

Original Research Article

Regulatory mechanisms associating innate leaves senescence of incongruent species

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ABSTRACT

Background: Senescence is the final developmental phase of a leaf which starts with nutrient salvage and ends with cell death. The first visible event during senescence is leaf yellowing, which typically starts at the leaf margins and progresses to the interior of the leaf blade. Though, regulators of senescence adopt a range of physiological and developmental mechanisms which undergo senescence of plant.

Methods: Leaves of different species were collected from the green house, and then rinsed several times with sterilized distilled water. For discs of leaves, two same sized leaves were collected and made the same sized discs. The samples were infiltrated with specific senescence inhibitor. The discs then kept in distilled water and placed under condition at 25⁰ C. Observed the phenotypes at two days interval, molecular based analysis was performed at 8th day of infiltration.

Results: In this study, innate senescence approach comparison to inhibitor based senescence has been performed in order to check its consequences on leaves of different crops such as; cauliflower, apple, tobacco, rose and Arabidopsis. Arabidopsis and apple have resulted in a narrative phenotype with high level of ion leakage. While in case of rose and cauliflower, the phenotype was characterized with yellow fading of leaves. Interestingly, in the tobacco plants, intense yellowing of leaves developed along bottom. Further, in order to confirm the efficiency and pattern of senescence, we had also assessed the changes occurred during leaf senescence via ion leakage and chlorophyll content, expression of SAG12 (a senescence associated gene) and (PSA) photosynthetic associated genes expression as markers.

Conclusions: It has been noted that progression of leaf senescence is a very critical and important factors affecting plant growth and development. It can be stated that initiation of leaves senescence can be controlled by using specific inhibitor.

Keywords: Leaves senescence, Inhibitor, Innate, Incongruent species

INTRODUCTION

Senescence is the final developmental phase of a plant which starts with nutrient salvage and ends with cell death. The first visible event during senescence is leaf yellowing, which typically starts at the leaf margins and

progresses to the interior of the leaf blade.¹ Leaf senescence is an integrated response of leaf cells to age information and other internal and environmental signals. This integrated senescence response provides plants with optimal fitness by incorporating the environmental and endogenous status of plants in a given ecological setting

by fine-tuning the initiation timing, progression rate, and nature of leaf senescence. The environmental factors that influence leaf senescence include both abiotic and biotic.² Thus in our present study we have hypothesized about the role of senescence approaches of leaves. Certain to that, leaf senescence is an active process involving the differential expressions, it is presumed that numerous factors are involved as central elements of the regulatory network. Genome-wide analyses of changes in expression have allowed the identification of many Arabidopsis encoding transcription factors up-regulation in senescing leaves.³⁻⁷ Only a few factors have been demonstrated to be involved in regulating leaf senescence by analyzing the leaf senescence phenotypes. Expression of photosynthesis-associated genes (PAGs) is down-regulated, while many other genes, designated as senescence-associated genes (SAGs), are up-regulated during senescence. Detailed studies on the SAG identities and their expression suggest a complex regulation of leaf senescence.⁸⁻¹¹ In Arabidopsis the age of individual leaves plays a prominent role in determining leaf longevity.¹²⁻¹⁴ Although floral initiation can influence plant longevity and whole-plant senescence.¹⁵ In present study; we investigated the regulation of innate senescence amongst different crops with link to inhibitor approach of senescence of leaves. Thus we had hypothesized to test the leaves senescence approaches significantly between different crops, and have regulate the senescence of leaves by using inhibitor. Moreover, promote leaf senescence involving in different aspects and had proved that inhibitor consistently inhibit the leaves senescence.

METHODS

Plant materials and growth condition

Leaves of different species were collected from the green houses, and then rinsed several times with sterilized distilled water. For discs of leaves, two same sized but opposite leaves were collected from plants and made the discs in same size. The discs then kept under distilled water and placed under condition at 25° C and observed the phenotypes after two days interval.

