Senescence Related Parameters

Leaf yellowing caused by chlorophyll degradation is a typical phenotype of leaf senescence, accompanied by degradation of biological macromolecules such as proteins and nucleic acid (Gan and Amasino, 1997). In order to further characterize the phenotype of *yl2*, we determined the total chlorophyll content (Chls), soluble protein, and the expression of senescence marker genes in *yl2* in comparison with ZY100 (Figure 4-3). Consistent with the premature leaf yellowing phenotype, Chls and soluble protein of the middle leaves in *yl2* were lower than in counterpart leaves of the ZY100 plants at the same developmental stages (Figure 4-3 A, B). The difference in Chls between *yl2* and ZY100 was more significant than the difference in macroscopic phenotype between them (Figure 4-3 A). The contents of Chls and soluble protein decreased rapidly in *yl2* from 65 DAT, whereas the ZY100 plants did not start losing chlorophyll until 80 DAT (Figure 4-3 A, B). At 65 DAT, Chls and soluble protein in the upper, middle and lower leaves were significantly lower in *yl2* than in the ZY100 plants (Figure 4-3 E, F). The expression of the marker genes *SAG12* and *RBCS* of the individual samples was determined. As shown in Figure 4-3 C and D, in *yl2*, expression of the senescence marker gene *SAG12* was first detected at 50 DAT and the photosynthetic gene *RBCS* became undetectable at 80 DAT. For ZY100 plants, *SAG12* expression was first detected at 65 DAT and *RBCS* expression became undetectable at 95 DAT. Significantly lower *SAG12* and higher *RBCS* expression were detected at different leaf positions of 65 DAT plants (Figure 4-3 G, H). The premature leaf senescence phenotype of *yl2* was well reflected in the expression patterns of *SAG12* and *RBCS*.

3.3. The premature leaf senescence of yl2 was controlled by a single recessive gene

To analyze the inheritance of *yl2* phenotype, two wild type tobacco varieties Cuibi1 (CB1) and Gexin3 (GX3) were crossed with *yl2*. All the F1 plants showed a normal green phenotype similar to wild type ZY100. 51 and 50 premature leaf senescence individuals were observed from the F2 populations of 198 and 203 plants separately, fitted to the expected segregation ratio of 3:1 (Table 4-1). These results indicated that

the premature leaf senescence phenotype of *yl2* was controlled by a single recessive gene.

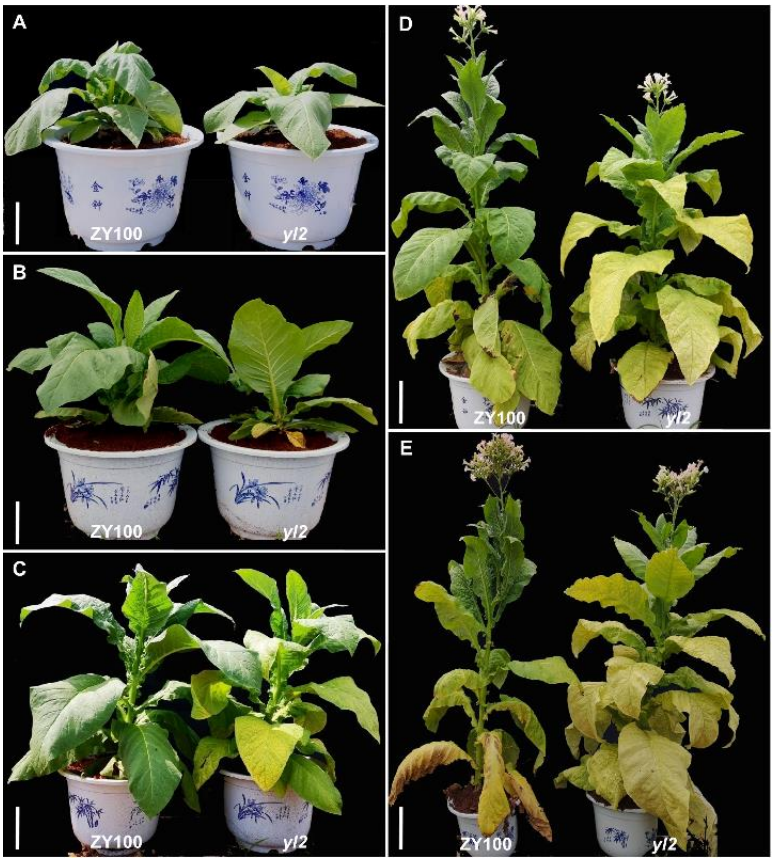

Figure 4-2 Phenotypic comparison between Zhongyang100 (ZY100) and *yl2*. The phenotype of ZY100 and *yl2* plants grown in the field. (A) 35 days after transplanting (DAT), bar 20 cm; (B) 50 DAT, bar 20 cm; (C) 65 DAT, bar 30 cm; (D) 80 DAT, bar 35 cm; and (E) 95 DAT, bar 40 cm.

This conclusion was demonstrated again by the segregation of the BC2F2 population for whole genome sequencing (WGS). To construct a population for WGS, single base mutations in *yl2* that were not associated with the leaf senescence phenotype should be removed as much as possible. To this end, *yl2* was backcrossed with its wild type background parent ZY100 several times (Figure 4-1). 54 premature leaf senescence individuals were observed from the BC2F2 population of 206 plants, fitted to the expected segregation ratio of 3:1 (Table 4-1).

