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Identification and characterization of contrasting sunflower genotypes to early leaf senescence process combining molecular and physiological studies (*Helianthus annuus L.*)

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Highlights

- First study on sunflower senescence genotypes integrating molecular, cytological and physiological approaches.
- Two senescence contrasting sunflower genotypes were selected.
- Relevant candidate genes were biologically validated.
- Cytological techniques (Tunel, DNA Ladder and Flow sorting) were optimized for this crop.

Abstract

Leaf senescence is a complex mechanism ruled by multiple genetic and environmental variables that affect crop yields. It is the last stage in leaf development, is characterized by an active decline in photosynthetic rate, nutrients recycling and cell death. The aim of this work was to identify contrasting sunflower inbred lines differing in leaf senescence and to deepen the study of this process in sunflower. Ten sunflower genotypes, previously selected by physiological analysis from 150 inbred genotypes, were evaluated under field conditions through physiological, cytological and molecular analysis. The physiological measurement allowed the identification of two contrasting senescence inbred lines, R453 and B481-6, with an increase in yield in the senescence delayed genotype. These findings were confirmed by cytological and molecular analysis using TUNEL, genomic DNA gel electrophoresis, flow sorting and gene expression analysis by qPCR. These results allowed the selection of the two most promising contrasting genotypes, which enables future studies and the identification of new biomarkers associated to early senescence in sunflower. In addition, they allowed the tuning of cytological techniques for a non-model species and its integration with molecular variables.

Abbreviations: GLA, green leaf area; GFD, grain filling degree; DAE, days after emergence; °CdAE, °C days after emergence; PPC, percentage of polyploidy.

Keywords: *Helianthus annuus L.*, leaf senescence, TUNEL, qPCR, flow cytometry, physiological analysis.

1. Introduction

Senescence is the last stage in plant development and a mechanism with high impact in crop yield that can be triggered by both internal (ageing, flowering, phytohormones) and environmental factors (temperature, drought, nutrient deficiency, shading, biotic stresses) [1–8]. In addition, this stage is an active, genetically controlled and reversible process until all nutrients have been recycled and ends in cell death [9–13]. During this process, changes in gene expression result in metabolic shift from anabolism to catabolism, which leads to decreased photosynthetic activity, active degradation of cellular structures and oxidative burst [14–16]. In annual plants, such as grain and oil crops, flowering induces senescence accompanied by nutrient remobilization from leaves to developing seeds [17]. Prematurely induced senescence can reduce crop yield. Thus, leaf senescence has an economic impact and affects the potential and real yields gap.

Sunflower (*Helianthus annuus L.*) is the third most important source of edible vegetable oil worldwide and the second in Argentina. This crop also provides an important source of biodiesel [18,19].

Sunflower is an annual monocarpic species in which reproductive organs exert a strong control on leaf senescence and nutrient remobilization, affecting grain weight by the source:sink ratio [20]. The age of a leaf and its position on the stem also affect the triggering of senescence and the rate of nutrient remobilization [8,21]. Moreover, a delay in senescence has a great impact in grain weight and yield of important crops, including sunflower [22,23] due to the maintenance of photosynthetically active leaf area during reproductive stage [24]. Hence, grain filling percentage per capitulum is a component that contributes to overall yield of sunflower crop [25] and it might be increased in crops that retain its photosynthetically leaf area for a longer period of time. This finding emphasizes the need to search for mutants that retain leaf greenness, also known as stay-green genotypes [26]. Functional stay-green shows delayed leaf senescence onset or altered senescence rate maintaining photosynthetic activity for longer periods, whereas cosmetic stay-green decreases the photosynthetic activity normally, but shows chlorophyll degradation problems, or higher chlorophyll content, without changes in senescence onset or rate [27]. In sunflower, senescence contributes to the gap between potential and real yield [28]. Such differences could be increased because of the inability of current hybrids to retain its green leaf area for a longer period, especially during the grain filling phase; therefore, senescence onset and rate are key determinants of yield and oil content in sunflower [29,30].

Although sunflower complete genome sequence is not available yet (http://sunflowergenome.org/early_access/repository/main/genome/index.html), functional genomics tools for cultivated sunflower have been developed, including transcriptional and metabolic profiling strategies as well as integrated bioinformatics analysis [18,21,31–41]. Recently, a characterization of leaf senescence process in sunflower was developed through a systems biology approach integrating transcriptomic and metabolomic analyses, thus allowing the detection of early metabolic changes prior to anthesis and before the onset of the first senescence symptoms [42].

Leaf senescence process induces changes at different organizational levels. At the morphological level, chlorophyll and chloroplast degradation are part of the earliest events taking place during senescence and this degradation results in a gradual yellowing that constitutes a visual marker of the process in leaves [4]. At the metabolic level, the photosynthetic activity is markedly reduced as consequence of plastidic protein degradation [7,43].

At the genetic level, many differentially expressed genes during senescence have been identified in *Arabidopsis thaliana* and recently validated as senescence associated genes (SAGs) and senescence down-regulated genes (SDGs) in sunflower [4,14,17,21], and other non-model species, such as rice [44,45], soybean [46], wheat [47], poplar [48], barley [49], rapeseed [50], maize [51], Petunia [52], tobacco [53] and cotton [54]. Some of these differentially expressed genes code for degradative proteins such as vacuolar processing enzymes (VPEs) [55], RNAses [56], lipases [57], and transport proteins (ABC transporters, aminoacid permease and cation exchanges) [14,58,59]. These findings evidence the metabolic shift from anabolism to catabolism and the recycling nature of senescence. Nucleic acid degradation also takes place during senescence, which has been studied by DNA laddering and TUNEL assays in *A. thaliana*, barley, wheat, cotton, tobacco, quinoa and sunflower [60–65]. Production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have also been reported during senescence [17,66,67], thus demonstrating the stressful environment of senescent tissues.

At the cytological level, non-mature cells endoreduplicate until they reach the mature state, when senescence takes place [68]. The existence of senescence associated vacuoles (SAVs) with intense proteolytic activity, in senescing leaves of soybean, Arabidopsis and tobacco, associated with chloroplast protein breakdown was recently reported; which suggests a potential role for autophagy in senescence [69,70].

Given the temporal gap between onset and phenotypic detection of senescence, molecular markers

are indispensible to enable the early detection of senescence [71] and, at the same time, determine if the stay-green genotypes are functional or not. Moreover, the senescence process should be measured with several markers that cover various aspects of senescence physiology. To this purpose, different physiological, cytological and molecular markers of senescence have been proposed in different species including quantification of chlorophyll content [21,72,73], photochemical efficiency [72], SAGs expression and DNA degradation detection (e.g. TUNEL [74]) [72], among others.

Given the importance of having contrasting genotypes for molecular studies of complex characters, the aim of this work was to identify contrasting sunflower genotypes associated to early leaf senescence process growing under field conditions through a physiological, cytological and molecular approach. The results from this study contribute to the knowledge of senescence process in sunflower and may facilitate future studies through the utilization of contrasting genotypes for this process.

2. Materials and methods

2.1. Plant material and experimental conditions

A field experiment was carried out at INTA Balcarce Experimental Station (37°45' S, 58°18' W) during 2012/13 growing season. During this period, 150 sunflower inbred genotypes from the Instituto Nacional de Tecnología Agropecuaria (INTA) Sunflower Breeding Program, previously characterized were sown and evaluated for senescence phenotype [75,76]. Ten of these genotypes had been previously selected as candidate contrasting genotypes (grouped in 5 contrasts according to plant phenology and architecture) by physiological measurements and were used at the present study.

Selected inbred lines from the INTA Sunflower Breeding Program, INTA Manfredi Sunflower Germplasm Collection and INTA Manfredi Sunflower Breeding Site were used (Table 1). Plants were sown at 7.2 plants/m^2 and cultivated under field conditions at the CNIA INTA Castelar (34°60'48"S, 58°67'33"W). Diseases, weeds as well as insects and bird were adequately controlled. Soil fertility assured maximum yields under non-limiting water conditions and soil water was maintained by irrigation.

Time was expressed on a thermal time basis by daily integration of air temperature with a threshold temperature of 6 °C and with plant emergence as thermal time origin [77].

The experiment was conducted with 3 randomized complete block design considering each one

with 3 replicates (plant–plots). Each plots composed of 10 plants of each genotype.

Additionally, a greenhouse experiment was carried out at the Biotechnology Institute of INTA. Four genotypes, selected because of their contrasting behavior during senescence in the field experiment, were sown in 10 liters pots with 10 biological replicates each genotype. Plants were cultivated under non-limiting water conditions and pot water content was maintained by irrigation. Pots contained a substrate composed by 50% of peat moss from Tierra del Fuego (Argentina), 20% of composted pine needle, 20% of common soil and 10% of perlite. Temperature was maintained between 20°C and 28 °C and the photoperiod was established in 16 h light (sodium lamp of 400 W) and 8 h darkness. Fertilization was performed by application of Hakaphos triple 18 (red container, 350 ppm) which provides nitrogen, phosphorous and potassium. Diseases and insects were adequately controlled.

