Chapter 3

Sunflower Leaf Senescence: A Complex Genetic Process with Economic Impact on Crop Production

Sebastián Moschen, Agustín I. López Gialdi, Norma Paniego, Paula Fernandez and Ruth Amelia Heinz

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Abstract

Leaf senescence is a complex process controlled by multiple genetic and environmental variables. In different crops, a delay in leaf senescence has an important impact on grain yield trough the maintenance of the photosynthetic leaf area during the reproductive stage. In sunflower (*Helianthus annuus* L.), the fourth largest oil crop worldwide, senescence reduces the capacity of plants to maintain their green leaf area for longer periods, especially during the grain filling phase, leading to important economic losses.

In crop species, taking into account the temporal gap between the onset and the phenotypic detection of senescence, identification of both, candidate genes and functional stay-green are indispensable to enable the early detection of senescence, the elucidation of molecular mechanisms and the development of tools for breeding applications.

In this chapter a comprehensive literature revision of leaf senescence process not only in model plant species but also in agronomical relevant crops is presented. Results derived from system biology approaches integrating transcriptomic, metabolomic and physiological data as well as those leading to the selection and characterization of stay green sunflower genotypes are included, making an important contribution to the knowledge of leaf senescence process and providing a valuable tool to assist in crop breeding.

Keywords: sunflower, candidate genes, leaf senescence, stay-green genotype, molecular markers

1. Introduction

Leaf senescence is an age-dependent complex process at the cellular, tissue, organ or organism level, leading to death at the end of the life span [1]. Annual plants as grain and oil crops undergo a visual process toward the end of the reproductive stage that is accompanied by nutrient remobilization from leaf to developing seeds [2]. Senescence process is controlled by multiple genetic and environmental variables, which has strong impact on crop yield [3]. Environmental factors such as light, nutrient availability, concentration of CO_2, abiotic and biotic stresses caused by disease may affect the rate of senescence. In this context, not only environmental conditions but also biotic factors influence senescence, being this an irreversible process prematurely induced under these adverse conditions [4]. Moreover, reproductive growth is mentioned as a factor that usually impacts on leaf senescence, and particularly in sunflower, the lack of sinks delays the onset of senescence [5]. During this process, changes in gene expression result in a metabolic shift from anabolism to catabolism, which leads to decreased photosynthetic activity, progressive degradation of cellular structures and oxidative burst [6–8]. It has been documented that a delay in leaf senescence has a substantial impact on grain yield through the maintenance of the photosynthetic leaf area during the reproductive stage in different crops [3, 9, 10].

In sunflower (*Helianthus annuus* L.), the largest important oil crop worldwide, the senescence process reduces the capacity of plants to maintain their green leaf area for longer periods, especially during the grain-filling phase, affecting the yield and thus leading to economic losses [11, 12]. This production constraint has deepened since sunflower crop production has been gradually moved to marginal areas due to the rapid change of agricultural practices in crops such as soybean and maize, which have greatly increased their cultivated areas as a consequence of favorable commodity prices and because farmers found more profitable to sow transgenic crops with resistance to herbicides and insects [13, 14].

During the last years, many efforts have been achieved to build up useful functional genomics tools for cultivated sunflower involving physiological, transcriptional and metabolic profiles [15–23].

In crop species, considering the temporal gap between onset and phenotypic detection of senescence process, the availability of candidate genes and molecular markers to the early detection of senescence is indispensable to discriminate between early-senescing and late-senescing lines to be applied in the different context of breeding activities [24]. For example, the identification of functional stay-green genotypes for breeding applications and/or for elucidating molecular mechanisms involved in this complex trait.

2. Senescence and crop yields: stay-green genotypes

Senescence is an essential process for the normal growth and development of plants, being an important mechanism for the adaptation to several environmental conditions.

The hypothesis that a delay leaf senescence increases the productivity may be valid for most crops with regard to total biomass production and tuber crops, but this assumption is more controversial with respect to seed yields [3]. However, it has been documented that a delay in leaf senescence has a high impact on grain weight and quality in different crops, including sunflower [3, 5, 25, 26].