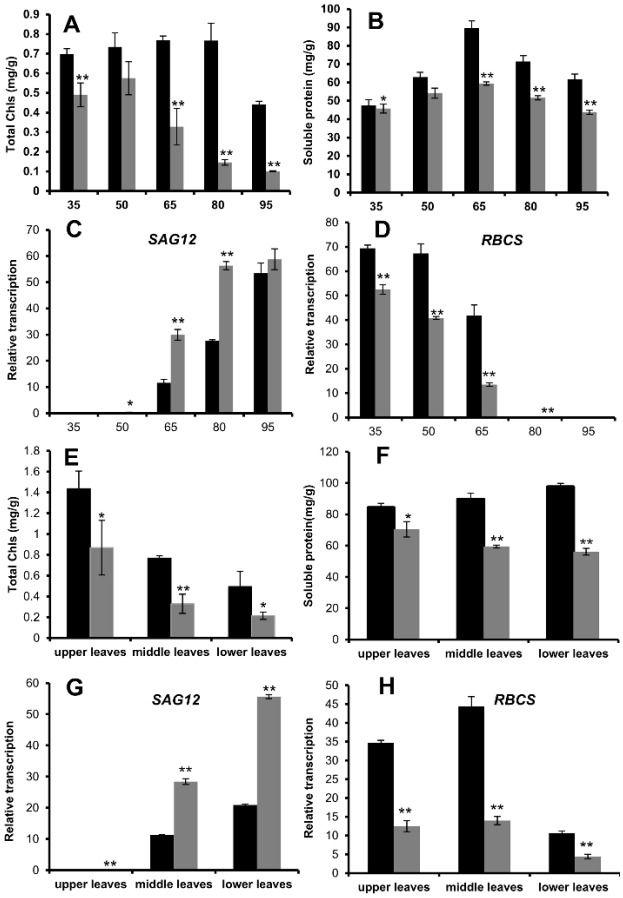

Figure 4-3 Leaf senescence related parameters of ZY100 and *yl2*. Black and grey column indicated ZY100 and *yl2*, respectively. (A) Total chlorophyll (Chls), (B) soluble protein, relative expression of (C) *SAG12* and (D) *RBCS* in middle leaves of the same leaf position of ZY100 and *yl2* at 35, 50, 65, 80 and 95 DAT. (E) Total chlorophyll (Chls), (F) soluble protein, relative expression of (G) *SAG12* and (H) *RBCS* in upper, middle, and lower leaves of the same leaf position of ZY100 and *yl2* at 65 DAT, respectively. Values are mean ± SD of three individual replicates. * and ** represent significant difference determined by the Student's t test at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Table 4-1 Genetic analysis of *yl2*

Population type	Cross	Total	Wild Type	Mutant Type	Segregation ratio	χ^{2} [1]
F2	CB1 × *yl2*	198	147	51	2.882	0.061
F2	GX3 × *yl2*	203	153	50	3.060	0.015
BC2F2*	((*yl2* × ZY100) × ZY100) × ZY100	206	152	54	2.815	0.162

CB1: Cuibi1, tobacco variety; GX3: Gexin3, tobacco variety; ZY100: Zhongyan100, tobacco variety; * BC2F2 was a population constructed for whole genome sequence. There are two types of BC2F2 population, with or without phenotypic segregated population (Figure 4-1). Here the table presents the segregated ratio of phenotypic segregated population. [1] Value for significant at 0.05 and *df* = 1 is 3.841.

3.4. Sequences assembly and SNPs detection

Two samples, including wild type ZY100, and 20 BC2F2 recessive individuals Sample 20, were sequenced. Taking tobacco variety Honghuadajinyuan (HD) genome in unpublished China tobacco genome database as a reference genome. The HD genome size is 4,416,194,378 bp, and the effective genome size without N is 4,317,259,121 bp. The 270,096,703,200 bp raw data (Table 4-2) were filtered to trim the low-quality sequences or adapter sequences of both ends, obtained 264,723,201,500 bp clean data (Table 4-3). The genome coverage rate of ZY100 and Sample 20 are 95.663% and 95.6541%, respectively. The mapped reads rate of ZY100 and Sample 20 are 98.06% and 97.35% (Table 4-4).

Table 4-2 Data production (Raw Data)

Sample Name	GC rate (%)	Q20 rate (%)	Reads	Bases
ZY100	41.25	98.23	900,178,288	135,026,743,200
Sample 20	41.54	98.06	900,466,400	135,069,960,000

ZY100: Zhongyan100, tobacco variety, genome DNA; Sample 20: DNA pool of 20 BC2F2 recessive individuals with premature leaf senescence phenotype; Q20 rate: The ratio of bases with quality value greater than 20 in all reads to total read length.

Table 4-3 Data production (Clean Data)

Sample Name	GC rate (%)	Q20 rate (%)	Reads	Bases
ZY100	41.55	98.45	888,846,714	133,327,007,100
Sample 20	41.43	98.41	882,641,296	132,396,194,400

ZY100: wild type parent genome DNA; Sample 20: DNA pool of 20 BC2F2 recessive individuals with premature leaf senescence phenotype; Q20 rate: The ratio of bases with quality value greater than 20 in all reads to total read length.

Table 4-4 Comparison result

Sample Name	Coverage rate (%)	Mapped reads rate (%)
ZY100	95.663	98.06
Sample 20	95.6541	97.35

ZY100: wild type parent genome DNA; Sample 20: DNA pool of 20 BC2F2 recessive individuals with premature leaf senescence phenotype.