2.2. Physiological parameters

2.2.1. Field experiment

Six plants of each genotype (two of each plot) were tagged and the evolution of green leaf area was assessed by periodic measurements of maximum width of each leaf of this plants. Percentage of greenness was estimated visually, always by the same observer, by comparing the relation between green and yellow parts of each leaf (from 100% to 0%). Green leaf area (GLA) was calculated per plant:

$$GLA\ (cm^2) = \frac{(Leaf\ area\ \times Percentage\ of\ greenness)}{100}$$

[25]

Where:

$$Leaf\ Area\ (cm^2) = 1.528\ \times\ (leaf\ width)^{1.7235}$$ [25]

When tagged plants reached the physiological maturity, their capitula were harvested. Seed number and seed weight per capitulum was measured. Yield per genotype was calculated as the weight of 1,000 seeds as follows:

$$Yield\ (g) = \left(\frac{FW\ (g)}{Number\ of\ seeds}\right) \times 1{,}000$$ [25]

Were FW refers to average seed fresh weight per plant.

Grain filling degree (GFD) is an estimation of grain filling percentage, and was estimated by

comparing harvested capitula using a sunflower grain filling scale (provided by Nidera S.A.), which enabled to assign values between 0 and 5.

2.2.2. Greenhouse experiment

Four genotypes were selected from the field experiment and cultivated under controlled conditions in a greenhouse experiment in order to evaluate phenology similarities. Three random plants of each genotype were tagged and physiological measurements such as maximum GLA, anthesis time, number of leaf and plant sizes were measured.

2.3. TUNEL assay

DNA strand breaks were detected by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) [74] using *in situ* Cell Death Detection Kit, TMR red (Roche, Basel, Switzerland). The youngest leaf (greater than 4 cm width) of 3 plants per genotype (field experiment) was sampled 10 days after anthesis and fixed in 4% PFA in 0.1 M PBS (pH 7.4) (for 4 h at 4 °C), dehydrated in an ethanol series (40% to 70%; 1 ml/15 min each concentration) and embedded in LR White® resin (Polysciences inc, Warrington, PA, USA). Semi-thin sections (1–2 μm thick) were obtained by cutting with a tungsten knife (Leica 2155 microtome, Leica Microsystems, Wetzlar, Germany). TUNEL labeling was performed according to the manufacturer's instructions. Briefly, sections were permeabilized with 20 μg ml^{-1} proteinase K for 20 min at room temperature and washed 4 times with PBS (0.1 M, pH 7.4). The labeling reaction was performed at 37 °C in a dark, humid chamber for 1 h. A negative control was included in each experiment without TdT enzyme in the reaction mixture. Permeabilized sections were incubated with DNase I (3 U ml^{-1}) for 15 min before the TUNEL assay as a positive control. Counter-staining was done with 0.02 mg ml^{-1} 4′,6-diamidino2-phenylindole (DAPI) staining. Sections were mounted using the Citifluor™ mounting medium (EMS, Hatfield, PA, USA).

TUNEL images were obtained by epifluorescence with an Axioskope 2 microscope (Carl Zeiss, Jena, Germany). The following filters were used to examine the fluorescent samples: DAPI filter (excitation 340–390 nm, emission 420–470 nm) and rhodamine filter (excitation 540–552 nm, emission 575–640 nm). Images were captured with a Cannon EOS 1000 D camera (Tokyo, Japan) and analyzed using the AxioVision 4.8.2 software package (Carl Zeiss, Jena, Germany).

2.4. DNA isolation and electrophoresis

Five days after anthesis, the tenth leaf (numbered from the bottom to the top of the plant) of 3 plants per genotype (field experiment) were harvested, frozen in liquid nitrogen and stored at -80 °C until

use. Genomic DNA was isolated from 100 µg of ground frozen samples using the NucleoSpin Plant II® kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions.

DNA was quantified in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the concentration was adjusted to 4 µg μl^{-1} by diluting with Chromasolv® water (Sigma-Aldrich, St. Louis, MO, USA) or concentrating with a Speed vac concentrator (Eppendorf AG, Hamburg, Germany).

The DNA from each sample was separated on a 0.8% (w/v) and 1.5% (w/v) agarose gel at 35 V for 20 h and stained with ethidium bromide. Images were obtained by a Gel Doc XR+ System (Bio-Rad, CA, USA).

2.5. RNA isolation and quality controls

The tenth leaf of 3 plants per genotype (field experiment) was harvested 5 and 15 days after anthesis, frozen in liquid nitrogen and stored at -80 °C until use. High quality total RNA was isolated from 100 mg of tissue using TriPure (Roche Diagnostics Inc, Basel, Switzerland) and following the manufacturer's instructions. Genomic DNA was eliminated by an enzymatic treatment using DNase I (Invitrogen, Argentina). RNA concentration was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The purity and integrity of total RNA was determined by 260/280 nm ratio and the integrity was assessed by electrophoresis in 1.5% (w/v) agarose gel.

2.6. Quantitative RT-PCR analysis

For each sample, 1 µg DNase treated RNA was reverse-transcribed using Superscript® III First-Strand Synthesis SuperMix kit (Invitrogen, USA) and random hexamer primers, according to the manufacturer's instructions.

qPCR experiments were carried out in a 12.5-µl reaction mix containing 200 nM of each primer 0.5 µl of cDNA sample and FastStart Universal SYBR Green Master kit (Roche Diagnostics Inc., Basel, Switzerland). Negative controls were incorporated in each assay. Reactions were performed using a 96-well plate thermocycler (StepOne Plus™ Real-Time PCR System and software, Life technologies, Waltham, MA, USA). Thermal profile was set to 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and hybridization temperature for 1 min. The optimization of hybridization temperature for each primer was previously tuned up [78]. Amplicon specificity was verified by melting curve analysis (60 to 95 °C) after 40 PCR cycles. The qPCR assay was carried out using 3 biological replicates for each condition and 3 technical replicates. The expression profile of each

candidate biomarker genes of leaf senescence (*HaNAC01, HaNAC03, HaNAC05* and *HaCAB2*) was estimated in relation to Elongation Factor-1α (EF-1α), which was previously selected as a reference gene [79]. 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 Analysis of quantitative RT-PCR data software (version 11.0) [80]. These profiles were estimated for the early senescence genotype in relation to its late senescence contrasting genotype using fgStatistic software [81], based on previously published algorithms [82].

2.7. Flow sorting analysis

The tenth leaf of 3 plants per genotype (greenhouse experiment) was harvested at 4 different times. Samplings were made 21 days post-emergence (juvenile phase), 49 days post-emergence (pre-anthesis phase), 70 days post-emergence (first post- anthesis phase) and 78 days post-emergence (second post- anthesis phase). A section of 1 cm^2 of each fresh leaf was cut and chopped with a razor blade in 0.5 ml of ice-cold Otto I extraction buffer [83]. The obtained nuclei suspension was then filtered in a 50-µm mesh and the filtered solution was stained by adding of 2 ml of Otto II buffer with DAPI (2 µg ml^{-1}) [83]. Stained nuclei suspension was analyzed in a flow cytometer (CyFlow, Partec, Germany), using an excitation wavelength of 350 nm and detecting an emission wavelength of 450 nm[84].

2.8. Statistical analysis

The analysis of variance was made with InfoStat software v.2014 [85], by applying the least significant difference test of Fisher (LSD Fisher test), 5% probability, to compare between the analyzed plants.

3. Results

3.1. Ecophysiological analysis

For this study, we selected 10 genotypes from a field experiment performed in 2012/2013 growing season and compared phenotypically genotypes with premature senescence against those with delayed senescence. For this analysis, similar development cycles, number of leaves and plant size were considered. Table 2 displays anthesis time and maximum leaf number for both genotypes. All lines showed intermediate phenological cycles.

To compare leaf senescence process between contrasting genotypes, we assessed green leaf area (GLA) evolution. The C154B and c977-b genotypes showed similar GLA evolution from

emergence to anthesis. At 600 °CdAE, GLA began to decline in c977-b genotype until it reached zero close to 1,250 ºCdAE. C154B showed an increase in GLA until 900 ºCdAE (post-anthesis) after which it started to decline until zero close to 1,400 ºCdAE (Fig. 1A). R453 and B481-6 displayed similar GLA evolution until 920 °CdAE. Then, GLA decreased abruptly in R453 and reached zero close to 1,250 ºCdAE. The decrease for B481-6, on the other hand, was gradual and, reached complete senescence at 1,400 ºCdAE (Fig. 1B). The maximum GLA in 2091 was significantly higher than that of 2021 until 1,000 ºCdAE. From this point, GLA started to decline abruptly and reached complete senescence approximately 1,250 ºCdAE and before 2021 (Fig. 1C). In B473-2, GLA was higher than in its contrasting genotype (C829B) in all analyzed times and reached zero simultaneously close to 1,250 ºCdAE (Fig. 1D). B10 showed higher GLA than its contrasting genotype (C818), from 600 °CdAE to 1,100 ºCdAE and reached zero at similar times close to 1,300 ºCdAE (Fig. 1E).