Stay-green is a regular term given to genotypes in which the senescence phenotype is delayed in comparison with a standard reference genotype. Stay-green genotypes could be classified into five different types taking into consideration functional or cosmetic stay-green [27, 28]. Functional stay-green genotypes have a photosynthetically active leaf area showing a delay in the onset of senescence (class A), or differing in the rate of the process (class B), whereas cosmetic stay-green genotypes are those in which the senescence proceeds normally but they show problems in chlorophyll degradation (class C), or the chlorophyll content does not decline due to rapid tissue death (class D), or they have a higher chlorophyll content with no change in onset or rate of senescence development (class E) [28]. Mature leaf is a net contributor of photosynthates to the whole plant. The carbon capture phase of the leaf is followed by a net organic nitrogen remobilization. The transition from carbon capture to nitrogen remobilization corresponds to the functional initiation of senescence [29]. In this sense, functional stay-green genotypes are present in those genotypes in which the C–N transition is delayed, or the transition occurs but the subsequent yellowing and N remobilization run slowly [29–31].

In this sense, functional stay-green genotypes could intercept more radiation, increasing photosynthesis and yield in crops with seeds rich in carbon compounds. However, a delay in the C–N transition could negatively affect the seed quality in crops with seed rich in protein compounds [30], such as soybean [32] and cowpea [33].

3. Study of leaf senescence process in sunflower

Sunflower is the fourth most important oil crop worldwide and the second one in the Argentine. Moreover, Argentina is the third largest exporter of crude oil and the second of protein and pellet flour. The added value of oilseed industrialization contributes in the economy with US$ 1400 million approximately, with a total production between 3.2 and 3.8 million tons of grain annually [34].

Sunflower is an annual monocarpic species in which reproductive phase exerts a strong control on leaf senescence and nutrient remobilization, affecting grain weight [35]. Potential yields of sunflower crop are far from the real ones in all Argentina productive regions. In Balcarce location, for instance (Southeast of Buenos Aires province), one of the best productive regions of Argentina, while the potential yields are estimated in 5000 kg ha^{-1}, those obtained by the best producers only reach 3000 kg ha^{-1}, and the average in the region ranges in 1800 kg ha^{-1} [36]. Among the factors that contribute to the productivity gap, one of the most important is the inability of current hybrids to keep their green leaf area for long periods, limiting the incident

radiation capture during the grain-filling period and impacting negatively on the yield and oil concentration [12, 37].

Sunflower genome complexity characterized by of long and highly similar repeats has slowed the pace of getting a complete genome reference. Recently, a high-quality assembly comprising 3.6 Gigabases has being achieved by means of PACBIO sequencing [38]. The reference genome together with extensive transcriptomic data from vegetative and floral organs is accessible at https://www.heliagene.org/HanXRQ-SUNRISE. In the last years, our group accomplished a series of transcriptional and metabolic profiling studies that were integrated into physiological, molecular and cytological analysis to contribute to the understanding the senescence process in this crop and breeding genotypes against abiotic constraints [19–23, 39–41].

Through a system biology approach and using a commercial sunflower hybrid, we characterized the leaf senescence process by integrating transcriptomic and metabolomic analyses using both glasshouse and field conditions [22]. Our results revealed early metabolic changes before to anthesis in the absence of the onset of the first visual senescence symptoms, with more pronounced changes observed when physiological and molecular variables were assessed under field conditions (**Figure 1**). Metabolite remobilization from mature and senescent leaves to the different sinks, particularly into seed development, affects their quality and quantity and is one of the most important aspects of crop improvement [3]. In this study, we showed a decrease of photosynthetic activity and cell growth before anthesis, whereas sucrose, fatty acid, nucleotide and amino acid metabolisms increased. The role of sugars in senescence has been widely discussed in recent years. Sugars are central elements of the source-sink relationships [42, 43] and have been reported as growth [44] and photosynthetic rate regulators [45]. However, the effect of sugars on senescence is controversial and differs between different species [1, 46, 47]. In sunflower, sugar content decreases during leaf development (**Figure 1**). This finding is in line with previous studies in tomato and higher plants, in which the photosynthetic rate dropped together with sugar levels in a mature leaf [48–50]. Furthermore, sunflower is a plant with a high demand for nutrient, especially sugars as substrate for oil synthesis, during the grain-filling phase. Likewise, low levels of sugars may increase production and/or ethylene sensitivity, which acts as senescence enhancer [51, 52].

Pathways related to nutrient recycling processes were also up-regulated. We found high expression levels of enzymes involved in recycling, such as asparagine synthetase and glutamine synthase, as well as the associated metabolites, asparagine and glutamine. These amino acids are involved in nitrogen and carbon transport between the different organs and are the most abundant amino acids in the xylem and phloem [53, 54], indicating a high recycling activity at early stages of leaf development.