3.5. Indels and SNPs detection

To identify indels, BWA alignments were used to obtain the results in BAM format. After identifying indels with GATK-Unified Genotyper and filtering low-quality loci, a high quality indel variation set was obtained. The statistical results of indels in ZY100 and Sample 20 are shown in Table 4-5.

Table 4-5 Statistic of indels

Sample Name	insert	insert rate (%)	deletion	deletion rate (%)
ZY100	77,873	30.116	180,703	69.884
Sample 20	70,224	29.131	170,839	70.869

ZY100: wild type parent genome DNA; Sample 20: DNA pool of 20 BC2F2 recessive individuals with premature leaf senescence phenotype.

SNPs were filtered by the quality value greater than 20, index greater than 0.9 given by SOAPsnp. The Q20 ratio of each sample was greater than 98%, indicating high-quality sequence. SNPs on the whole genome of the two samples were identified separately (Table 4-6). Compared with the reference genome, 3,868,622 SNPs in ZY100 and 3,496,832 SNPs in Sample 20 were detected, respectively. Among these, only 0.70% of ZY100 SNPs and 0.72% of Sample 20 SNPs were located on coding sequence (CDS). SNPs with quality value greater than 20 were taken for subsequent analysis. Unique SNPs with read depth greater than 10 were considered reliable. SNPs with homozygous parent genotype and the non-synonymous changes in CDS regions were chosen for further analysis. The SNPs in Sample 20 with mutant type G to A or C to T, and the site was homozygous in wild type, and the index greater than 0.9 were taken. Finally, 18 candidate SNPs located on CDS involving 17 genes were taken (Table 4-7), 14 of which were non-synonymous mutations, and four of which were synonymous mutations. The homologous genes in *Arabidopsis* of the 17 genes were analyzed. None of them had been reported related to leaf senescence.

Table 4-6 Statistic of SNPs

Sample Name	Total SNP	Homozygous	Heterozygous	Synonymous CDS	Non-synonymous CDS
ZY100	3,868,622	1,300,966 (33.63%)	2,567,656 (66.37%)	11,790 (0.30%)	15,347 (0.40%)
Sample 20	3,496,832	1,141,391 (32.64%)	2,355,441 (67.36%)	11,012 (0.31%)	14,411 (0.41%)

CDS: coding sequence.

Table 4-7 Statistic of candidate SNPs in Sample 20

No.	Chr [1]	Location [1]	Type	Amino acid Change	Tobacco Gene [1]	Homologous genes in *Arabidopsis*	Protein Name	Gene Function
SNP1	chr2	141133223	G->A	D->N	Ntab0499100.1	AT3G42170	DAYSLEEPER	development
SNP2	chr2	144623236	C->T	Q->U	Ntab0148250.1	None	None	None
SNP3	chr2	146694972	G->A	R->Q	Ntab0148790.1	AT4G30100	P-loop	None
SNP4	chr2	148417217	C->T	R->C	Ntab0707780.1	AT5G65683	Zinc finger	None
SNP5	chr5	45696504	C->T	S->L	Ntab0006490.1	AT1G71730	None	None
SNP6	chr5	42181447	G->A	G->R	Ntab0126350.1	AT1G51580	RNA-binding	None
SNP7	chr5	39210895	G->A	P->P	Ntab0322910.1	AT4G24380	dihydrofolate reductase	None
SNP8	chr5	16022542	C->T	L->F	Ntab0871680.1	AT1G71810	Protein kinase	None
SNP9	chr5	19889973	C->T	A->V	Ntab0941330.1	AT5G07900	Mitochondrial transcription termination factor	None
SNP10	chr8	57493297	C->T	A->V	Ntab0583840.1	AT1G28570	SGNH hydrolase-type esterase	None
SNP11	chr9	46336532	C->T	S->L	Ntab0464420.1	AT2G47820	arginine-glutamic acid dipeptide repeat	None
SNP12	chr11	45575938	C->T	S->F	Ntab0064080.1	AT5G54660	HSP20-like chaperones	None
SNP13	chr11	40177186	C->T	P->P	Ntab0438040.1	None	None	None
SNP14	chr11	40177253	G->A	E->K	Ntab0438040.1	None	None	None

SNP15	chr16	74215465	G->A	L->L	Ntab0163950.1	AT5G37710	alpha/beta-Hydrolases	calmodulin binding
SNP16	chr19	53842783	G->A	S->N	Ntab0941490.1	AT3G04030	Homeodomain-like	None
SNP17	sf583	341022	G->A	V->I	Ntab0771730.1	AT5G55820	inner centromere protein	seed development
SNP18	sf1301	826066	G->A	E->E	Ntab0116670.1	AT1G27760	similarity to human interferon-related developmental regulator	salt tolerance

Chr: Chromosome; sf: scaffold; [1] The chromosome, scaffold, location and accession number were obtained from the China Tobacco Genome Database (unpublished data).