Disinfestations of leaves and discs preparations

After making discs of all crops, the discs were pre-sterilized with 5% sodium hypochlorite for about 15 minutes then rinsed with sterilized distilled water for three times and kept under control conditions until phenotype was observed.

Ion leakage measurement

The suspended solution of treated disc was collected at two days interval and measured the quantity of ion leakage. Although, for rose leaves and Arabidopsis, leaves were collected then boiled it into hot water, and suspended solution was then analyzed.

Measurement of chlorophyll contents

The chlorophyll contents were measured according to the method used by Ayumi and Makino.¹⁶ The fresh material was handled immediately after collection. After chopping well, the pieces (0.5 g) of samples were standardized with the help of a homogenizer by adding 10ml of 80% acetone. Although, primary acetone extract containing all chloroplast pigments was obtained in this way. This extract was then centrifuged at 2500 rpm for about 5min. Since the concentration of pigments was in most cases too great for reading to be performed on a spectrophotometer, the obtained extract was diluted by adding 9 ml of 80% acetone/ml of extract. The extract produced in this way was subjected to reading on a spectrophotometer.

Extraction of DNA and PCR analyses

The DNA was extracted from leaves after the ten days of infiltration. 10 ml of 2 X CTAB solutions was pre-heated at 65-70° C in water bath. Mortar and pestle were pre-cooled at -80° C. About 5g of leaves were frozen in the liquid nitrogen. After that, pulverized powder was transferred to a pre-heated 50 ml and centrifuged the tube containing 10 ml of 2 X CTAB solutions. Then placed it on ice bath and 1 ml of β -mercaptethanol were added in the tube. All materials were mixed thoroughly with a stainless steel spoon. Then CIA liquid was added and incubated at 37°C for 20 minutes with shaking at 120 rpm. After that, centrifuged at 3000 rpm for 20 minutes at room temperature and transfer the supernatant to a new 1 ml plastic tube. Slowly added precipitation buffer gently reverse the shake 15-20 times until the precipitation of DNA together. Added pre-cooled ethanol at -20°C, followed by immersion in the following ethanol solution: 70% ethanol, 7 min, 70% ethanol, 7 min, 100% ethanol, 5 min.

Phenotypic observation of noticeable yellowing

Observations were made at two days interval of infiltration of leaves discs, and yellowing crakes of leaves were investigated The yellowing of leaves could be initiated at the discs tips, at the petiole side of the leaf, or in the middle of the leaf blade, which did not always resemble the wild-type developmental yellowing pattern of leaf senescence.

Statistical analysis

Data so obtained was statistically analyzed according to the technique of analysis of variance (ANOVA). The treatment means were compared using least significant difference (LSD) at 5% level of probability. All computational and statistical analysis was performed using student edition software package 8.1.

RESULTS

Regulatory mechanisms involving leaves senescence of apple at different time intervals

Apple (*Malus domestica*), a deciduous temperate climate species, is trigger by a rather abrupt temperature drop, down to the lower teens. The aim of the present study was to elucidate the effects of seasonal leaf senescence. We hypothesized that leaves senescence can be delayed by the use of senescence inhibitor. Phenotypic analysis of discs leaves of apple was measured at two days interval till the entire experiment. Slight yellowing of leaves was observed at the beginning of infiltrations. Contrast to that we also treated leaves with inhibitor hormones of senescence and we found that by using hormonal activity, senescence approach was outstandingly inhibited and significantly reduced yellowing process of leaves. Mostly after 10 to 15 days of infiltration of discs leaves, the leaves flatter to yellow faded was observed in innate leaf senescence instead using inhibitor, which slightly reduced senescence activity (Figure 1). In order to confirm the efficiency and effectiveness, we had also assessed the changes happened via expression, ion leakage measurement, chlorophyll content and RhSAG12 (a senescence associated gene) expression as markers for senescence progression. Interestingly, we had found significantly exceptional differences compare to that of innate leaves senescence with using inhibitor of senescence. Similarly, increase in ion leakage and RhSAG12 expression, and decrease in chlorophyll concentration was observed during senescence of leaves (Figure 1).