Transcription factors (TFs) are key proteins involved in the regulation of gene expression and signal transduction networks, regulating different biological processes and their function is crucial for triggering and/or controlling the different aspect of senescence process. Members of the NAC, AP2-EREBP, HB, bZIP and MYB transcription factor families showed high expression levels, and their expression level was highly correlated, suggesting their involvement in sunflower senescence. These results are in agreement with previous results described for *Arabidopsis thaliana* [55]. Particularly, we found a transcript with high sequence identity

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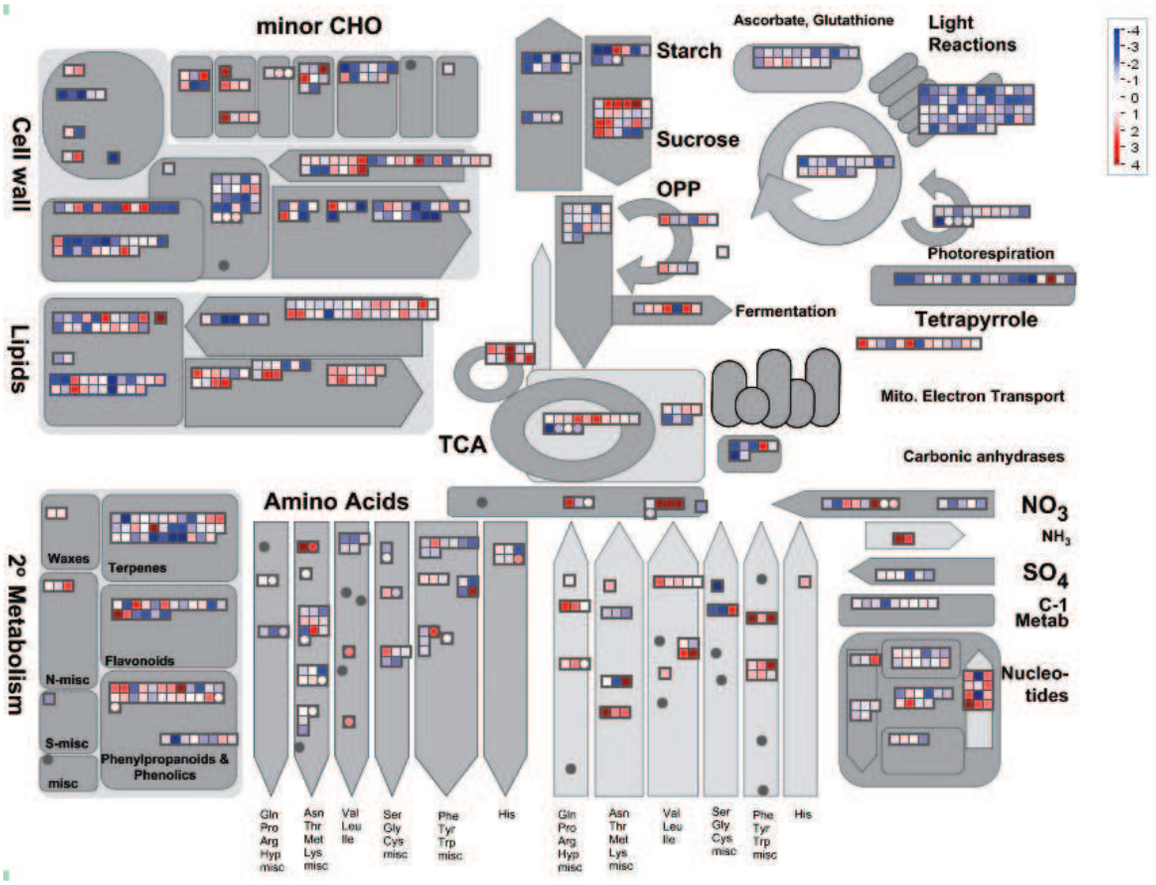

Figure 1. Metabolism overview in the field experiment at pre-anthesis time. Genes and metabolites are represented by squares and circles, respectively. Color intensity corresponds to the expression ratio at logarithmic scale (red: up-regulated, blue: down-regulated) [22].

to *ANAC02* or *ATAF1*. This transcript might be associated with an upstream regulation of the signaling pathway involving ORE1 and EIN2 [56], thus activating their expression and inhibiting the expression of Golden2-like (GLKs) genes, which are necessary for chloroplast development and maintenance [57]. ORE1 also acts as an antagonist of GLK protein, adding more complexity to this regulation pathway [57]. In *A. thaliana*, ORE1 TF induces leaf senescence [58]. In addition, the micro-RNA *miR164* suppress *ORE1* transcript levels; *miR164* and *ORE1* may be regulated in a loop that would also involve EIN2, where EIN2 would promote the expression of ORE1 and would inhibit *miR164* [59]. In a previous work conducted in sunflower, expression profiles of candidate genes *Ha-EIN2* and *Ha-NAC01* (with high sequence identity to *ORE1*) were evaluated together with *miR164* levels [21] showing similar expression patterns to Arabidopsis and in line with the increase in the nutrient remobilization rate.