3.6. SNPs validation

To verify the accuracy of candidate SNPs identified by bioinformatic analysis, SNPs were verified by PCR amplification. The primer sequences for each SNP are shown in Table 4-8. Due to the presence of highly homologous genes, the specific primers were unable to design for SNP3, SNP15 and SNP18. PCR amplifications were performed using genomic DNA of *yl2*, and genomic DNA of ZY100 and 20 BC2F2 recessive individuals used for sequencing as templates, respectively, and the specific amplification products were sequenced (Table 4-9). Of the 15 SNPs verified, only SNP10 and SNP16 were consistent with the WGS results. For SNP2, SNP4 and SNP13, they were identical in parents and 20 offspring. For the other 10 SNPs, although 20 offspring were different from ZY100 at the SNP positions, no mutations were shown in *yl2*. Even in the small population of 20 offspring, the sequencing results showed that not all individuals were homozygous at SNP10 and SNP16 positions. It can be speculated that SNP10 or SNP16 were not the SNP that cause the leaf senescence phenotype of *yl2*. From this we initially believed that the 18 candidate SNPs located on CDS obtained by WGS were not point mutations leading to *yl2* phenotype.

Based on the linkage frequency of SNP10 and SNP16 in 20 offspring, we can preliminarily speculate that SNP16 located on chromosome 19 may be the closest mutation to the target gene.

Table 4-8 The primer sequences for SNPs validation

No.	Forward Primer Sequence [1]	Reverse Primer Sequence [1]
SNP1	CACGCAGAGAACTTGCTCATGC	GTTGTAATAGCCATGAACCC
SNP2	GCAGGGAGTAAAGTGAGTAC	CAGGTAACAATGGCTGACCG
SNP3	-	-
SNP4	GAAGGTTGGTGACCCATTC	GAACTGCACCTAAGCTCACC
SNP5	GCTCAGCTTCCGGGTCACGGCC	CACTCCTTTTTCACTCTCTGATAG
SNP6	GAGCGTAATGTAATTTAACACGAGC	CCAAGACATACATATATCATCAG
SNP7	CTATTGGTATATTTTAACG	TGTTATTGTTGTTAACGACATTGA
SNP8	CACACTCCAGATGTCCAGTC	TAATATTAAGAAAGGACCTTGCT
SNP9	GGAATGCATAACGTCTTGTTTC	CGAAACAGAGCTCGTACGTCCG
SNP10	AACAGGTGATGAAATACGGG	GTTCCACAAACTCTATCCTCG
SNP11	GCCAAGAAAAGTGATAAAAGCCCAT	ATCCTCAGACGAAGCTATCT
SNP12	GAAGTTCAATTTGAAGAAGCAAC	GTGGGTACACCACTGCCTGCAAC
SNP13	CACACATCTGCGTCAACAC	CAGACTGCTTGTCGTCGTT
SNP14	CACACATCTGCGTCAACAC	CAGACTGCTTGTCGTCGTT
SNP15	-	-
SNP16	GTTTCATTTAAGCAGCTGCC	GGTTTAAATGAAGGATCAAATGT
SNP17	CAATCGTAATGATCTAGACCG	GTATGACTTTTCGCACAG
SNP18	-	-

[1] The chromosome, location and sequence were obtained from the China Tobacco Genome Database (unpublished data); -: Due to the high similarity between homologous genes in tobacco, specific primers for this SNP cannot be designed.

Table 4-9 SNPs validation

No.	Type	*yl2*	ZY100	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	**P11**	**P12**	**P13**	**P14**	**P15**	**P16**	**P17**	**P18**	**P19**	**P20**
SNP1	G->A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
SNP2	C->T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
SNP3	G->A											-											
SNP4	C->T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
SNP5	C->T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
SNP6	G->A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
SNP7	G->A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
SNP8	C->T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
SNP9	C->T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
SNP10	C->T	T	C	C/T	C	T	T	C/T	C	C/T	T	T	T	T	C	C/T	C/T	C	T	C/T	C/T	C/T	C
SNP11	C->T	C	C	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T
SNP12	C->T	C	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
SNP13	C->T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
SNP14	G->A	G	G	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A
SNP15	G->A											-											
SNP16	G->A	A	G	A	A	A	A	A	A	A	A	A	A	G/A	A	A	A	A	A	A	A	A	A
SNP17	G->A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
SNP18	G->A											-											

P1-P20: 20 BC2F2 recessive individuals. -: Due to the high similarity between homologous genes in tobacco, specific primers for this SNP cannot be designed.

3.7. Preliminary Mapping of YL2

The F2 population developed between CB1, GX3 and *yl2* was used to map *YL2*. Based on the hypothesis that *YL2* was closest to SNP16 located on chromosome 19, the polymorphism of 53 SSR markers by Bindler et al. (Bindler et al., 2011; Bindler et al., 2007) and 3 SSR markers by Tong et al. (Tong et al., 2012) randomly distributed on linkage group 24 (LG24) were analyzed using the parental *yl2*, CB1 and GX3 (Table 4-7, Figure 4-4, 4-5). Six SSR markers (24-27, 24-34, 24-44, 24-53, X24-5, X24-12) with polymorphism between CB1 and *yl2* were selected to screen 46 recessive individuals from the F2 population of CB1 and *yl2*. Nine SSR markers (24-15, 24-25, 24-27, 24-29, 24-30, 24-44, 24-53, X24-5, X24-12) with polymorphism between GX3 and *yl2* were selected to screen 46 recessive individuals from the F2 population of GX3 and *yl2*. The isolation of each SSR marker and *YL2* was converted into genetic distance by the QTL Ici Mapping software (V4.0). The results showed that *YL2* was located on one end of LG24, with a distance of 14.2 cM from the polymorphic marker PT60487 (Figure 4-7). There are 17 additional SSR markers within the mapping interval of the *YL2*. Except only one marker PT60410a was not among the randomly selected SSR markers described above, another 16 SSR markers have been shown have no polymorphism between wild type and *yl2* in the above experiments. Moreover, the forward and reverse sequences of PT60487 primers were used in BLAST search against all the currently available genome sequences of *Nicotiana* species, including *N. tabacum* cultivars K326 (China Tobacco Genome Database), TN90 and Bsama Xanthi (Sierro et al., 2014), *N. benthamiana* (Bombarely et al., 2012), and even the diploid ancestor *N. sylvestris* and *N. tomentosiformis* (Sierro et al., 2013). We did not find any match between PT60487 primers and the aforementioned genomes using the BLAST search. Then, the forward and reverse sequences of another 16 SSR markers were used in BLAST search against all the available genome sequences of *Nicotiana* species, same with PT60487. We found that only one SSR marker PT53716 was physically located on Ntab0912020, a gene encoding GDSL esterase, similar to At2G38180. However, there is no research evidence that it is related to the regulation of leaf senescence. We were also unable to determine the relative location and genetic distance between PT53716 and *YL2*. SNPs located on chromosome 19, which were obtained by WGS, will be developed as new molecular markers. Subsequently, more SNPs will be used to narrow down the interval and fine mapping of *YL2*.

