

## Sunflower (*Helianthus annuus* L.) leaf senescence analysis by a physiological and molecular approach

*Sebastián Moschen*<sup>1,2</sup>, *Paula Fernández*<sup>1,3</sup>, *Sofía Bengoa Luoni*<sup>1</sup>, *Norma Paniego*<sup>1,3</sup>, *Luis Aguirrezábal*<sup>2,3</sup>, *Guillermo Dosio*<sup>2,3</sup> and *Ruth A. Heinz*<sup>1,3</sup>. <sup>1</sup>Instituto de Biotecnología, CICVyA, INTA Castelar, N. Repetto y Los Reseros S/N (1686) Hurlingham, Buenos Aires, Argentina, [rheinz@cnia.inta.gov.ar](mailto:rheinz@cnia.inta.gov.ar); <sup>2</sup>Unidad Integrada (FCA/INTA) Balcarce, Ruta 226 km. 73.5 (7620) Balcarce, Argentina, [gdosio@mdp.edu.ar](mailto:gdosio@mdp.edu.ar); <sup>3</sup>CONICET, Argentina

### ABSTRACT

- Leaf senescence is a complex mechanism controlled by multiple variables, either from genetic and environmental origin that strongly impacts on crop yield. It has been previously described that water stress accelerates leaf senescence in sunflower while head removal delays the process. Even when senescence has been extensively studied in model plants, the molecular aspects that take place at the onset of leaf senescence in sunflower are still unknown. Visual parameters as yellowing or necrosis of leaves are detectable long after the signaling cascade of senescence process is activated. Recently, different transcription factors with NAC domain have been characterized as early SAGs (Senescence Associated Genes) which might play a key role in leaf senescence and program cell death. The aims of this work were: (i) the evaluation of physiological and biochemical parameters associated to senescence in a time frame, for different growing and leaf-age conditions, (ii) the early detection of leaf senescence by the molecular isolation of a potential SAG, (iii) the study of the differential expression of these candidates along the development of leaf senescence.
- Two field experiments were performed at INTA Balcarce, in two growing seasons. Sunflower plants (VDH487, Advanta) were grown under good water and nutrients conditions. Treatments were: (i) head excision, (ii) a moderate water stress during all developmental period, and (iii) control. The evolution along a thermal time scale of physiological (nitrogen, leaf area), biochemical (soluble sugars, chlorophyll) and molecular (NAC transcription factor expression) events related to senescence process were evaluated. Parameters evaluated to detect leaf senescence were 80% (nitrogen, leaf area, soluble sugars and chlorophyll), 20 % (chlorophyll) and 0% (chlorophyll) of the maximal value reached for each variable. Identification of candidate sunflower SAGs was performed by searching tentative orthologous to described SAGs in different plant species. Transcriptional profile of candidate SAGs was done by qPCR in leaves at different ages and growing conditions, using reference genes with stable expression pattern, previously identified and characterized by our group.
- The time course of the evaluated parameters showed differences among treatments but also a strong interaction of growing conditions and leaf ages was detected. An early water stress showed a delay in the occurrence of the events in the oldest measured leaf, while in the youngest, the opposite was observed. Nitrogen fall was the very first physiological marker detected, whereas other relevant parameters were detected later in the cascade of events in the senescence process. The expression profile of one of the identified sunflower orthologous SAGs, an ORE1 transcription factor containing a NAC domain, was assessed through qPCR. Expression patterns of ORE1 showed an early activation in relation to physiological and biochemical events, which makes this gene a potential indicator of the triggering of the process.
- Knowing the time of the onset of the sequence of events related to senescence could determine the causes of this process and generate physiological and molecular tools to facilitate future interventions on it. This can be useful for application in assisted breeding of this crop with great economic importance. According to the results of this study, the transcription factor ORE1 appears as a potential candidate by qPCR with an early expression pattern, followed by an increase of expression prior to anthesis.
- The main contribution of this work was the identification and characterization of genetic sequences involved in the onset and evolution of the leaf senescence process through the study of physiological and transcriptional profiles in sunflower leaves from plants challenged to treatments that alter the senescence rate.