Moreover, using bioinformatic approaches and evaluating two different approaches for gene expression correlation analysis: Weighted Gene Correlation Network Analysis (WGCNA) and BioSignature Discoverer (BioSD, Gnosis Data Analysis, Heraklion, Greece), we integrated transcriptomic and metabolomic data [39]. WGCNA allowed the detection of 10 metabolites and 13 TFs (**Figure 2**), whereas BioSD allowed the detection of one metabolite and six TFs as potential biomarkers. Comparative analysis demonstrated that three transcription factors were detected

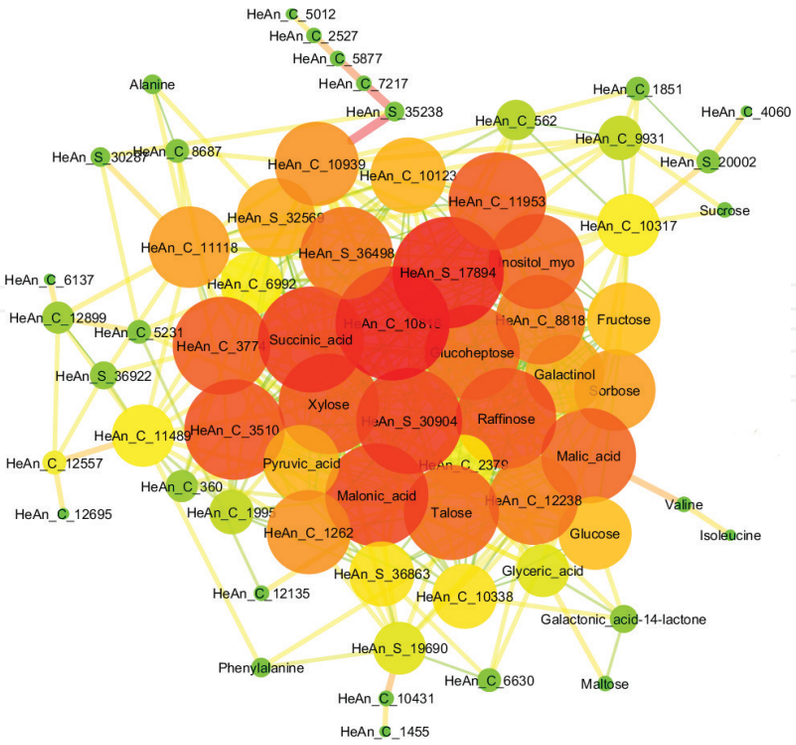

Figure 2. Integrated network of hubs metabolites and transcription factors detected by WGCNA [39]. 24 selected hubs metabolites and 82 TFs statistically significant during senescence were correlated (p-value < 0.0001) and visualized in Cytoscape [60] by degree (node size and color) and edge weight (edge size and color).

by both approaches, highlighting them as potentially robust biomarkers associated with leaf senescence in sunflower. This complementary use of network and BioSignature Discoverer analysis of transcriptomic and metabolomic data provided a useful tool for identifying candidate genes and metabolites, which may have a role in the triggering and development of the leaf senescence process.

Transcriptomic analysis in combination with metabolic profile is a comprenhensive tool for the study of leaf senescence. These results suggest a complex regulatory network underlying this process. Thus, the identification of regulatory networks based on expression profiling is an important starting point for the detection of new key genes involved in the triggering of the senescence process in this crop.

4. Selection of stay-green genotypes in sunflower

Given the importance of the stay-green genotypes selection for studies of this complex character, we performed a screening and selection of contrasting sunflower genotypes associated with early leaf senescence process evaluating 135 different genotypes from the INTA Sunflower Breeding Program (INTA Manfredi Sunflower Germplasm Collection) [61] growing under field conditions through a physiological, cytological and molecular approach.

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Physiological measurement of growing cycle, anthesis time, number of leaves, plant size, evolution of total/dry leaf, green leaf area at anthesis time and SPAD was performed to select pairs of contrasting genotypes with very similar plant architecture, phenology and leaf area until anthesis, but with different senescence rate. This analysis allowed us to select 10 genotypes that were further evaluated, allowing the identification of two contrasting senescence inbred lines, R453 (early senescence genotype) and B481-6 (putative stay-green genotype) [23] (**Figure 3**).