Table 4-10 56 SSR randomly selected markers on linkage group 24

No.	SSR Marker	No.	SSR Marker	No.	SSR Marker
24-1	PT51967	24-20	PT60877	24-39	PT61631
24-2	PT51349	24-21	PT55218	24-40	PT54206
24-3	PT51934	24-22	PT61457	24-41	PT50736
24-4	PT54559	24-23	PT54881	24-42	PT61456
24-5	PT61280	24-24	PT61308	24-43	PT30327

24-6	PT55251	24-25	PT20163	24-44	PT61494
24-7	PT52080	24-26	PT51878	24-45	PT61649
24-8	PT54601	24-27	PT60487	24-46	PT51339
24-9	PT52059	24-28	PT51212	24-47	PT61345
24-10	PT53701	24-29	PT52944	24-48	PT54107
24-11	PT50083	24-30	PT52828	24-49	PT20168
24-12	PT61583	24-31	PT52988	24-50	PT53266
24-13	PT52640	24-32	PT53530	24-51	PT30375
24-14	PT30067	24-33	PT60373	24-52	PT53716
24-15	PT50541	24-34	PT60792	24-53	PT60870
24-16	PT53089	24-35	PT52610	X24-4	TM10641a
24-17	PT51624	24-36	PT53522	X24-5	TME2076
24-18	PT50226	24-37	PT60540	X24-12	TM11187
24-19	PT50969	24-38	PT51357		

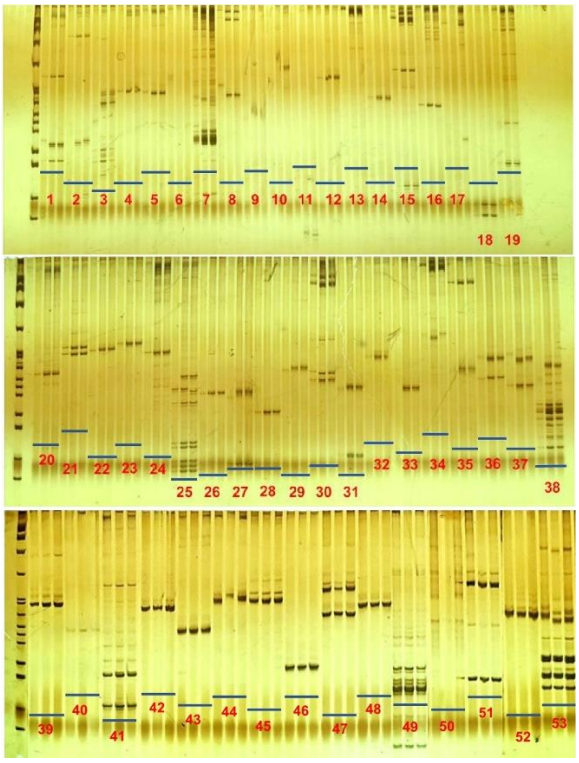

Figure 4-4 Polymorphism analysis of SSR markers from Bindler et al. (Bindler et al., 2011; Bindler et al., 2007). The numbers correspond to SSR markers 24-1 to 24-53, respectively (Table 4-7). For each SSR marker corresponds to 3 lanes, from left to right are *yl2*, CB1 and GX3.

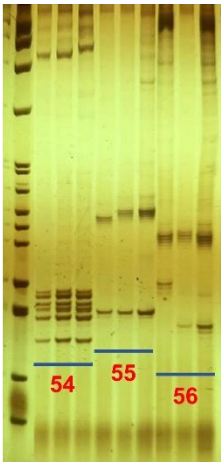

Figure 4-5 Polymorphism analysis of SSR markers from Tong et al. (Tong et al., 2012). The numbers correspond to SSR markers X24-4, X24-5 and X24-12, respectively (Table 4-7). For each SSR marker corresponds to 3 lanes, from left to right are *yl2*, CB1 and GX3.