Key words: senescence – chlorophyll – nitrogen - SAG – Transcription factor - ORE1

## INTRODUCTION:

The maintenance of green and functional leaves during long periods allows to intercept more radiation during the grain filling and thus influence weight and oil content of sunflower seeds (Dosio et al. 2000; Aguirrezábal et al. 2003). Leaf senescence is a complex and highly coordinated process, controlled by multiple variables, either from genetical and environmental origin that has strong impact on crop yield (Noodén et al. 1997). In certain regions, such as the SE of Buenos Aires, the sunflower crop senescence takes place abruptly, coinciding with adverse environmental conditions and the incidence of foliar diseases (Dosio & Quiroz, 2010). Therefore, it is considered as a process with high economic impact involved in the gap between potential and real yield observed.

Although senescence symptoms have been explored in different plant species, the involved mechanisms that control it have not been characterized yet (Buchanan-Wollaston et al. 2003). The distinctive symptom of leaf senescence is the breakdown of chloroplasts and therefore, the decrease in chlorophyll content becomes a key indicator of the process (Hörtensteiner 2006). Both the beginning and the rate of senescence may be affected by autonomous and environmental signals.

Environmental factors such as light (Weaver & Amasino 2001), nutrient availability, CO<sub>2</sub> concentration, abiotic and biotic stresses (Sadras et al. 2000), affect the rate of senescence. Pic et al. (2002) showed that the succession of certain events at macroscopic, biochemical and molecular level in pea leaf senescence were not modified in leaves of different age, or under conditions of moderate water stress. Since some of the environmental conditions that affect senescence have important effects on carbon metabolism, Wingler et al., (2006) assigned to sugar content in leaves an integrating role of environmental signals regulating leaf senescence. Reproductive growth is mentioned as a factor that normally affects leaf senescence, and particularly in sunflower, the lack of sinks delays the onset of senescence (Sadras et al. 2000). In contrast, control of senescence by growth of reproductive structures was not observed in *Arabidopsis thaliana* (Noodén & Penny 2001).

Determining the onset of senescence is complex because there is not a "symptom" indicating this moment. During the leaf senescence two main stages can be distinguished: an initial process, when up or down regulation of gene expression occurs for a number of genes involved in nutrient export and degradation of structures, and a progression phase involving the evolution of the process. Both, the variation in chlorophyll content and yellowing or the necrosis of leaves, are detectable long after the signaling cascade of the senescence process is activated.

Molecular studies of leaf senescence are generally based on the accumulation of messenger RNA coding for enzymes involved in degradation of structures, however, this process is highly dependent on interaction between endogenous and environmental signals, involving different genes whose expression is induced or inhibited at different stages along the process (Gan & Amasino 1997).

NAC transcription factors have been recently identified in model species and they play a relevant role in the regulation of progression of leaf senescence, related to programmed cell death (Kim et al. 2009; Balazadeh et al. 2010; Hu et al. 2010; Nuruzzaman et al. 2010; Balazadeh et al. 2011). In sunflower, we identify a transcript similar to the *ORE1* gene described in *Arabidopsis thaliana*, a NAC transcription like factor, mining the *Sunflower Unigene Resource (SUR v1.0) database* developed at INTA (<http://bioinformatica.inta.gov.ar/ATGC/>). In this work we aim to: (i) evaluate the physiological and biochemical parameters associated to senescence in a time frame for different growing and leaf-age conditions, (ii) identify potential SAGs as early molecular markers for the senescence process (iii) study the differential expression profile of these candidate genes along the development of leaf senescence.