In addition, we assessed the yield components for all genotypes (Table 3). Two pairs of contrasting genotypes R453/B481-6 and C818/B10 displayed significant differences in yield, with higher seed weight in the delayed senescence genotypes. Moreover, in c977-b/C154B; 2091/2012 and B473-2/C829B contrasting genotypes, those with delayed senescence showed lower seed weight, although these differences were not significant (Fig. 1F). Regarding the evaluation of the grain filling, no significant differences were observed in GFD between candidate contrasting genotypes, except for C818 and B10 genotypes (Fig. 1F).

These results suggested that c977-b/C154B and R453/B481-6 genotypes were the best candidates for contrasting senescence phenotype under field conditions. Therefore, we selected these 2 pairs of genotypes for further analysis. Considering that field experiment may incorporate highly uncontrolled variation sources in the assay, we performed a greenhouse experiment with these 4 genotypes to compare their phenology (anthesis time, maximum leaf number, and phenological cycle) (Table 2).

Under greenhouse conditions, c977-b and C154B flowered with 3 DAE of difference, whereas in R453 and B481-6, the difference was of only 1 DAE (Table 2). GLA and maximum leaf number were measured at anthesis time. No significant differences were observed in GLA at anthesis time remained in both pairs of contrasting genotypes. However, both pairs showed very similar number of leaves, although c977-b and C154B significantly differed in maximum leaf number.

Altogether, these results indicate that c977-b/C154B and R453/B481-6 are the best candidate contrasting genotypes. In this sense, we selected them for further molecular analysis.

3.2. Cytological and molecular analysis

To confirm the senescence phenotype of the selected genotypes, we also analyzed these genotypes by cytological and molecular approaches.

As senescence involves PCD, nuclear DNA degradation associated with PCD can be detected *in situ* by TUNEL assay. For this reason, we analyzed mesophyll cell nuclei, corresponding to 4 selected genotypes, by visualizing them with DAPI staining (Fig. 3A, 3C, 3E and 3G). Apparently only c977-b and R453, with premature senescence phenotype, showed TUNEL-positive nuclei, whereas C154B and B481-6, with delayed senescence phenotype, had TUNEL-negative nuclei (Fig. 3B, 3D, 3F and 3H). No TUNEL-labeling was detected in negative control (Fig. 3I). To complement TUNEL assay, we then performed DNA gel electrophoresis assays. Candidate contrasting genotypes displayed differential DNA migration patterns, by showing more DNA degradation in premature senescence genotypes (c977-b and R453), (Fig. 3J and 3K).The percentage of polyploid cells (PPC) was compared by flow sorting and at different times between candidate contrasting genotypes. The genotypes R453 and B481-6 showed decrease in PPC over time but, at pre-anthesis and first post-anthesis phases, B481-6 exhibited significantly higher PPC (Fig. 4A); which indicates higher number of viable cells in this genotype with delayed senescence. Moreover, no difference was observed between c977-b and C154B; these genotypes decreased their PPC as senescence progressed (Fig. 4B). By qPCR assays, we analyzed the expression profiles of *HaNAC01*, *HaNAC03* and *HaNAC05* transcription factor candidate genes at 2 different times for the 4 selected genotypes. R453 showed higher expression levels of NAC transcription factor in relation to B481-6, and its expression increased by 15 days post- anthesis (Fig. 5A, 5B and 5C). NAC genes showed higher transcript levels in c977-b in relation to C154B but its expression decreased by the second sampling time, except for the *HaNAC05* gene (Fig. 5A, 5B and 5C). Differences in *HaCAB2* expression levels between contrasting genotypes were not statistically significant at the evaluated sampling times (Fig. 5D).

4. Discussion

4.1. Phenotypic and physiological analysis

Leaf senescence is a complex and highly coordinated mechanism with substantial effects on crop yield [21] and has been reported in diverse plant species including sunflower [22–24]. The onset and progression of leaf senescence process is genetically controlled and involves changes in gene expression [21]. In this sense, gene expression analysis is a useful method to identify candidate genes with high impact in crop improvement [17,18,21,58,59,86].

In this study, we evaluated the evolution of senescence process in 10 sunflower contrasting inbred genotypes by physiological, cytological and molecular approaches in order to select the best contrasting pair of genotypes for further senescence experiments. We found that c977-b and R453 exhibited premature senescence, whereas C154B and B481-6 showed a delayed senescence phenotype, in agreement with a previous phenotypic field assay.

At the physiological level, GLA evolution is an indirect measurement of photosynthetically active leaf area [87] and its decrease has been reported as product of active chloroplast degeneration and chlorophyll degradation [4,8]. In monocarpic species, such as sunflower, 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 [4,22,25,38,88]. According to these reports, c977-b/C154B and R453/B481-6, which are candidate contrasting genotypes, showed similar GLA at anthesis time, but with significant differences in GLA decrease after the anthesis period, both characteristics being very important to be classified as senescence contrasting genotypes.

Delayed leaf senescence may have a positive impact in grain yield by maintaining photosynthetically active leaf area during reproductive stage of economically important crops [24] including sunflower [22,23]. These observations are in agreement with the grain yield increase observed in B481-6 compared to R453 and suggest a type B stay-green phenotype [27]. However, this effect was not observed in C154B/ c977-b, suggesting that C154B could be a cosmetic stay green phenotype [26,27,89].

Several environmental factors affect seed development, floral initiation and the number of empty seeds [26,29,90–97]. In this study, plants were grown under the same conditions and consequently grain filling differences could be due to genetic variations among genotypes. Indeed, a high source-sink relation (number of leaves, or green area, per seed) has an indirect impact on the number of empty seeds [98].

The phenotypic and physiological results showed R453/B481-6 as the best candidates for senescence trait, with similar phenology and an increase of grain yield in the delayed senescence genotype. Other genotypes were discarded for the lack of phenological similarities and inconsistency with a previous phenotypic field assay.

4.2. Cytological analysis

TUNEL technique allows the detection of *in situ* DNA fragmentation at individual cell level [99]. In this study, we found TUNEL-positive nuclei in mesophyll cells of both c977-b and R453 premature

senescence genotypes and TUNEL-negative nuclei in C154B and B481-6 delayed senescence genotypes. However, TUNEL technique does not discriminate between random fragmentation and oligonucleosome-sized cleavage of DNA as result of PCD [60]. Mesophyll is the most active photosynthetic tissue of higher plants, having cells with high chloroplast and chlorophyll content [100]. In parallel, photosynthetic cells, and specifically mesophyll cells were reported to be affected firstly during senescence [4,8,101,102]. Therefore, detection of TUNEL-positive nuclei in mesophyll cells might indicate that DNA fragmentation could be due to senescence process. The TUNEL results were complemented by genomic DNA gel electrophoresis. In this assay, c977-b and R453 premature senescing genotypes showed more degradation than their candidate contrasting genotypes, C154B and B481-6 respectively. Alternatively, the TUNEL results can be validated by recognizing characteristics of PCD process, such as presence of SAV´s, membrane "blebbing", membrane ion leakage and analysis of both mitochondria and central vacuole morphology and integrity [60,61,72,103,104]. Altogether, these results indicate that c977-b/C154B and R453/B481-6 are contrasting genotypes at a cytological level.

During leaf development, 3 phases can be distinguished: proliferation (high cells division rate), expansion (cells stop dividing but continue to expand) and maturity (cells no longer expand) [105]. Once leaf senescence process is triggered, cells show nuclear and cellular division arrest, whereas the cells that do not exhibit such process will be in endocycle phase and will increase their ploidy levels [68,106]. However, during the transition between proliferation and expansion phases, cells occasionally exit the normal cell cycle and enter an endocycle phase [107]. In *A. thaliana*, an increase in polyploid cellular fraction during the transition between first and second phases has been reported and this fraction was maintained during maturity phase [105]. In contrast, during quinoa leaf senescence, nuclei progressively undergo a process of PCD while endoreduplicating [65].

Moreover, this polyploidization occurs widely in metabolically active tissues of plants and animals [108,109] and cells retain their viability until most nutrients have been remobilized during leaf senescence process [110].

In this work, we found a higher polyploidy cellular content in B481-6 than in R453, with significant differences towards pre-anthesis and post-anthesis phases. These results indicate that B481-6 may have viable and metabolically active cells for longer time than its candidate contrasting genotype, especially during the beginning of grain filling phase. This result is consistent with the previous analysis in this work in which we assessed differences in senescence process evolution between R453/B481-6 genotypes. In contrast, the polyploid cellular content of C154B was similar to its contrasting genotype; which indicates no difference in cellular viability between these genotypes.