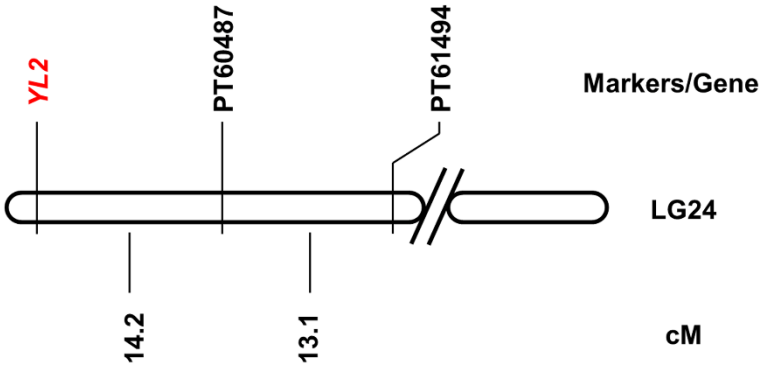

Figure 4-6 Preliminary mapping of *YL2*. Genetic map of *YL2* and SSR markers on tobacco linkage group 24 (LG24). cM indicates centimorgan.

3.8. Plant Hormone Treatments

To investigate whether *YL2* was involved in the regulation of hormone signaling pathways in leaf senescence, four plant hormones that promote leaf senescence were used to do treatments in ZY100 and *yl2* detached leaf discs (Figure 4-7). Based on visual observation, the results showed that without plant hormone treatment, the yellowing of the *yl2* leaves was faster than that of ZY100 (Figure 4-8 Control). Treatments of abscisic acid (ABA) and methyl jasmonate (MeJA) accelerated yellowing of leaf discs. Treatments of 1 - aminocyclopropane - 1 - carboxylic acid

(ACC) and salicylic acid (SA) did not appear to accelerate yellowing of leaf discs. No significant difference shown between ACC/SA treatments and control.

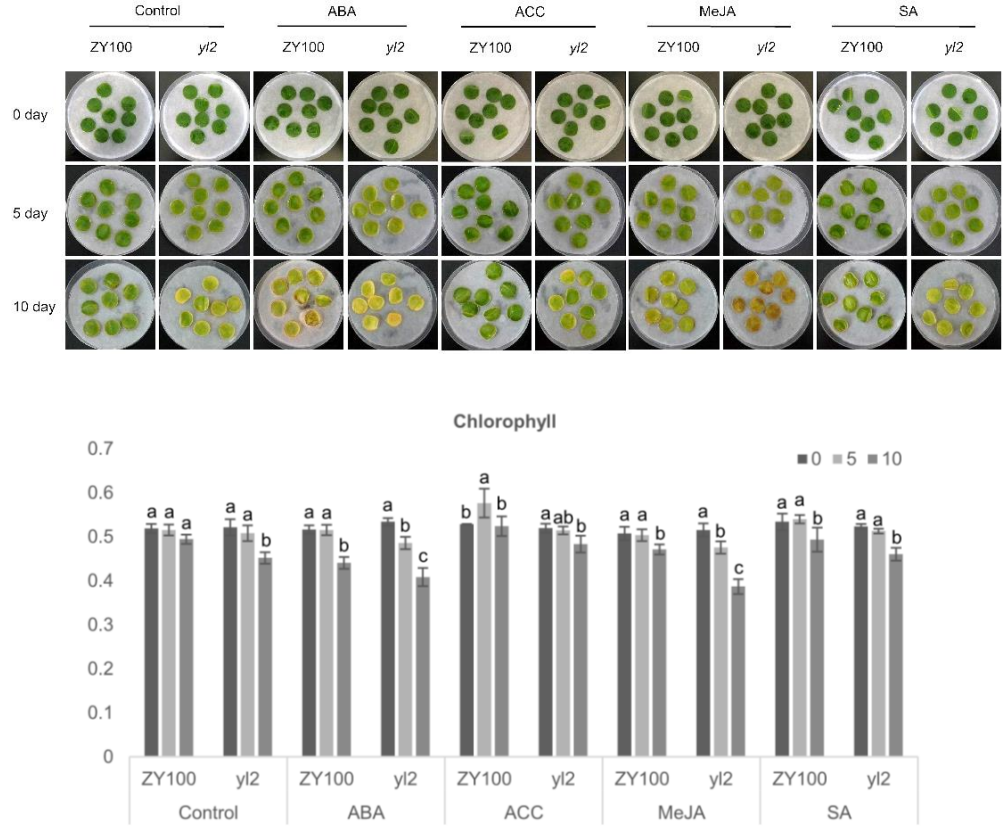

Figure 4-7 Plant hormone treatments of ZY100 and *yl2* leaf discs. Control was treated with the incubation buffer without hormone. ABA: abscisic acid; ACC: 1 - aminocyclopropane - 1 - carboxylic acid; MeJA: methyl jasmonate; SA: salicylic acid

4. Conclusion

In this study, a novel tobacco EMS mutagenesis induced premature leaf senescence mutant controlled by a single recessive gene was identified. 18 candidate SNPs that may lead to premature leaf senescence phenotype of *yl2* were obtained by whole genome sequencing (WGS). The validation results confirmed that 18 candidate SNPs were not associated with *yl2* phenotype. The published SSR molecular markers were used to map the target gene to one end of the tobacco LG24, and the genetic distance from PT60487 was 14.2 cM. SNPs obtained by WGS can be used as new molecular markers for further localization of *YL2*. The induction of plant hormones on leaf

senescence of *yl2* was initially explored. The *yl2* was more sensitive to ABA and JA than the wild type. However, the current results were still unable to determine whether the target was involved in the signaling pathway of hormone induced leaf senescence. Further cloning and characterization of the target gene will lead to a better understanding of the molecular mechanisms underlining leaf senescence in tobacco and provide useful information in the genetic manipulation of tobacco leaf senescence.