## MATERIALS AND METHODS

Two field experiments were carried out at INTA Balcarce Experimental Station (37°45' S, 58°18' W) during the 2009/10 and 2010/11 crop season. Sunflower hybrid VDH 487 (Advanta Seeds) was sown at a 7.2 plants/m<sup>2</sup> density. Emergence occurred 11 days later. Diseases, weeds and insects were adequately controlled. Soil fertility assured maximum yields under non limiting water conditions. Rainfall was complemented with irrigation when necessary, to avoid water deficit in control plants. Soil volumetric humidity was measured periodically using the time domain reflectometry technique (Trase System, Model 6050X1, Soil moisture Equipment Corp., Santa Barbara, CA, USA). Leaves 10, 15 and 25 (numbered from the bottom to the top of

the plant) were considered as initiated when their primordia were visible (about 40  $\mu\text{m}$  long) on the apical meristem under a stereo microscope using magnification 80x (Olympus SZX12).

The following experimental conditions were applied to either accelerate or delay leaf senescence in comparison with control plants:

Water deficit (WD): a mild water deficit was achieved in the experiment 2009/2010 by covering the soil with a 200  $\mu\text{m}$  plastic mesh installed 35 days before flowering, and in the experiment 2010/2011 by a shelter installed prior to sow. Volumetric humidity of the soil was reduced about 40%. Controlled irrigation helped to maintain the deficit level from treatment application to the sample harvest day.

Head excision (HE): the head of all plants of the plot was cut with a fine scalpel 4 days before flowering to suppress the reproductive sink.

Control (C). Untreated plots were kept as controls.

The experiment was conducted as a randomized complete block design with three replicates (plant-plots). Each experimental unit was integrated by three randomly selected plants from each plot.

At several times, samples were taken from different leaves and treatments, immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction. In parallel to the sampling, measurements were performed on the same plants to assess the ecophysiological variables related to the progress of senescence process as chlorophyll content, soluble carbohydrates and nitrogen content.

Chlorophyll quantification was performed through direct extraction with N, N-dimethylformamide, and subsequent quantification based on spectrophotometer absorbance readings (Inskeep & Bloom 1985). The soluble sugar content was measured by the phenol-sulfuric acid method proposed by Dubois et al. (1956), and total nitrogen (%) with the Dumas method (TrunSpec CN, Leco, Michigan, USA).

High quality total RNA was isolated from 100 mg of frozen tissue using Trizol® following manufacturer's instructions (Invitrogen, Argentina). Genomic DNA was eliminated after treatment with Dnase I for 60 min (Invitrogen, Argentina). RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA).

For each sample, 500 ng DNase treated RNA was reverse-transcribed using SuperscriptIII first strand synthesis system (Invitrogen, USA) and random hexamer primers according to manufacturer's instructions. qPCR reaction was carried out in a 25- $\mu\text{l}$  reaction mix containing 200 nM of each primer, 1  $\mu\text{l}$  of cDNA sample and FastStart Universal SYBR Green Master (Roche Applied Science). Negative RT RNA control and non-template controls were incorporated in the assays. qPCR were performed using a 96-well plate thermocycler (ABI Prism 7000 Sequence Detection System and software, PE Applied Biosystems, USA). The thermal profile was set to  $95^{\circ}\text{C}$  for 10 min, and 40 cycles of  $95^{\circ}\text{C}$  for 15 s, and hybridization temperature for 1 min. Amplicon specificity was verified by melting curve analysis (60 to  $95^{\circ}\text{C}$ ) after 40 PCR cycles. The qPCR assay was carried out using two biological replicates for each treatment and two technical replicates for each biological replicate, derived from independent cDNA synthesis.