4.3. Molecular analysis

To confirm contrasting senescence process between different selected genotypes, we evaluated the expression levels of 3 NAC transcription factors previously detected as candidate genes associated to leaf senescence in sunflower, *HaNAC01, HaNAC03* and HaNAC05 [21,78]. We found higher expression of all analyzed SAGs in premature senescing genotypes (c977-b and R453) in comparison to their contrasting delayed senescence genotypes. This result confirms, also at a transcriptomic level, that the senescence process was accelerated in these genotypes.

In a previous study, the *HaNAC01, HaNAC03* and *HaNAC05* transcription factors were validated as SAG's, and *HaCAB2* as SDG, in sunflower [21,78]. Moreover, expression profiles of sunflower candidate gene *Ha-NAC01* (with high sequence identity to Arabidopsis ORE1) was evaluated together with miR164 levels, which suppress *ORE1* transcript levels [21]. The induction of leaf senescence by *ORE1* transcription factor has been demonstrated in Arabidopsis [111,112] and has been widely studied in different non-model species. *HaNAC01* displayed the highest expression level among the 3 analyzed candidate genes and this difference was detected in R453/B481-6 contrasting genotypes. This finding highlights these genotypes as the best candidates for further analysis of leaf senescence in sunflower. These results are similar to those of a previous study with contrasting genotypes of cotton for leaf senescence phenotype [54], in which NAC FTs were overexpressed in the premature senescing genotype. The expression of *HaCAB02,* with high sequence similarity to a chlorophyll A/B-binding protein 2 [72], was similar in both pairs of contrasting genotypes without significant differences, probably because of the sampling time. This gene is an indicator of advanced senescence phases and consequently differences in expression patterns between contrasting genotypes might arise in later sampling times (e.g. 25 days post-anthesis).

Conclusion

This work constitutes the first study of the early leaf senescence process in sunflower contrasting genotypes by a combination of physiological, cytological and molecular techniques, also identifying PCD biomarkers. We identified two senescence contrasting inbred lines, R453 and B481-6, with an impact in yield increase in the senescence delayed genotype, thus arising as a very interesting line for further studies of senescence process in sunflower. The B481-6 genotype showed a delayed senescence phenotype, also evidenced by cytological and molecular analysis and an increase of seed weight which makes this genotype a potential candidate for functional stay-green phenotype in comparison with R453 genotype.

Relevant candidate genes were biologically validated, confirming that the selected contrasting genotypes constitute valuable material for future analysis through high throughput methodologies. In this context, high throughput transcriptomic analysis of these selected contrasting sunflower lines by microarrays or NGS technologies (e.g. RNA-seq) could contribute to revealing new differentially expressed genes between both genotypes increasing candidate genes number. These results and characterization of local germplasm will be useful in dissecting this complex trait in sunflower, thus providing a valuable tool to assist in crop breeding.

Conflict of interest statement

The authors declare that they have no competing interests.

Authors' contributions

PF, ALG and SM conceived and designed the work, contributed to the implementation and wrote the first draft of the manuscript, CSV performed flow sorting analysis, MPLF and SM designed and conceived TUNEL and DNA gel electrophoresis assays, RH and NP initiated the project and contributed to the work by the interpretation, discussion of the data and critically revised the manuscript. All authors read and approved the final manuscript.

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References

[1] D.B. Egli, J.E. Leggett, W.G. Duncan, Influence of N stress on leaf senescence and N redistribution in soybeans., Agron. J. 70 (1976) 43–47. doi:10.2134/agronj1978.00021962007000010011x.

[2] L.D. Nooden, Whole plant senescence, in: A.C. Leopold, L.D. Nooden (Eds.), Senescence and Aging in Plants, First, Academic Press Inc., San Diego, California, USA, 1988: pp. 392–439. doi:10.1016/B978-0-12-520920-5.50018-3.

[3] K. Al-Khatib, G.M. Paulsen, Photosynthesis and Productivity during High-Temperature Stress of Wheat Genotypes from Major World Regions, Crop Sci. 30 (1990) 1127–1132. doi:10.2135/cropsci1990.0011183X003000050034x.

[4] S. Gan, R.M. Amasino, Making sense of senescence, Plant Physiol. 113 (1997) 313–319.

[5] A. Wingler, A. von Schaewen, R.C. Leegood, P.J. Lea, W. Paul Quick, Regulation of Leaf Senescence by Cytokinin, Sugars, and Light, Plant Physiol. 116 (1998) 329–335. doi:10.1104/pp.116.1.329.

[6] V. Rubio, R. Bustos, M.L. Irigoyen, X. Cardona-Lopez, M. Rojas-Triana, J. Paz-Ares, Plant hormones and nutrient signaling., Plant Mol Biol. 69 (2009) 361–373. doi:10.1007/s11103-008-9380-y.

[7] P.L. Gregersen, A. Culetic, L. Boschian, K. Krupinska, Plant senescence and crop productivity., Plant Mol. Biol. 82 (2013) 603–22. doi:10.1007/s11103-013-0013-8.

[8] H. Thomas, Senescence , ageing and death of the whole plant, New Phytol. 197 (2013) 696–711. doi:10.1111/nph.12047.

[9] B.F. Quirino, Y.S. Noh, E. Himelblau, R.M. Amasino, Molecular aspects of leaf senescence., Trends Plant Sci. 5 (2000) 278–82.

[10] Y. Nishizawa, M. Saruta, K. Nakazono, Z. Nishio, M. Soma, T. Yoshida, et al., Characterization of transgenic rice plants over-expressing the stress-inducible beta-glucanase gene Gns1, Plant Mol Biol. 51 (2003) 143–152.

[11] K.D. Edwards, M. Humphry, J.P. Sanchez-Tamburrino, Advances in plant senescence, in: T. Nagata (Ed.), Senescence, InTech Open Access Publisher, 2012: pp. 117 – 136. doi:10.5772/34735.

[12] S. Balazadeh, J. Schildhauer, W.L. Araújo, S. Munné-Bosch, A.R. Fernie, S. Proost, et al., Reversal of senescence by N resupply to N-starved Arabidopsis thaliana: transcriptomic and metabolomic consequences., J. Exp. Bot. 65 (2014) 3975–3992. doi:10.1093/jxb/eru119.

[13] S. del Duca, D. Serafini-Fracassini, G. Cai, Senescence and programmed cell death in plants: polyamine action mediated by transglutaminase., Front. Plant Sci. 5 (2014) 120. doi:10.3389/fpls.2014.00120.

[14] Y. Guo, Z. Cai, S. Gan, Transcriptome of Arabidopsis leaf senescence, Plant, Cell Environ. 27 (2004) 521–549. doi:10.1111/j.1365-3040.2003.01158.x.

[15] B. Srivalli, R. Khanna-Chopra, The developing reproductive “sink” induces oxidative stress to mediate nitrogen mobilization during monocarpic senescence in wheat., Biochem Biophys Res Commun. 325 (2004) 198–202. doi:10.1016/j.bbrc.2004.09.221.

[16] E. Agüera, P. Cabello, P. de la Haba, Induction of leaf senescence by low nitrogen nutrition in sunflower (Helianthus annuus) plants, Physiol Plant. 138 (2010) 256–267. doi:10.1111/j.1399-3054.2009.01336.x.

[17] V. Buchanan-Wollaston, S. Earl, E. Harrison, E. Mathas, S. Navabpour, T. Page, et al., The molecular analysis of leaf senescence -a genomic approach, Plant Biotechnol J. 1 (2003) 3–22. doi:10.1046/j.1467-7652.2003.00004.x.

[18] P. Fernandez, J. Di Rienzo, L. Fernandez, H.E. Hopp, N. Paniego, H. R.A., Transcriptomic identification of candidate genes involved in sunflower responses to chilling and salt stresses based on cDNA microarray analysis, BMC Plant Biol. 8 (2008). doi:10.1186/1471-2229-8-11.

[19] I. Schuster, C. Cruz, Estatística genômica aplicada a populações derivadas de cruzamentos controlados, Second, Editora UFV, Viçosa, Minas Gerais, Brazil, 2008.

[20] M. López Pereira, N. Trápani, V.O. Sadras, Genetic improvement of sunflower in Argentina between 1930 and 1995, F. Crop. Res. 67 (2000) 215–221. doi:10.1016/S0378-4290(00)00096-4.

[21] S. Moschen, S. Bengoa Luoni, N.B. Paniego, H.E. Hopp, G.A.A. Dosio, P. Fernandez, et al., Identification of Candidate Genes Associated with Leaf Senescence in Cultivated Sunflower (Helianthus annuus L.), PLoS One. 9 (2014) e104379. doi:10.1371/journal.pone.0104379.

[22] V.O. Sadras, L. Echarte, F.H. Andrade, Profiles of leaf senescence during reproductive growth of sunflower and maize, Ann. Bot. 85 (2000) 187–195. doi:10.1006/anbo.1999.1013.

[23] A. de la Vega, Cantore M. A., Sposaro N. N., Trapani N., Lopez Pereira M., A.J. Hall, Canopy stay green and yield in non stressed sunflower, Fields Crop Res. 121 (2011) 175–185. doi:10.1016/j.fcr.2010.12.015.