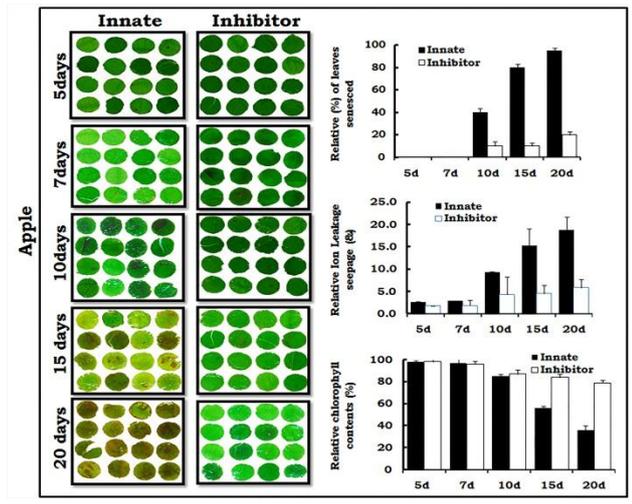


Figure 1: Phenotypic analysis and regulatory mechanisms associated with leaves senescence of apple.

Encountering of leaves senescence, average of ion leakage measurement, relative chlorophyll content was observed in innate and inhibitor affected leaves of apple. Data are the average of three different repeats. Error bar represented as standard deviations and treatment means were compared using least significant difference (LSD) at 5% level of probability.

Regulatory mechanisms involving leaves senescence of cauliflower at different time intervals

Further we had tested the leaves senescence approach in cauliflower (*Brassica oleracea*) leaves. Although, compare to that of leaves infiltrated with inhibitor, senescence started early in innate leaves, usually the yellow shading were initiated after 7days in infiltration. Further, slight yellowing of leaves was also observed at the beginning days of infiltrations. In spite to that, we also had found that during innate phenomenal senescence after 20 days i.e. most of leaves were became yellow. Contrasted to that finding, treated leaves with inhibitor hormones of senescence was exceptionally inhibited and significantly reduced yellowing process of leaves till 15 days. Generally, after 7 days of infiltration of discs leaves, the leaves flatter to yellow faded was observed in innate leaf senescence and going rapidly to be yellow till 20 days, instead using inhibitor the leaves consistently stayed green till 15 days (Figure 2). Moreover, further confirmation was done by assessing the changes happened via expression, ion leakage measurement, chlorophyll content and RhSAG12 (a senescence associated gene) expression for senescence progression. Unexpectedly, we found exceptionally diverse results compare to that of innate leaves senescence with using inhibitor of senescence. Similarly, increase in ion leakage and RhSAG12 expression, and decrease in chlorophyll concentration were observed during senescence of leaves (Figure 2).

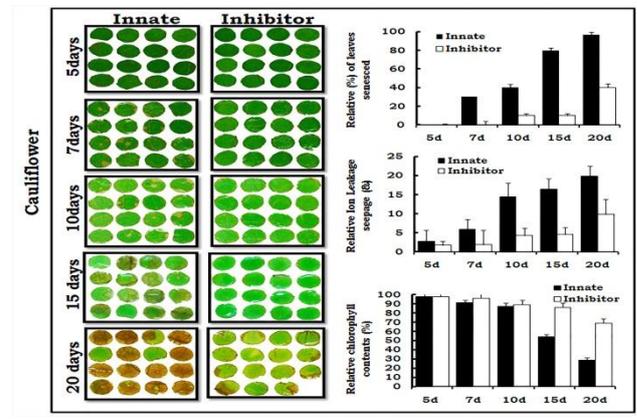


Figure 2: Phenotypic analysis and regulatory mechanisms associated with leaves senescence of cauliflower.

Encountering of leaves senescence, average of ion leakage measurement, relative chlorophyll content was observed in innate and inhibitor affected leaves of cauliflower. Data are the average of three different repeats. Error bar represented as standard deviations and treatment means were compared using least significant difference (LSD) at 5% level of probability.