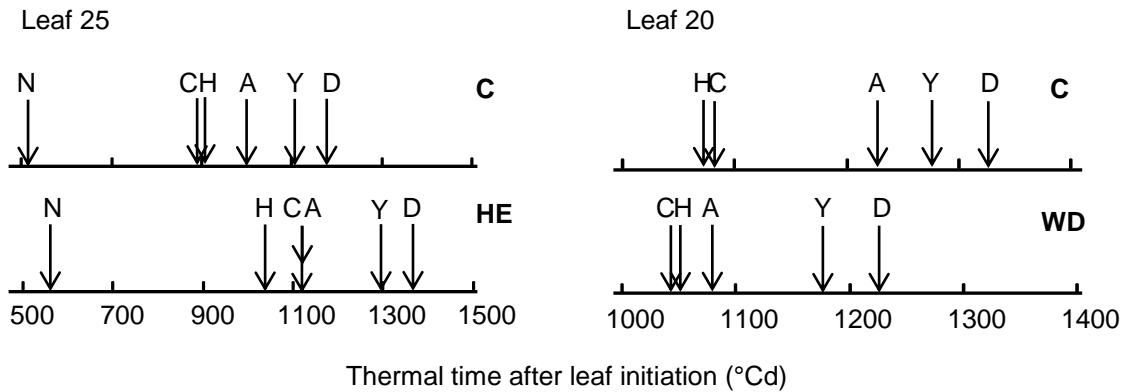
Two reference genes previously characterized in sunflower, EF-1 $\alpha$  and  $\alpha$ -TUB, were used as endogenous control of expression level (Fernandez et al. 2011). Amplification efficiencies and Ct values were determined for each gene and each tested condition, with the slope of a linear regression model using the *LinRegPCR* (Ruijter et al. 2009). The expression profiles of these genes were estimated in relation to reference genes using *fgStatistic* software, which uses previously published algorithms (Pfaffl 2001).

## RESULTS

To assess the development of leaf senescence under different experimental conditions and leaf ages, ecophysiological measurements were performed: leaf area, nitrogen, chlorophyll and soluble sugars content. Parameters evaluated to detect indicators of leaf senescence were 80% (nitrogen, leaf area, soluble sugars and chlorophyll), 20 % (chlorophyll: yellowing) and 0% (chlorophyll: desiccation) of the maximal value reached for each variable. Figure 1 shows the evolution of different physiological parameters in different leaves assessed and compared with different treatments.

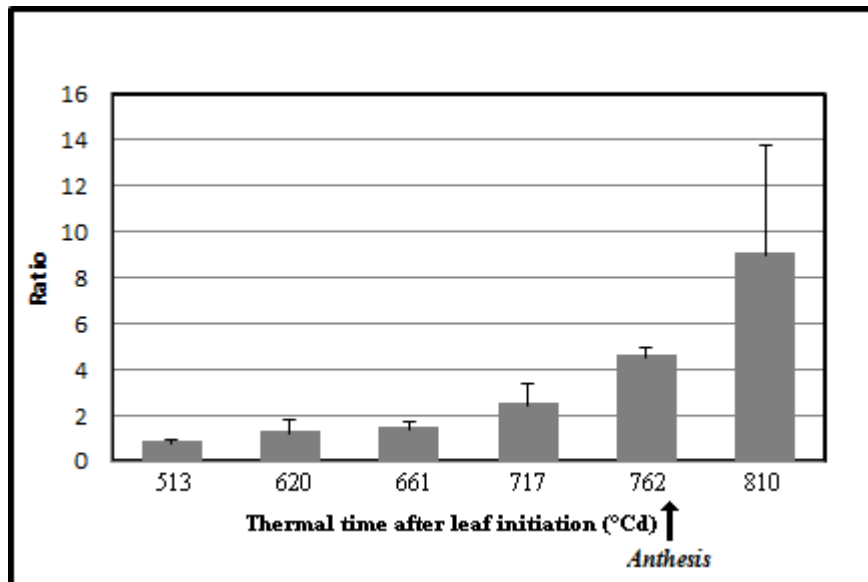
The evaluated parameters showed differences among treatments, but also a strong interaction of growing conditions and leaf ages was detected. An early water stress anticipated the occurrence of the events in the oldest measured leaf (Fig. 1). The opposite was observed in the youngest one (data not shown). On the other hand, head excision showed a delay in all physiological indicators of leaf senescence for all the evaluated leaves.