[24] R.M. Ewing, J.M. Claverie, EST databases as multi-conditional gene expression datasets, Pacific Symp. Biocomput. (2000) 430–442.

[25] L. Aguirrezábal, G. Orioli, Girasol: Aspectos fisiológicos que determinan el rendimiento, First, Unidad Integrada Balcarce, Balcarce, Buenos Aires, Argentina, 2001.

[26] S. Balazadeh, Stay-green not always stays green, Mol. Plant. 7 (2014) 1264–1266. doi:10.1093/mp/ssu076.

[27] H. Thomas, C.J. Howarth, Five ways to stay green, J. Exp. Bot. 51 (2000) 329–337. doi:10.1093/jexbot/51.suppl_1.329.

[28] G.A.A. Dosio, L.A.N. Aguirrezábal, Variaciones del rendimiento en girasol. Identificando las causas, Rev. Agromercado, Cuad. Girasol. 90 (2004) 7–10.

[29] G.A.A. Dosio, L.A.N. Aguirrezábal, F.H. Andrade, V.R. Pereyra, Solar radiation intercepted during seed filling and oil production in two sunflower hybrids, Crop Sci. 40 (2000) 1637–1644. doi:10.2135/cropsci2000.4061637x.

[30] L.A.N. Aguirrezábal, Y. Lavaud, G.A.A. Dosio, N.G. Izquierdo, F.H. Andrade, L.M. González, Intercepted Solar Radiation during Seed Filling Determines Sunflower Weight per Seed and Oil Concentration, Crop Sci. 43 (2003) 152–161. doi:10.2135/cropsci2003.1520.

[31] P. Fernandez, N. Paniego, S. Lew, H.E. Hopp, R.A. Heinz, Differential representation of sunflower ESTs in enriched organ-specific cDNA libraries in a small scale sequencing project, BMC Genomics. 4 (2003). doi:10.1186/1471-2164-4-40.

[32] P. Cabello, E. Agüera, Metabolic changes during natural ageing in sunflower (Helianthus annuus) leaves: expression and activity of glutamine synthetase isoforms are regulated differently during senescence, Physiol Plant. 128 (2006) 175–185. doi:10.1111/j.1399-3054.2006.00722.x.

[33] N.B. Paniego, R.A. Heinz, P. Fernandez, P. Talia, V. Nishinakamasu, H.E. Hopp, Sunflower, in: C. Kole (Ed.), Genome Mapp. Mol. Breed. Plants, Springer-Verlag, Berlin Heidelberg, 2007: pp. 153–177. doi:10.1007/978-3-540-34388-2_4.

[34] L. Peluffo, V. Lia, C. Troglia, C. Maringolo, P. Norma, A. Escande, et al., Metabolic profiles of sunflower genotypes with contrasting response to Sclerotinia sclerotiorum infection, Phytochemistry. 71 (2010) 70–80. doi:10.1016/j.phytochem.2009.09.018.

[35] J. Bazin, N. Langlade, P. Vincourt, S. Arribat, S. Balzergue, H. El-Maarouf-Bouteau, et al., Targeted mRNA oxidation regulates sunflower seed dormancy alleviation during dry after-ripening, Plant Cell. 23 (2011) 2196–2208. doi:10.1105/tpc.111.086694.

[36] E. Agüera, P. Cabello, L. de la Mata, E. Molina, P. de la Haba, Metabolic Regulation of Leaf Senescence in Sunflower (Helianthus annuus L.) Plants, in: T. Nagata (Ed.), Senescence, InTech Open Access Publisher, 2012: pp. 51–68. doi:10.5772/33671.

[37] E. Bachlava, C. Taylor, S. Tang, J.E. Bowers, J.R. Mandel, J.M. Burke, et al., SNP discovery and development of a high-density genotyping array for sunflower, PLoS One. 7 (2012) e29814. doi:10.1371/journal.pone.0029814.

[38] P. Fernandez, S. Moschen, N. Paniego, R.A. Heinz, Functional approaches to study leaf senescence in sunflower, in: T. Nagata (Ed.), Senescence, InTech Open Access Publisher, 2012: pp. 69–88. doi:10.5772/34785.

[39] Z. Lai, N.C. Kane, A. Kozik, K.A. Hodgins, K.M. Dlugosch, M.S. Barker, et al., Genomics of Composi tae weeds: EST libraries, microarrays, and e vidence of introgression, Am J Bot. 99 (2012) 209–218. doi:10.3732/ajb.1100313.

[40] P. Fernandez, M. Soria, D. Blesa, J. di Rienzo, S. Moschen, M. Rivarola, et al., Development, characterization and experimental validation of a cultivated sunflower (Helianthus annuus L.) gene expression oligonucleotide microarray, PLoS One. 7 (2012) e45899. doi:10.1371/journal.pone.0045899.

[41] S. Moschen, L.M. Radonic, G.F. Ehrenbolger, P. Fernández, V. Lıa, N.B. Paniego, et al., Functional Genomics and Transgenesis Applied to Sunflower Breeding, in: J.I. Arribas (Ed.), Sunflowers Growth Dev. Environ. Influ. Pests/Diseases, Nova Science Publishers Inc., 2014: pp. 131–164.

[42] S. Moschen, S. Bengoa Luoni, J.A. Di Rienzo, M. del P. Caro, T. Tohge, M. Watanabe, et al., Integrating transcriptomic and metabolomic analysis to understand

natural leaf senescence in sunflower, Plant Biotechnol. J. (2015). doi:10.1111/pbi.12422.

[43] H. Thomas, Defining senescence and death, J. Exp. Bot. 54 (2003) 1127–1132. doi:10.1093/jxb/erg133.

[44] H. Hu, M. Dai, J. Yao, B. Xiao, X. Li, Q. Zhang, et al., Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice, Proc Natl Acad Sci U S A. 103 (2006) 12987–12992. doi:10.1073/pnas.0604882103.

[45] H. Hu, J. You, J. Fang, X. Zhu, Z. Qi, L. Xiong, Characterization of transcription factor gene SNAC2 conferring cold and salt tolerance in rice, Plant Mol Biol. 67 (2008) 169–181. doi:10.1007/s11103-008-9309-5.

[46] D.T. Le, R. Nishiyama, Y. Watanabe, K. Mochida, K. Yamaguchi-Shinozaki, K. Shinozaki, et al., Genome-wide survey and expression analysis of the plant-specific NAC transcription factor family in soybean during development and dehydration stress, DNA Res. 18 (2011) 263–276. doi:10.1093/dnares/dsr015.

[47] D. Cantu, S.P. Pearce, A. Distelfeld, M.W. Christiansen, C. Uauy, E. Akhunov, et al., Effect of the down-regulation of the high Grain Protein Content (GPC) genes on the wheat transcriptome during monocarpic senescence, BMC Genomics. 12 (2011) 492. doi:10.1186/1471-2164-12-492.

[48] R. Hu, G. Qi, Y. Kong, D. Kong, Q. Gao, G. Zhou, Comprehensive analysis of NAC domain transcription factor gene family in Populus trichocarpa, BMC Plant Biol. 10 (2010) 145. doi:10.1186/1471-2229-10-145.

[49] M.W. Christiansen, P.B. Holm, P.L. Gregersen, Characterization of barley (Hordeum vulgare L.) NAC transcription factors suggests conserved functions compared to both monocots and dicots, BMC Res. Notes. 4 (2011). doi:10.1186/1756-0500-4-302.

[50] V. Buchanan-Wollaston, C. Ainsworth, Leaf senescence in Brassica napus: cloning of senescence-related genes by subtractive hybridization, J Exp Bot. 33 (1997) 821–834.

[51] R.S. Sekhon, K.L. Childs, N. Santoro, C.E. Foster, C.R. Buell, N. de Leon, et al., Transcriptional and metabolic analysis of senescence induced by preventing pollination in maize, Plant Physiol. 159 (2012) 1730–1744.

doi:10.1104/pp.112.199224.

[52] K.C. Snowden, A.J. Simkin, B.J. Janssen, K.R. Templeton, H.M. Loucas, J.L. Simons, et al., The Decreased apical dominance1/Petunia hybrida CAROTENOID CLEAVAGE DIOXYGENASE8 gene affects branch production and plays a role in leaf senescence, root growth, and flower development, Plant Cell. 17 (2005) 746–759. doi:10.1105/tpc.104.027714.

[53] J.M. Zapata, A. Guéra, A. Esteban-Carrasco, M. Martín, B. Sabater, Chloroplasts regulate leaf senescence: delayed senescence in transgenic ndhF-defective tobacco, Cell Death Differ. 12 (2005) 1277–84. doi:10.1038/sj.cdd.4401657.

[54] X. Kong, Z. Luo, H. Dong, a E. Eneji, W. Li, H. Lu, Gene expression profiles deciphering leaf senescence variation between early- and late-senescence cotton lines, PLoS One. 8 (2013) e69847. doi:10.1371/journal.pone.0069847.