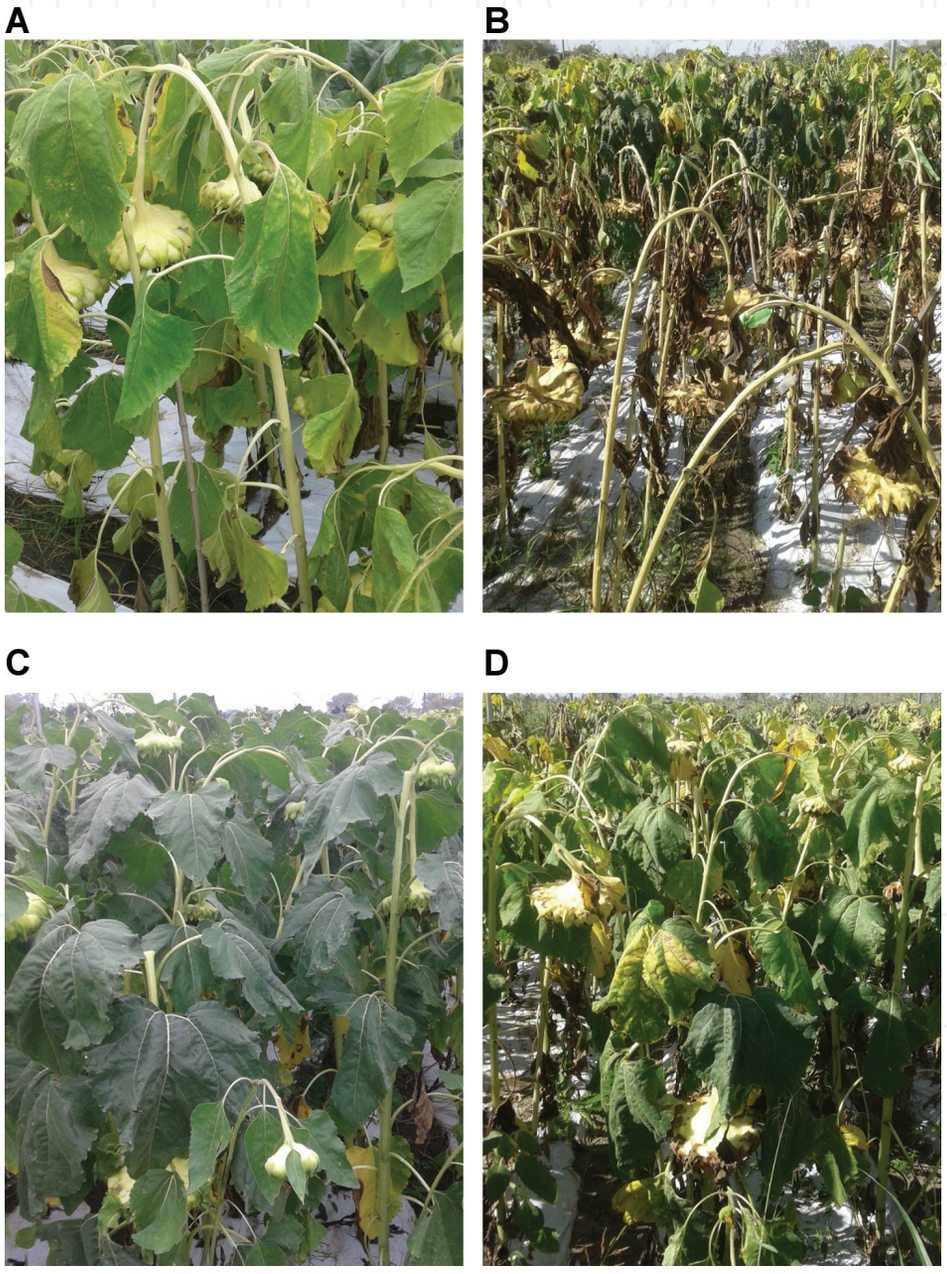

Figure 3. Phenotypic analysis of sunflower genotypes under field experiment. (A) and (B) early senescence genotype R453. (C) and (D) Stay-green genotype B481-6. Images (A) and (C) correspond to 15 days after anthesis and images (B) and (D) 30 days after anthesis.

At the physiological level, green leaf area evolution (GLA) is an indirect measurement of photosynthetically active leaf area, and its decrease has been reported as product of active chloroplast degeneration and chlorophyll degradation.