Regulatory mechanisms involving leaves senescence of rose at different time intervals

Rose (*Rosa hybrida*) is one of the most important and economic ornamental plant in the world. The family of

rose (*Rosaceae*) is classified as an astonishingly large family of flowering plants. As we know that, the process of senescence is the final stage of flower development that significantly precedes the termination of the floral organ of any plant. Consequently, natural senescence is governed by endogenous signals at the developmental age. Even though, genetically controlled and environmental stresses such as heat, cold, drought, pollination and as well as pathogen attack may consistently accelerate senescence. Meanwhile, this may lead us to understand the mechanism of promoting the senescence approach. In present study we had assessed that Photosynthesis is a highly regulated, multistep process and plays an important role in growth and development of plants. Moreover, in present findings we observed the chlorophyll concentrations in innate leaves and inhibitor treated leaves which were significantly elaborated (Figure 3). The chlorophyll concentration remarkably decreased in innate senesced leaves. Compare to that of innate senescence, the inhibitor of senescence had same concentration of chlorophyll. In order to make confirm these alternations, we tested the expression of Photosynthetic Associated Gene (PAGs) in comparisons with SAG12. The results had been confirmed by finding significantly higher expression levels of SAG12 and slightly lower expression of PAGs in innate senesced leaves contrast to that of SAG12 lower and PAGs higher expression in inhibitor of senescence.

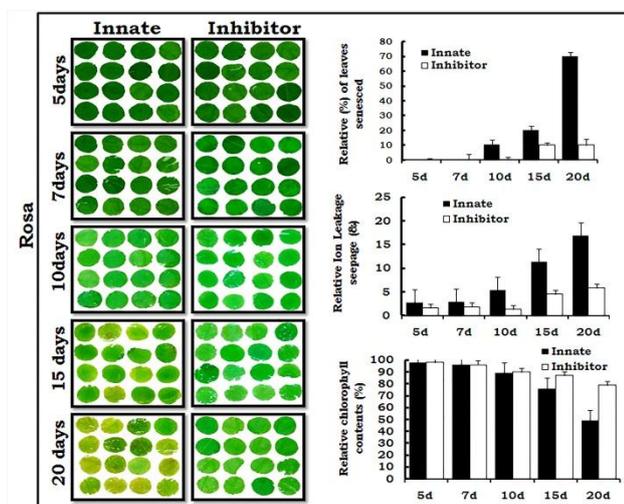


Figure 3: Phenotypic analysis and regulatory mechanisms associated with leaves senescence of rose. Encountering of leaves senescence, Average of ion leakage measurement, relative chlorophyll content was observed in innate and inhibitor affected leaves of rose. Data are the average of three different repeats. Error bar represented as standard deviations and treatment means were compared using least significant difference (LSD) at 5% level of probability.

Regulatory mechanisms involving leaves senescence of tobacco at different time intervals

Tobacco (*Nicotiana tabacum*), is one of the most agronomical crop used in many kind of latest research as

model plant. Although in present study, the phenotypic observation of leaves senescence was measured at two days interval. Interestingly, in contrast to other crops we found, the senescence start slightly in the entire experiment. Slight yellowing of leaves was observed from the beginning days till end of experiments, mostly after 7 days to 20 days (Figure 4). Moreover, leaves flatter to yellow faded were observed in innate leaf senescence instead using inhibitor, which slightly reduced senescence activity. Furthermore, confirmation of changes happened via expression, ion leakage measurement, chlorophyll content and RhSAG12 were also observed.