[55] G.L. Müller, M.F. Drincovich, C.S. Andreo, M.V. Lara, Role of photosynthesis and analysis of key enzymes involved in primary metabolism throughout the lifespan of the tobacco flower, J. Exp. Bot. 61 (2010) 3675–3688. doi:10.1093/jxb/erq187.

[56] C.B. Taylor, P.A. Bariola, S.B. DelCardayré, R.T. Raines, P.J. Green, RNS2: a senescence-associated RNase of Arabidopsis that diverged from the S-RNases before speciation, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5118–22.

[57] S.B. Ryu, X. Wang, Expression of Phospholipase D during Castor Bean Leaf Senescence, Plant Physiol. 108 (1995) 713–719.

[58] S. Gepstein, G. Sabehi, M.J. Carp, T. Hajouj, M.F.O. Nesher, I. Yariv, et al., Large-scale identification of leaf senescence-associated genes, Plant J. 36 (2003) 629–642. doi:10.1046/j.1365-313X.2003.01908.x.

[59] V. Buchanan-Wollaston, T. Page, E. Harrison, E. Breeze, P.O. Lim, H.G. Nam, et al., Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis, Plant J. 42 (2005) 567–585. doi:10.1111/j.1365-313X.2005.02399.x.

[60] J. Balk, C.J. Leaver, The PET1-CMS mitochondrial mutation in sunflower is associated with premature programmed cell death and cytochrome c release, Plant Cell. 13 (2001) 1803–1818. doi:10.1105/TPC.010116.

[61] E. Liljeroth, T. Bryngelsson, DNA fragmentation in cereal roots indicative of programmed root cortical cell death, Physiol. Plant. 111 (2001) 365–372. doi:10.1034/j.1399-3054.2001.1110314.x.

[62] M.C. Roche, A study of programmed cell death in cotton (Gossypium hirsutum), Texas A&M University, 2007.

[63] B. Uzelac, D. Janosević, S. Budimir, In situ detection of programmed cell death in Nicotiana tabacum leaves during senescence, J. Microsc. 230 (2008) 1–3. doi:10.1111/j.1365-2818.2008.01947.x.

[64] H. El-Maarouf-Bouteau, C. Mazuy, F. Corbineau, C. Bailly, DNA alteration and programmed cell death during ageing of sunflower seed, J. Exp. Bot. 62 (2011) 5003–5011. doi:10.1093/jxb/err198.

[65] M.P. López-Fernández, H.P. Burrieza, A.J. Rizzo, L.J. Martínez-Tosar, S. Maldonado, Cellular and molecular aspects of quinoa leaf senescence, Plant Sci. (2015). doi:10.1016/j.plantsci.2015.06.003.

[66] Y.Y. Leshem, E. Haramaty, The Characterization and Contrasting Effects of the Nitric Oxide Free Radical in Vegetative Stress and Senescence of Pisum sativum Linn. Foliage, J. Plant Physiol. 148 (1996) 258–263. doi:10.1016/S0176-1617(96)80251-3.

[67] I. Gadjev, J.M. Stone, T.S. Gechev, Programmed cell death in plants: new insights into redox regulation and the role of hydrogen peroxide, Int. Rev. Cell Mol. Biol. 270 (2008) 87–144. doi:10.1016/S1937-6448(08)01403-2.

[68] C. Gutierrez, The Arabidopsis cell division cycle., Arabidopsis Book. 7 (2009) e0120. doi:10.1199/tab.0120.

[69] M.S. Otegui, Y.-S. Noh, D.E. Martínez, M.G. Vila Petroff, L.A. Staehelin, R.M. Amasino, et al., Senescence-associated vacuoles with intense proteolytic activity develop in leaves of Arabidopsis and soybean, Plant J. 41 (2005) 831–844. doi:10.1111/j.1365-313X.2005.02346.x.

[70] C.A. Carrión, M.L. Costa, D.E. Martínez, C. Mohr, K. Humbeck, J.J. Guiamet, In vivo inhibition of cysteine proteases provides evidence for the involvement of “senescence-associated vacuoles” in chloroplast protein degradation during dark-induced senescence of tobacco leaves, J. Exp. Bot. 64 (2013) 4967–4980. doi:10.1093/jxb/ert285.

[71] C. Diaz, S. Purdy, A. Christ, J.-F. Morot-Gaudry, A. Wingler, C. Masclaux-Daubresse, Characterization of markers to determine the extent and variability of leaf senescence in Arabidopsis. A metabolic profiling approach, Plant Physiol. 138 (2005) 898–908. doi:10.1104/pp.105.060764.

[72] P.O. Lim, H.J. Kim, H.G. Nam, Leaf Senescence, Annu. Rev. Plant Biol. 58 (2007) 115–136. doi:10.1146/annurev.arplant.57.032905.105316.

[73] H.R. Woo, J.H. Kim, J. Kim, J. Kim, U. Lee, I.-J. Song, et al., The RAV1 transcription factor positively regulates leaf senescence in Arabidopsis, J. Exp. Bot. 61 (2010) 3947–57. doi:10.1093/jxb/erq206.

[74] Y. Gavrieli, Y. Sherman, S.A. Ben-Sasson, Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation, J. Cell Biol. 119 (1992) 493–501.

[75] C.M. Fusari, J.A. Di Rienzo, C. Troglia, V. Nishinakamasu, M.V. Moreno, C. Maringolo, et al., Association mapping in sunflower for sclerotinia head rot resistance., BMC Plant Biol. 12 (2012) 93. doi:10.1186/1471-2229-12-93.

[76] C. V Filippi, N. Aguirre, J.G. Rivas, J. Zubrzycki, A. Puebla, D. Cordes, et al., Population structure and genetic diversity characterization of a sunflower association mapping population using SSR and SNP markers., BMC Plant Biol. 15 (2015) 52. doi:10.1186/s12870-014-0360-x.

[77] J.R. Kiniry, R. Blanchet, J.R. Williams, V. Texier, K. Jones, M. Cabelguenne, Sunflower simulation using the EPIC and ALMANAC models, F. Crop. Res. 30 (1992) 403–423. doi:10.1016/0378-4290(92)90008-W.

[78] S. Bengoa Luoni, Análisis, identificación y validación de factores de transcripción asociados a senescencia foliar temprana en girasol (Helianthus annuus L.), Universidad Nacional de General San Martín (UNSAM), 2014.

[79] P. Fernandez, J.A. di Rienzo, S. Moschen, G.A. Dosio, L.A. Aguirrezabal, H.E. Hopp, et al., Comparison of predictive methods and biological validation for qPCR reference genes in sunflower leaf senescence transcript analysis, Plant Cell Rep. 30 (2011) 63–74. doi:10.1007/s00299-010-0944-3.

[80] J.M. Ruijter, C. Ramakers, W.M. Hoogaars, Y. Karlen, O. Bakker, M.J. van den Hoff, et al., Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data, Nucleic Acids Res. 37 (2009) e45. doi:10.1093/nar/gkp045.

[81] fgStatistics, fg Statistics, (2009) https://sites.google.com/site/fgstatistics/Home.

[82] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acid Res. 29 (2001) e45. doi:10.1093/nar/29.9.e45.

[83] F. Otto, DAPI Staining of Fixed Cells for High-Resolution Flow Cytometly of Nuclear DNA, Methods Cell Biol. 33 (1990) 105–110.

[84] J.S. Johnston, M.D. Bennett, A.L. Rayburn, D.W. Galbraith, H.J. Price, Reference standards for determination of DNA content of plant nuclei, Am. J. Bot. 86 (1999) 609–613.

[85] J.A. Di Rienzo, F. Casanoves, M.G. Balzarini, L. Gonzalez, M. Tablada, C.W. Robledo, InfoStat, (2014) http://www.infostat.com.ar/.

[86] E. Breeze, E. Harrison, S. McHattie, L. Hughes, R. Hickman, C. Hill, et al., High-resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals a distinct chronology of processes and regulation, Plant Cell. 23 (2011) 873–94. doi:10.1105/tpc.111.083345.

[87] M.M. Al-Amery, J.H. Hamza, M.P. Fuller, Effect of Boron Foliar Application on Reproductive Growth of Sunflower (Helianthus annuus L.), Int. J. Agron. 2011 (2011) 1–5. doi:10.1155/2011/230712.

[88] S. Moschen, P. Fernández, S. Bengoa Luoni, N. Paniego, L. Aguirrezábal, G.A.A. Dosio, et al., Sunflower (Helianthus annuus L.) leaf senescence analysis by a physiological and molecular approach, 18th Sunflower Int. Conf. (2012).

[89] H. Thomas, H. Ougham, The stay-green trait, J. Exp. Bot. 65 (2014) 3889–900. doi:10.1093/jxb/eru037.

[90] I. Nur, Sunflower and the problem of unfilled seeds under Sudan conditions, Acta Agron. Acad. Sci. Hungarica. 27 (1978) 339 – 340.

[91] T.M. Yegappan, D.M. Paton, C.T. Gates, W.J. Muller, Water Stress in Sunflower (Helianthus annuus L.) 3. Responses of Cypsela Size, Ann. Bot. 49 (1982) 69–75.