R453 and B481-6 displayed similar GLA evolution until anthesis. Then, GLA decreased abruptly in R453 and faster than the B481-6 genotype. GLA decline in B481-6, on the other hand, was gradual and reached complete senescence at 200 ºCdAE later (**Figure 4A**). In sunflower, and many other monocarpic species, this senescence symptom is evident after anthesis, during grain-filling period, and is mainly due to source-sink relationships established at this stage of development [5, 20, 36, 62]. Radiation interception at the canopy level showed similar patterns with an early decrease in the early senescence genotype (**Figure 4C**). In grain crops, a delay in leaf senescence should have a positive impact on grain yield [3, 26]. Yield components were evaluated displaying significant differences in yield, with higher seed weight in the stay-green genotype (**Figure 4B**). These observations are in agreement with the expected for this trait suggesting a type B stay-green phenotype [28]. Moreover, photosynthesis measurement was performed 15 days after anthesis showing higher photosynthesis rate in the stay-green genotype (**Figure 4D**), supporting this finding.

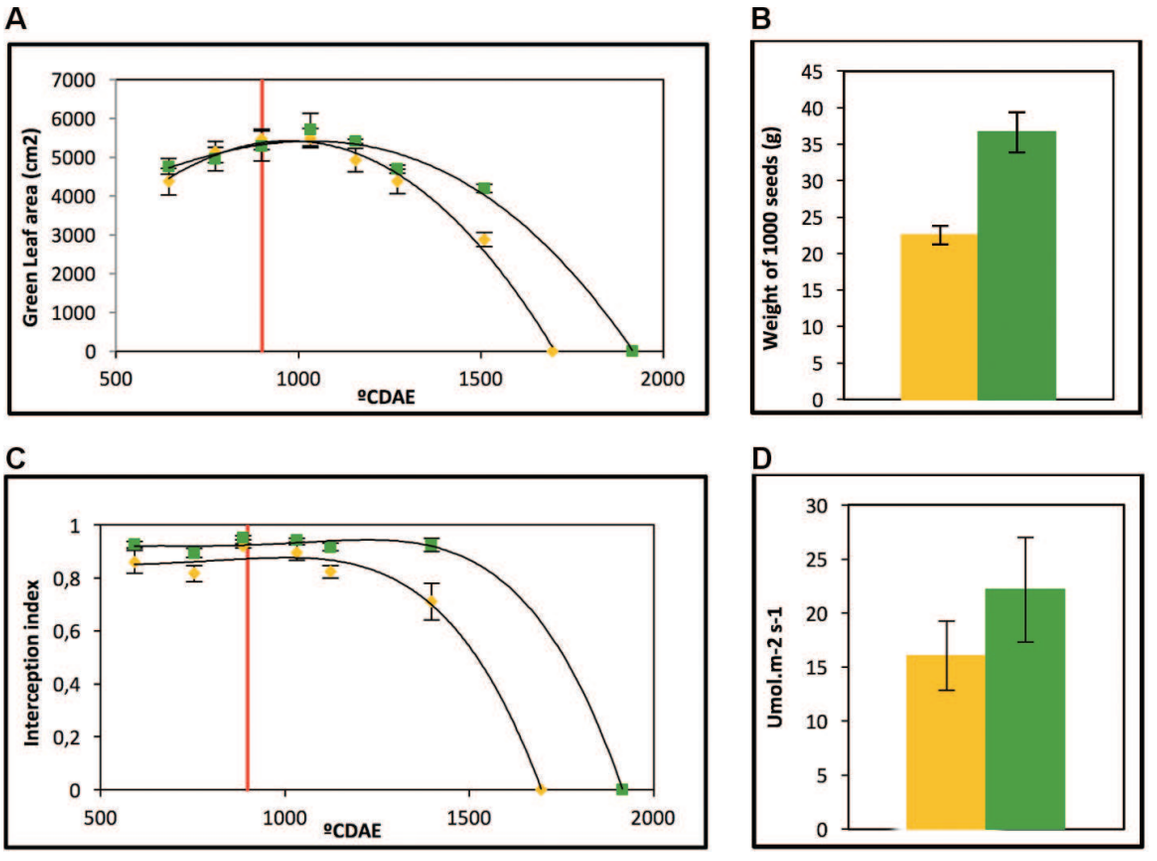

Figure 4. Physiological analysis of contrasting genotypes. R453 in orange and B481-6 in green colors. (A) Green leaf area (GLA); (B) yield; (C) radiation interception and (D) photosynthesis. ºCdAE indicates thermal time after emergence. Red line in (A) and (C) indicates anthesis time for both genotypes.