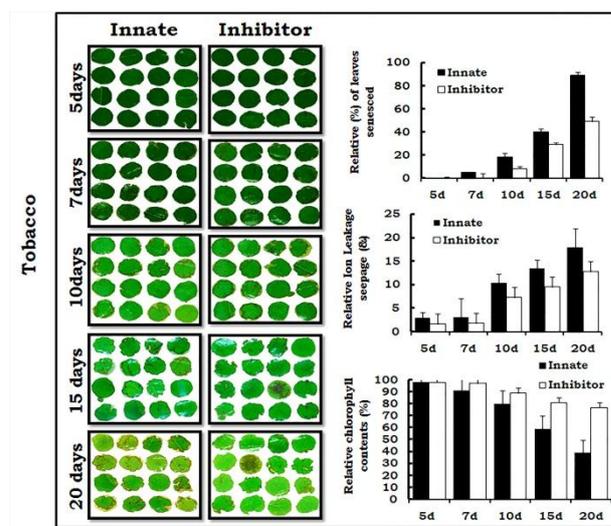


Figure 4: Phenotypic analysis and regulatory mechanisms associated with leaves senescence of tobacco.

Encountering of leaves senescence, Average of ion leakage measurement, relative chlorophyll content was observed in innate and inhibitor affected leaves of apple. Data are the average of three different repeats. Error bar represented as standard deviations and treatment means were compared using least significant difference (LSD) at 5% level of probability

Regulatory mechanisms involving leaves senescence of Arabidopsis at different time intervals

Certainty, leaf senescence approach considered as highly complex process that involves multiple functions, via endogenous and exogenous signals throughout the leaf life cycle. It is amazing that leaf senescence is controlled with numerous inhibitors of regulation. Additionally, leaves senescence is not a lone process, but it involves continuous time dependent transitions of cellular physiology and metabolism. Therefore, senescence needs to be understood through its dynamic perspective. Thus so far, Arabidopsis is a key model plant in all over the world, most novel research is focusing on this crop. Although in present study we found surprisingly results that innate leaves were significantly senesced earlier than treated with inhibitor. After 15 days most of leaves were died. Even though, with treatment with inhibitor the

leaves were stayed green by containing chlorophyll higher than innate senesced leaves (Figure 5).

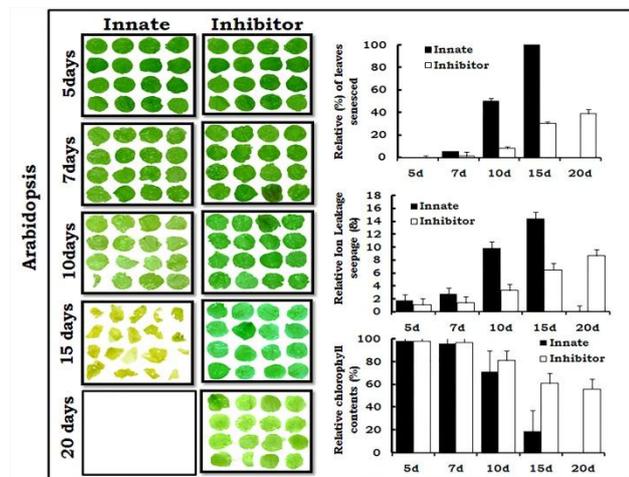


Figure 5: Phenotypic analysis and regulatory mechanism associated with leaves senescence of Arabidopsis.

Encountering of leaves senescence, average of ion leakage measurement, Relative chlorophyll content was observed in innate and inhibitor affected leaves of Arabidopsis. Data are the average of three different repeats. Error bar represented as standard deviations and treatment means were compared using least significant difference (LSD) at 5% level of probability.

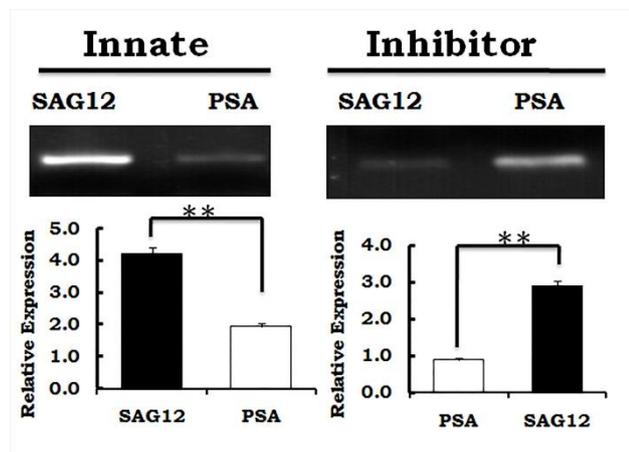


Figure 6: Relative expression measured between inhibitor related and innate senesced leaves with markers genes by semi quantitative PCR analysis.