[92] H.M. Rawson, R.L. Dunstone, M.J. Long, J.E. Begg, Canopy Development, Light Interception and Seed Production in Sunflower as Influenced by Temperature and Radiation, Aust. J. Plant Physiol. 11 (1984) 255–265. doi:10.1071/PP9840255.

[93] J.H. Palmer, B.T. Steer, The generative area as the site of floret initiation in the sunflower capitulum and its integration to predict floret number, F. Crop. Res. 11

(1985) 1–12. doi:10.1016/0378-4290(85)90088-7.

[94] Y. Saranga, P. Horcicka, S. Wolf, Effect of source–sink relationship on yield components and yield of confection sunflower, Helia. 19 (1996) 29 – 38.

[95] S.N. Vasudevan, K. Virupakshappa, S. Bhaskar, M. Udayakumar, Influence of growth regulators on some productive parameters and oil content in sunflower (Helianthus annuus L.), Indian J. Plant Physiol. 1 (1996) 277–280.

[96] C.A. Chimenti, A.J. Hall, Grain number responses to temperature during floret differentiation in sunflower, F. Crop. Res. 72 (2001) 177–184. doi:10.1016/S0378-4290(01)00175-7.

[97] J.E. Cantagallo, A.J. Hall, Seed number in sunflower as affected by light stress during the floret differentiation interval, F. Crop. Res. 74 (2002) 173–181. doi:10.1016/S0378-4290(01)00209-X.

[98] M. Alkio, A. Schubert, W. Diepenbrock, E. Grimm, Effect of source – sink ratio on seed set and filling in sunflower (Helianthus annuus L .), Plant, Cell Environ. 26 (2003) 1609–1619.

[99] M.P. López-Fernández, S. Maldonado, Programmed cell death during quinoa perisperm development., J. Exp. Bot. 64 (2013) 3313–25. doi:10.1093/jxb/ert170.

[100] L. Taiz, E. Zeiger, Photosynthesis: The Light Reactions, in: Plant Physiol., 4° ed., Sinauer Associates, 2006: p. 672.

[101] S. Hortensteiner, U. Feller, Nitrogen metabolism and remobilization during senescence, J. Exp. Bot. 53 (2002) 927–937. doi:10.1093/jexbot/53.370.927.

[102] S. Hörtensteiner, Chlorophyll degradation during senescence, Annu. Rev. Plant Biol. 57 (2006) 55–77.

[103] S.W. Yang, S.K. Kim, W.T. Kim, Perturbation of NgTRF1 expression induces apoptosis-like cell death in tobacco BY-2 cells and implicates NgTRF1 in the control of telomere length and stability., Plant Cell. 16 (2004) 3370–3385. doi:10.1105/tpc.104.026278.

[104] A. Kuthanova, Z. Opatrny, L. Fischer, Is internucleosomal DNA fragmentation an indicator of programmed death in plant cells?, J. Exp. Bot. 59 (2008) 2233–2240. doi:10.1093/jxb/ern090.

[105] G.T.S. Beemster, L. De Veylder, S. Vercruysse, G. West, D. Rombaut, P. Van

Hummelen, et al., Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis, Plant Physiol. 138 (2005) 734–743. doi:10.1104/pp.104.053884.

[106] k. k. Imai, Y. Ohashi, T. Tsuge, T. Yoshizumi, M. Matsui, A. Oka, et al., The A-type cyclin CYCA2;3 is a key regulator of ploidy levels in Arabidopsis endoreduplication, Plant Cell. 18 (2006) 382–396. doi:10.1105/tpc.105.037309.

[107] J. Dolezel, S. Lucretti, High-resolution flow karyotyping and chromosome sorting in Vicia faba lines with standard and reconstructed karyotypes, Theor. App. Genet. 90 (1995) 797–802. doi:10.1007/BF00222014.

[108] W.Y. Brodsky, I. V. Uryvaeva, Cell polyploidy: its relation to tissue growth and function, Int. Rev. Cytol. 50 (1977) 275–332.

[109] F. D'Amato, Role of polyploidy in reproductive organs and tissues, in: B.M. Johri (Ed.), Embryol. Angiosperms., Springer, 1984: pp. 519–566. doi:10.1007/978-3-642-69302-1_11.

[110] Y. Guo, Towards systems biological understanding of leaf senescence., Plant Mol. Biol. 82 (2013) 519–28. doi:10.1007/s11103-012-9974-2.

[111] S. Balazadeh, H. Siddiqui, A.D. Allu, L.P. Matallana-Ramirez, C. Caldana, M. Mehrnia, et al., A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence, Plant J. 62 (2010) 250–264. doi:TPJ4151 [pii] 10.1111/j.1365-313X.2010.04151.x.

[112] J.H. Kim, H.R. Woo, J. Kim, P.O. Lim, I.C. Lee, S.H. Choi, et al., Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis, Science (80-.). 323 (2009) 1053–1057. doi:323/5917/1053 [pii] 10.1126/science.1166386.

Figure 1: Physiological analysis of plants grown under field conditions. A–E: Average green leaf area (GLA) against thermal time after emergence (°CdAE), **(A)** c977-b/C154B, **(B)** R453/B481-6, **(C)** 2021/2091, **(D)** B473-2/C829B and **(E)** C818/B10 candidate contrasting genotypes. Solid curve shows delayed senescence genotypes and dotted curve premature senescence ones. Vertical dotted lines denote average anthesis of both premature (light grey) and delayed (dark grey) senescence genotypes (values in Table 1). **(F)** Yield reached by the ten analyzed genotypes, expressed as weight of 1,000 seeds. Same color bars indicate candidate contrasting genotypes. Upper values of each bar correspond to grain filling degree (GFD; relative units), error bars correspond to standard errors. In all figures, standard errors are show by error bars, and asterisks indicate significant differences according to LSD Fisher test ($P \leqslant 0.05$).

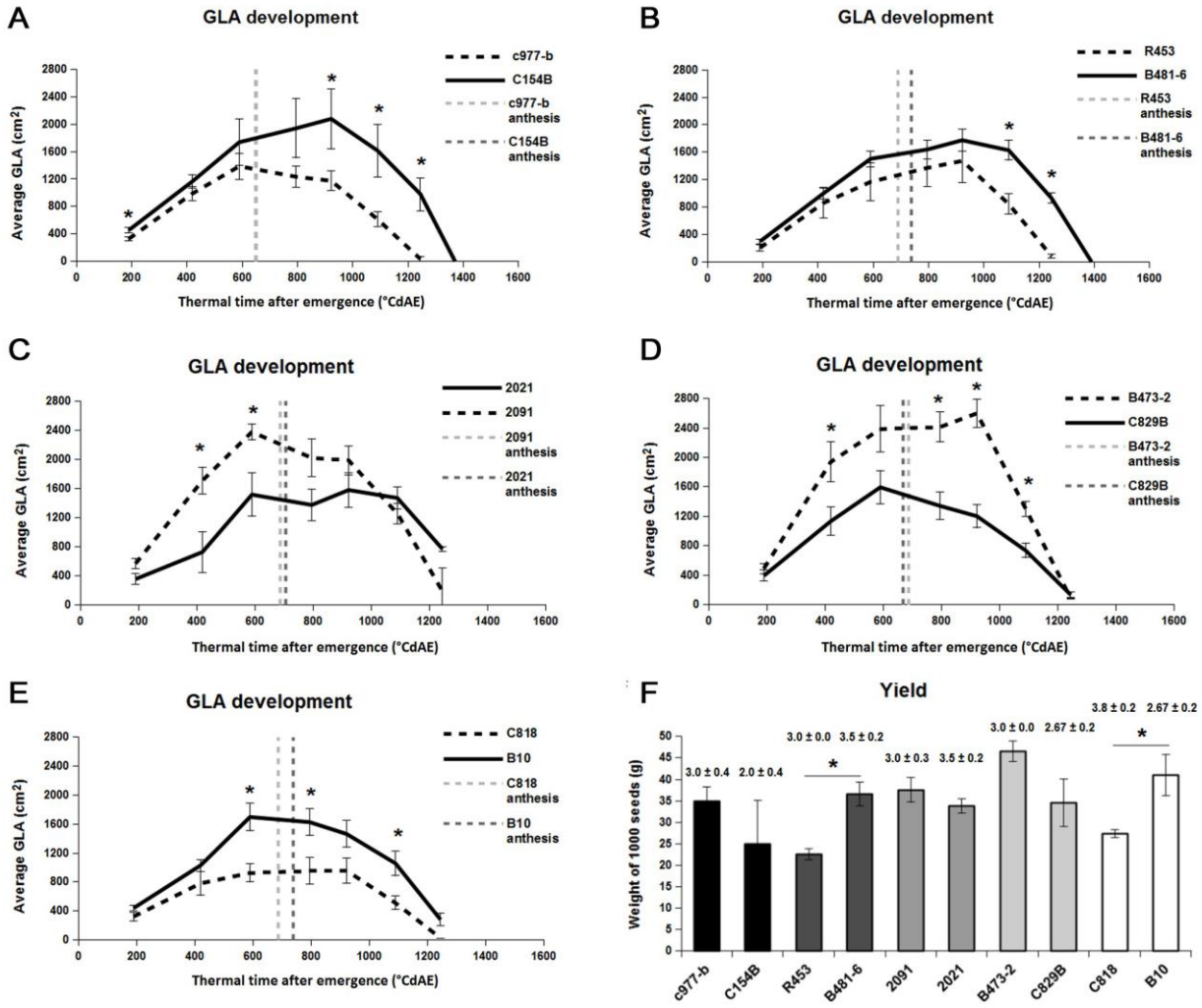

Figure 2: Physiological analysis under greenhouse conditions. Average maximum GLA at anthesis, reached by c977-b (black), C154B (dark grey), R453 (light grey) and B481-6 (white) selected contrasting genotypes. Standard errors are shown by error bars.