Nitrogen fall was the very first physiological marker detected, whereas other relevant parameters were detected later on in the cascade of events, along the senescence process.



**Fig. 1.** Thermal time scale (°Cd) of events related to leaf senescence. N: nitrogen, C: chlorophyll, H: soluble carbohydrates, A: leaf area, Y: yellowing, and D: desiccation. Treatments: C: control, HE: head excision, and WD: water deficit. Figures on the left show leaf 25 in experiment 2009/2010, and figures on the right: leaf 20 in experiment 2010/2011.

In order to find early molecular markers related to the senescence process, we designed a qPCR assays to detect and to monitor the expression profile of an Arabidopsis putative orthologous SAG in sunflower, the *ORE1* transcription factor containing a NAC domain. Figure 2 shows the expression level of *HaORE1* gene in leaf 10 in control condition. Expression patterns of *HaORE1* showed a basal early activation in relation to physiological and biochemical events, with an increase of the expression level prior to anthesis, coinciding with the beginning of the remobilization of nutrients and the increase of the leaf senescence rate. Under this assumption, *HaORE1* becomes a potential leaf senescence functional molecular marker.



**Fig. 2.** Gene expression ratio of candidate gen *HaORE1* in relation to the reference gene Elongation Factor *EF1α*. RNA quantification from leaf 10 of plants grown in (C) conditions, sampled at different time points in experiment 2010/2011, was performed by qPCR.

## DISCUSSION

The main contribution of this work was the identification and characterization of genetic sequences involved at the onset and evolution of the leaf senescence process through the study of physiological and transcriptional profiles in sunflower leaves from plants challenged to treatments that alter the senescence rate. Knowing the onset of the cascade of events that trigger senescence, could help to determine the causes of this process and generate physiological and molecular strategies to facilitate future interventions on it. In this study we assessed the physiological variables related to the progress of senescence. When an early water-stressed was applied, a different response of young and adult leaves was observed; while young leaf showed an anticipated occurrence of senescence events, in the oldest measured leaf, the opposite was observed. This could be mainly due to an alteration in plant growth derived from an adaptation to water deficit, leading to smaller plants with higher chlorophyll concentration and a delay in the evolution of senescence in the oldest leaves, relative to control plants. On the other hand, the situation was reversed in younger leaves, showing acceleration in the senescence rate in water-stressed plants. Nitrogen fall was the very first physiological marker detected, making it a good physiological indicator of senescence progress, whereas other relevant parameters were detected later in the cascade of events along the senescence process. Head excision conduced to a delay in all senescence related parameters. Grains are the main sink for carbohydrates and nutrient remobilization along senescence. Therefore, the lack of grains in the HE treatment led to a delay of these events.

The transcription factor Ha*ORE1* appears as a potential molecular functional marker, showing an early activation in relation to physiological and biochemical events, with an early expression pattern, followed by an increase of expression prior to anthesis, which makes this gene a potential indicator of the triggering of the process. *ORE1*, a NAC transcription like factor that has been extensively studied recently in model plants, has been described as strongly related to leaf senescence, showing a complex expression pattern, being under control of the ethylene signaling pathway and subjected to negative regulation by miRNA164.

The execution of senescence process consists of multiple interconnecting pathways which regulate and/or modulate this series of orderly steps; therefore, different transcription factors can play an important role as regulators of these pathways. Recently, a list of transcription factors that regulate leaf senescence in *Arabidopsis* were reported (Balazadeh et al. 2008). The search for tentative orthologous genes in the *Sunflower Unigene Resource (SUR v1.0)*, using BlastX algorithm (Altschul et al. 1990), led to the identification of 42 genes with a significant score value to transcription factors like NAC, MYB, WRKY, ARP among others. Many of these genes are being studied by qPCR, in order to bring new insights for putative new candidate genes acting as possible TFs regulators of the leaf senescence cascade.

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