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5

Conclusion and Discussion

Conclusion

In this study, two premature leaf senescence mutants of tetraploid common tobacco (*Nicotiana tabacum* L.) *yl1* and *yl2* obtained by EMS mutagenesis were identified and the target genes responsible for the leaf senescence phenotype were preliminary mapped. The main conclusion of the dissertation are as follows.

yl1 is a premature leaf senescence mutant. At 50 days after transplanting (DAT), the lower leaves of *yl1* have begun to turn yellow. Chlorophyll in leaves of *yl1* degraded earlier than that in wild type, and degraded more rapidly in the late stage of leaf senescence. At the molecular level, the expression level of photosynthesis related gene *RBCS* of *yl1* was lower than that of wild type, while the expression level of senescence induced gene *SAG12* was higher than that of wild type. Genetic analysis indicated that the phenotype of *yl1* was controlled by a single recessive gene. The target gene was located on the tobacco linkage group 11 (LG11), between PT60305 and PT53066, with a genetic distance of 3.51 and 1.08 cM, respectively. Under the condition of ethylene and jasmonic acid induction, mutation of the target gene accelerates leaf senescence.

yl2 is a premature leaf senescence mutant. The chlorophyll content and protein content of *yl2* were lower than that of wild type throughout the developmental stage. However, the lower leaves of *yl2* showed a visible yellowing phenotype at 65 DAT. The degradation of chlorophyll in *yl2* occurred earlier and declined rapidly in the late stage of leaf senescence. Genetic analysis indicated that the phenotype of *yl2* was controlled by a single recessive gene. Using the MutMap combined with whole genome sequencing (WGS), 18 candidate SNPs associated with *yl2* phenotype were predicted. SNPs validation results indicated that all of them were not associated with *yl2* phenotype. The target gene was located on one end of the tobacco LG24, with a genetic distance of 14.2 cM from the polymorphic marker PT60487. Under the condition of abscisic acid and jasmonic acid induction, mutation of the target gene accelerates leaf senescence.

Although the shortcoming such as the huge tobacco genome, more than 70% repetitive sequences (Renny-Byfield et al., 2011), an extremely narrow genetic diversity (Yang et al., 2007; Zhang et al., 2006), and insufficient molecular markers, have led to the limited preliminary results of this study at present, this study is still the first report on the discovery and identification of leaf senescence regulating genes using tobacco EMS mutants.

Discussion

In addition to the results mentioned above, there are still a lot of unanswered and further analysis need to be done in future.

P|ant leaf senescence is the last stage of leaf growth and development. Accompanied by chlorophyll degradation and a decrease in photosynthetic efficiency, the leaves

realize the function transformation from nutrient assimilation to nutrient reactivation, which affects the quality and yield of plants (Guo and Gan, 2014). Studying the molecular regulation mechanism of leaf senescence is of great significance for the improvement of important agronomic traits such as crop yield and quality. The tobacco leaf premature senescence mutant induced by EMS is not only a good material to study the molecular mechanism of tobacco leaf senescence regulation, but also an excellent breeding material.

The effect of leaf senescence on plant growth has two opposite aspects. On the one hand, delayed leaf senescence prolongs the time when the leaves have the ability of photosynthesis, so as to improve yield. On the other hand, the existing studies in wheat and barley, as well as the prediction of the theoretical framework of leaf senescence, have shown that delayed leaf senescence will affect the recovery and reuse of nutrients from senescencing organs to young tissues and harvested organs, resulting in adverse effects on yield (Distelfeld et al., 2014; Have et al., 2017; Wu et al., 2012). For most crops, especially those with seeds as the main harvesting target, there is a positive correlation between delayed leaf senescence and high yield, and premature leaf senescence is unfavorable (del Pozo et al., 2016; Vadez et al., 2011).

In addition to being used as a model plant for scientific research, tobacco is an important leaf cash crop. In tobacco industry, yellowing senescent leaves are harvesting target. The yellowing and senescencing process of tobacco leaves is very important to the yield and quality of tobacco leaves. Accelerating the senescent process of tobacco leaves can shorten the tobacco leaves production cycle, so as to reduce the time cost. However, during tobacco leaf senescence, the metabolic process in leaf will change significantly. The strengthening of secondary metabolic pathways leads to the accumulation of specific metabolites. Han (1994) study showed that the content of some aroma substances produced by secondary metabolism increased with the improvement of tobacco maturity (Han, 1994). Chakrabarti et al. (2008) study showed that in high demethylated tobacco, *CYP82E4*, the coding gene of P450 protein that catalyzes nicotine demethylation, has senescence-specific expression, and demethylated nicotine, the product of this demethylation reaction, is the precursor of N-nitrosodemethyl nicotine, one of the most important carcinogens in tobacco and flue gas (Chakrabarti et al., 2008). It can be seen that the senescent process of tobacco leaves is also related to the formation and accumulation of chemical components, aroma substances and harmful components of tobacco leaves,which will directly affect the curing process of tobacco leaves, and then affect the quality of cigarettes. In addition, in the author's previous studies on leaf senescence of *Arabidopsis*, it was also observed that plants with premature leaf senescence are sometimes accompanied with adverse characteristics such as short plants and small leaf area. In production, tobacco is a kind of plant that mainly uses its leaves. If the premature leaf senescence mutant shows the characteristics of short plant and small leaf area, it will lead to the reduction of tobacco leaf yield, thus offsetting the advantage of reducing production costs caused by the shortening of the leaf production cycle. In this study, although the plant height, leaf number and leaf area of *yl1* and *yl2* were not significantly different

from those of wild type, they still deserved further attention. Moreover, once the plant initiates the leaf senescence process, the metabolism and transfer of the substances in the plant will change, plants thus ensure the completion of the life cycle (Guo, 2013; Guo and Gan, 2012). The leaf senescence of *yl1* and *yl2* initiate at about 50 DAT, which remains the vigorous vegetative growth stage of tobacco. How the leaf senescence process initiated during the vegetative growth stage affects the accumulation, degradation and transfer of metabolites related to tobacco leaf quality in leaves, is also a problem worthy of attention and further research.