To confirm the senescence phenotype of the selected genotypes, we also performed cytological and molecular analysis. As senescence involves programed Cell Death (PCD), nuclear DNA degradation associated with PCD can be detected in situ by TUNEL assay. In this sense, we analyzed mesophyll cells nuclei of both genotypes 10 days after anthesis. R453, with premature senescence phenotype, also showed TUNEL-positive nuclei, whereas B481-6, with stay-green phenotype, had TUNEL-negative nuclei (**Figure 5**). Mesophyll is the most photosynthetically active tissue of higher plants, having cells with high chloroplast and chlorophyll content and the cells in this tissue are affected firstly during senescence [9, 62, 63]. TUNEL-positive nuclei detection indicates that senescence process has already started in the early senescence genotype.

The gene expression pattern of the senescence-associated candidate transcription factors was evaluated for differences in the timing of senescence pathways activation between the early senescence and stay-green genotype [21, 22]. HaNAC01, HaNAC03 and HaNAC05 transcription factors were evaluated at two different times, 5 days after anthesis (DAA) and 15 days after anthesis (**Figure 6**). R453 showed higher expression levels of the three NAC transcription factor than B481-6, and its expression increased by 15 days post-anthesis.

Altogether, these findings highlight these genotypes as interesting potential candidates for further analysis of leaf senescence in sunflower. The B481-6 genotype showed a stay-green phenotype, also evidenced by cytological and molecular analysis and an increase of seed weight,