Samples after 8th day of infiltration were used for measuring the expression. Error bar represented as standard deviations and treatment means were compared using least significant difference (LSD) at 5% level of probability.

DISCUSSION

It has been reported that leaf senescence is a genetically controlled developmental process, eventually leading to cell death. Apparently, senescence does not occur in young leaves under normal growth conditions. Perhaps, senescence repressors efficiently suppress the onset of senescence during early leaf development, and/or

activators are switched on as a leaf ages.¹⁷ Interestingly, the effect of innate was significantly different in all selected congruent species. The overall observations of leaves of all selected species suggested that during whole experiments, there was slight yellowing of leaves at the beginning days, if there is sufficient infiltration in all senesced leaves and inhibitor of senescence. But later days, leaves were fluttering to yellow faded. This perception suggested that efficiency and effectiveness of senescence was highly significant. Although assessed changes confirmed by measuring of ion leakage activity, chlorophyll concentration, RhSAG12 (senescence associated gene) and Photosynthetic related genes (PRGs) expressions. We had found significantly outstanding differences between innate senescence and inhibitor used senescence. Similarly, augmentation of ion leakage and RhSAG12 expression, contrast to that reductions in chlorophyll concentration were observed during senescence of leaves that also plays critical role in leaf senescence. Leaf senescence is specifically regulated to maximize plant fitness by remobilizing nutrients from senescing leaves, so that its onset, progression, and completion should be finely controlled by the differential expressions. The application of recent genomics technology has enabled the isolation of a category of genes, so-called senescence-associated genes (SAGs), which shows increased expression in senescing leaves.¹⁸⁻²⁰

Chlorophyll reduction in innate leaves

In present study we had observed chlorophyll concentrations in innate senesced leaves and senescence inhibitor treated leaves of all selected crops. As we know that photosynthesis is a highly regulated, multistep process and plays an important role in growth and development of plants.²¹ The chlorophyll concentration remarkably decreased in all selected crops innate senesced leaves while the senescence inhibitor treated leaves had consistent concentration of chlorophyll. In order to make confirm these alternations, we had tested the photosynthetic associated gene (PAGs) in comparisons with SAG12 (a Senescence Associated Gene). The results had been confirmed by finding significantly higher expression with SAG12 and slightly lower expression of PAGs in innate senesced leaves in contrast to that of SAG12 lower and PAGs higher expression in inhibitor of senescence.

Leaf senescence was determined by comparing the level of yellowing in the innate and inhibitor senesced leaves during age-dependent leaf senescence and as well as of discs of leaves. At 10 days after inflation, the leaves were initiated and started to turn yellow, but inhibitor treated leaves remained green. At 20 days after infiltration, the leaves had turned completely yellow and showed signs of death with drying. In contrast, inhibitor leaves retained their integrity and showed only partial yellowing. In order to observe its severity, the Leaf senescence symptoms were also analyzed by measuring typical

senescence-associated physiological markers, such as chlorophyll content, expression of photosynthetic related and senescence associated genes. After one week of infiltration, the chlorophyll concentration in leaves started to decline, whereas after 15 days innate senesced leaves had already lost 40-50% of their chlorophyll content. Similar findings had also been reported in relation to acceleration, affection and initiation of senescence of leaves, photosynthetic activities in order to decline the content of chlorophyll in silenced plants.^{22, 23} Moreover, we had reviewed that factors of the others family often have similar functions.²⁴ More than 20% *Arabidopsis* are specifically induced during developmentally triggered senescence.^{25,26} Taken together all of these observations, It can be stated that initiation of leaf natural senescence and control senescence by using specific inhibitor is highly regulated process.

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Conflict of interest: None declared

Ethical approval: The study was approved by the institutional ethics committee

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