Gene mapping is the basic content of scientific and research. Gene mapping methods are constantly updated with the emergence of various new technologies. The classical gene mapping methods represented by map-based cloning has identified many important genes. Map-based cloning can be divided into two stages: preliminary mapping and fine mapping. Preliminary mapping is to screen polymorphic molecular markers to determine chromosome intervals. Fine mapping used genetic map to analyze the high-density linkage of the preliminary mapping interval. High density genetic map and large mapping population are the main limiting factors of fine mapping.

Tobacco (*Nicotiana tabacum* L.) is an allotetraploid, which evolved through interspecific hybridization between the diploid *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. Although map-based cloning technology has been wildly used in plants such as *Arabidopsis* and rice, it is a challenge for allotetraploid tobacco with 4.7 Gb genome and more than 70% repetitive sequences (Renny-Byfield et al., 2011). Therefore, for a long time, we have been exploring efficient methods of tobacco gene mapping.

Gene mapping method based on high-throughput sequencing is a new mapping technology in recent years. It has the advantages of small mapping population, independent of genetic map and accurate mapping interval. In the case of small genome, simple genome structure and few mutation sites, the mutant can be directly sequenced and the mutation sites corresponding to the phenotype can be found, so as to achieve the purpose of finding genes. For species with large genome and complex genome structure, bulk segregant analysis (BSA) were usually used. BSA is to pooling-sequence the individuals with mutant phenotype and wild type phenotype in the segregation population, and detect the variation respectively. Based on the principle that mutant genes should be co-separated from adjacent loci, the allele frequency in the population is detected, so as to narrow the mapping region and identify candidate genes.

However, we also encountered various problems in the study of tobacco gene mapping using BSA method. For example, the allotetraploid tobacco has a large number of highly homologous genes. In the process of verifying candidate SNPs, it is even impossible to design primers that can specifically amplify the SNP regions. To solve this problem, in future experiments, PCR should be done to amplify the SNP region using non-specific primers. The PCR products should be connected to T-vector and transformed to *E.coli*, and then a large number of single colonies should be

sequenced to screen the amplification fragments in the region where we focus on, so as to verify SNP.

For *yl1*, the interval can be narrow down and the target gene can be fine mapped by expanding the population and developing SSR and SNP molecular markers. With the continuous development of sequencing technology in recent years, SNP molecular markers in tobacco genome have been extensively developed. In addition to the published SSR molecular markers by Bindler et al. and Tong et al. (Bindler et al., 2011; Bindler et al., 2007; Tong et al., 2012), four sets of SNP molecular markers of tobacco have been published and integrated into the 24 tobacco linkage groups by Bindler et al. (Bindler et al., 2011). In the process of narrow down the interval, searching for possible candidate genes for sequencing, finally finding the target gene.

For *yl2*, although we did not obtain the SNP that leads to *yl2* phenotype by MutMap and WGS, the SNPs obtained by sequencing can be verified to be developed as new molecular markers. In particular, the results of WGS indicated the physical location of the target gene in the genome, which can provide references for screening SNPs.

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Appendix – Publications

1. **Gao, X.M.**; Wu, X.R.; Liu, G.S.; Zhang, Z.L.; Chao, J.T.; Li, Z.Y.; Guo, Y.F.; Sun, Y.H. (2019). Characterization and Mapping of a Novel Premature Leaf Senescence Mutant in Common Tobacco (*Nicotiana tabacum* L.). *Plants*, 8, 415.

2. **Gao, X.M.**; Guo, Y.F. (2018). Research progress of leaf senescence related genes in tobacco. *Journal of Plant Sciences*, 6, 87-92.

3. Ali, A.; **Gao, X.M.**; Guo, Y.F. (2018) Plant Senescence-Methods and Protocols (Chapter 2: Initiation, Progression, and Genetic Manipulation of Leaf Senescence). Humana Press, New York.

4. Li, W.; Zhang, H.L.; Li, X.X.; Zhang, F.X.; Liu, C.; Du, Y.M.; **Gao, X.M.**; Zhang, Z.L.; Zhang, X.B.; Hou, Z.H.; Zhou, H.; Sheng, X.F.; Wang, G.D.; Guo, Y.F. (2017) Integrative metabolomic and transcriptomic analyses unveil nutrient remobilization events in leaf senescence of tobacco. *Scientific Reports*, 7, 12126-12142.

5. Wang, D.W.; Wang S.M.; Chao, J.T.; Wu, X.R.; Sun, Y.H.; Li, F.X.; Lv, J.; **Gao, X.M.**; Liu, G.S.; Wang, Y.Y. (2017) Morphological phenotyping and genetic analyses of a new chemical-mutagenized population of tobacco (*Nicotiana tabacum* L.). *Plata*, 246, 149-163.