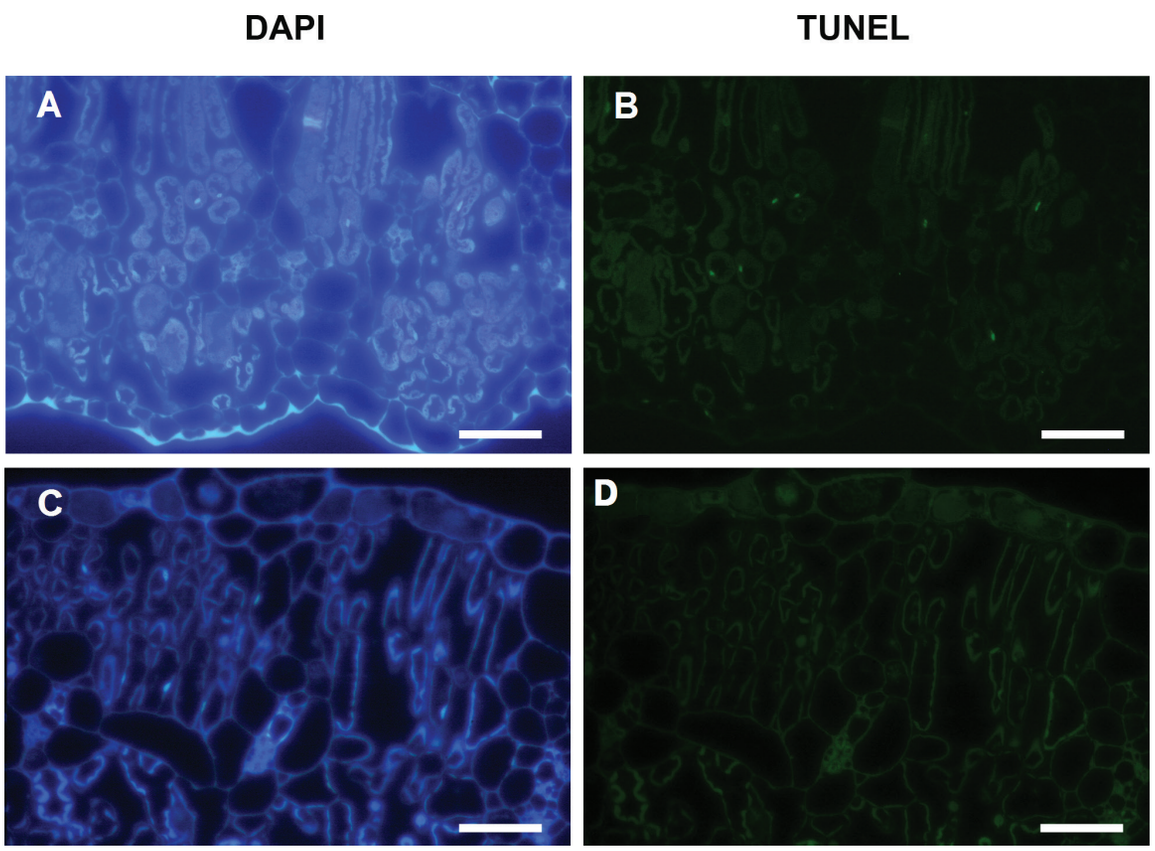

Figure 5. TUNEL assays of selected contrasting genotypes. (A) and (C): Nuclei visualization by DAPI staining. (B): TUNEL-positive nuclei in mesophyll cells of premature senescence genotypes (R453). (D): TUNEL-negative nuclei in stay-green genotype (B481-6) [23].

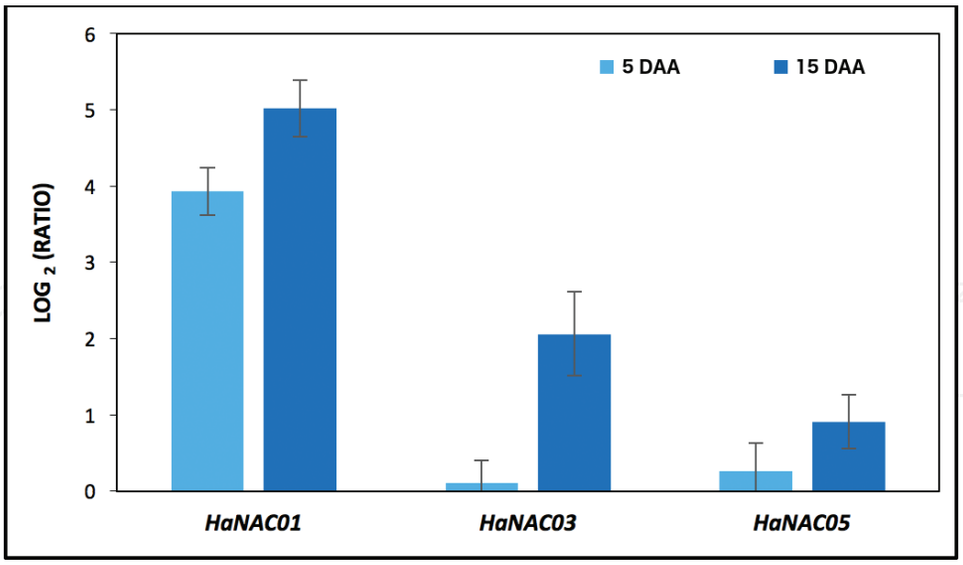

Figure 6. qPCR analysis of NAC transcription factors candidate genes. Relative transcript levels are shown as the ratio (log2 scale) between the expression in the early senescence genotype (R453) in relation to the stay-green genotype (B481-6) in two different times 5 days after anthesis (DAA) and 15 days after anthesis [23].

which makes this genotype a potential candidate for the functional stay-green phenotype in comparison with R453 genotype.

5. Conclusions and perspectives

Integration of transcriptomic and metabolomics data arises as a powerful approach to identify pathways and candidate genes related to the senescence process in sunflower, an economically important oil crop without previous molecular information about this process. The results discussed in this chapter provide an important start point for understanding the senescence process and open new insights to explore alternative strategies and possibilities.

Moreover, by a combination of physiological, cytological and molecular analysis, we identified two senescence contrasting genotypes. B481-6 genotype showed a delay in senescence symptom evaluated both, under physiological and molecular measurement. This senescence delay, together with an increase in photosynthesis rate, leads to an increase in yield, highlighting this genotype as functional stay-green. These results together with a better understanding of the onset of the process will in turn impact on the development of different senescence management strategies and could help controlling the grain-filling process. All in all, these advances provide a valuable tool to assist in crop breeding, which represents a significant challenge for the future of agriculture attending to the increase in both, world population and climate risks that affect productivity.

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Author details

Sebastián Moschen[1,2,3], Agustín I. López Gialdi[2], Norma Paniego[1,2], Paula Fernandez[1,2,3] and Ruth Amelia Heinz[1,2,4]*

*Address all correspondence to: heinz.ruth@inta.gob.ar

1 Instituto de Biotecnología, CICVyA, Instituto Nacional de Tecnología Agropecuaria, Hurlingham, Buenos Aires, Argentina

2 Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, Ciudad Autónoma de Buenos Aires, Argentina

3 Escuela de Ciencia y Tecnología, Universidad Nacional de San Martín, San Martín, Buenos Aires, Argentina

4 Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina

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