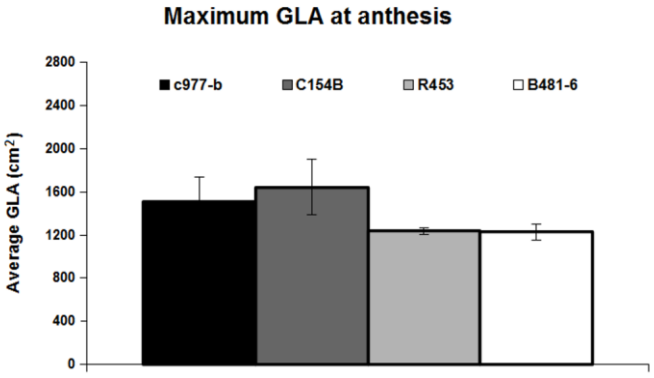

Figure 3: TUNEL and DNA gel electrophoresis assays of selected contrasting genotypes, grown under field conditions. A–I: *In situ* nuclear DNA fragmentation of sunflower leaf cells analyzed by TUNEL assay. **(A), (C), (E)** and **(G)**: Nuclei visualization by DAPI staining. **(B)** and **(F)**: TUNEL-positive nuclei in mesophyll cells of premature senescence genotypes, as seen by fluorescent signal. **(D)** and **(H)**: TUNEL-negative nuclei in delayed senescence genotypes. **(I)**: Negative control. **(J)** and **(K)**: Agarose gel electrophoresis assays; **(J)** 0.8% w/v and **(K)** 1.5% w/v. Lane 1: 1 Kbp molecular marker, 2-4: c977-b genotype, 5-7: R453, 8-10:B481-6 and 11-12: C154B.

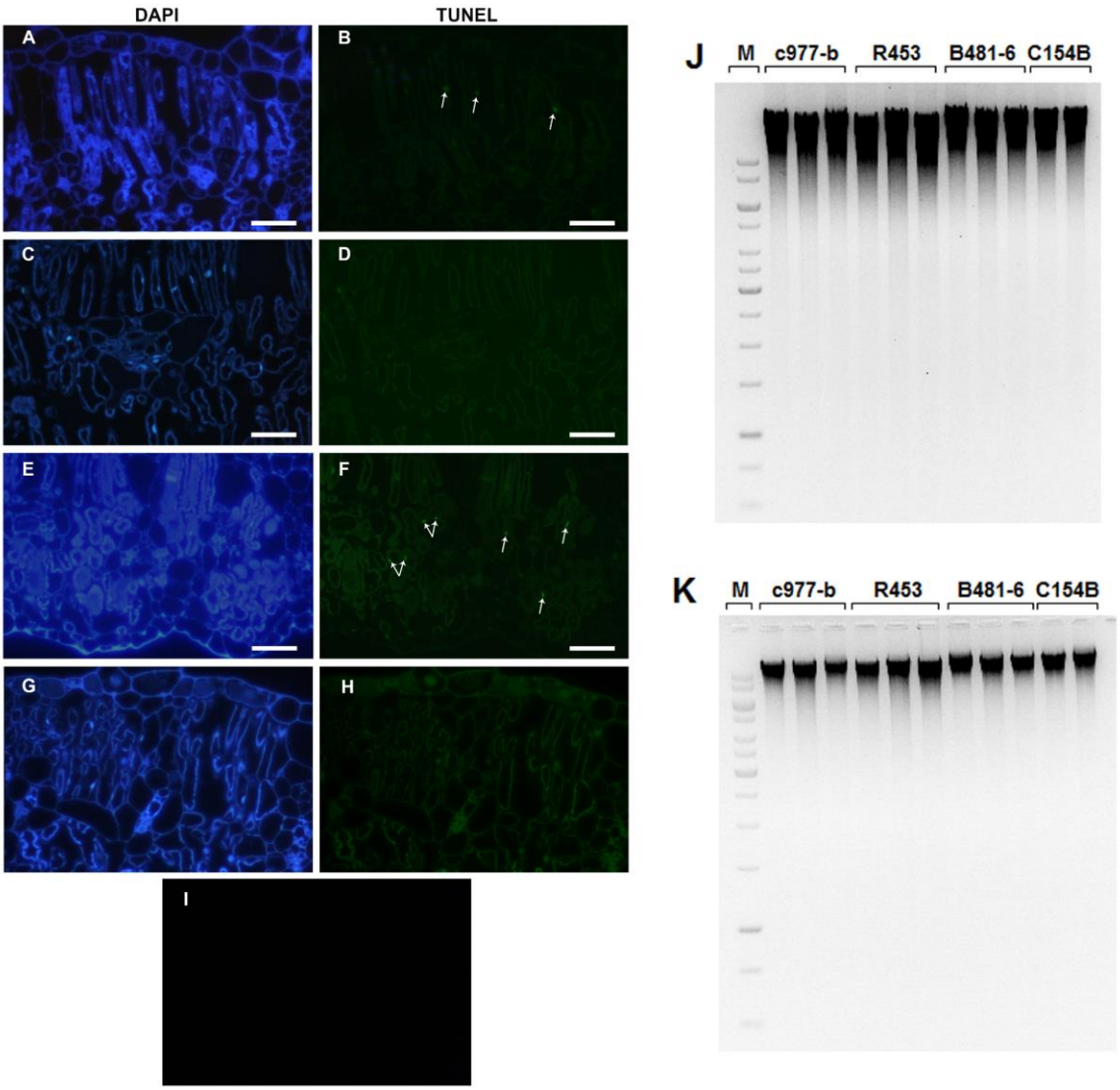

Figure 4: Flow sorting. Percentage of polyploid cells (PPC) compared between **(A)** R543 and B481-6 and **(B)** c977-b and C154B contrasting genotypes, grown under greenhouse conditions. DAE: days after emergence. Dotted curve shows premature senescence genotype and solid curve delayed senescence one. In both figures, standard errors are shown by error bars, and asterisks indicate significant differences according to LSD Fisher test ($P \leqslant 0.05$).

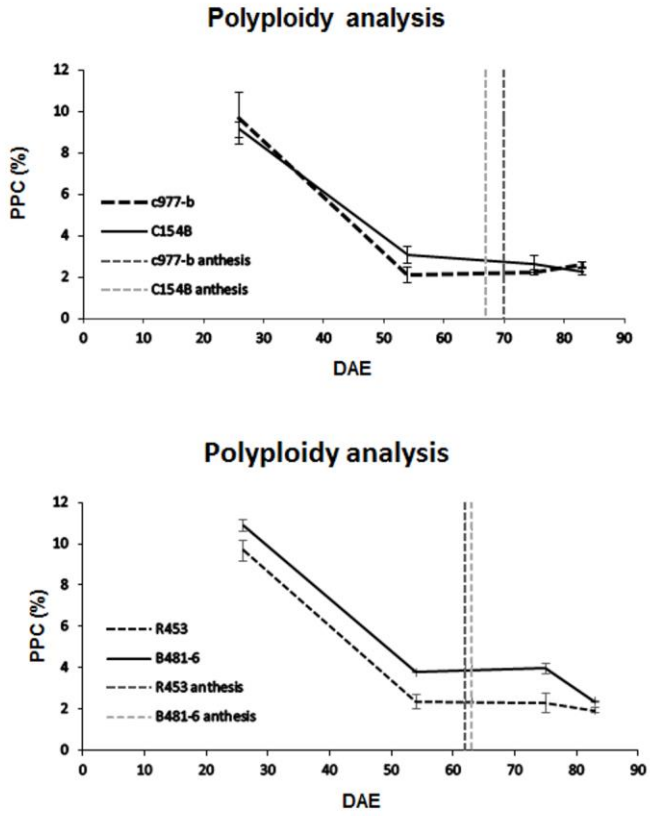

Figure 5: qPCR assays. Expression profiles of transcription factors. **A**, **B** and **C** – SAGs in sunflower. **(A)** *HaNAC01*, **(B)** *HaNAC03* and **(C)** *HaNAC05*. **(D)** *HaCAB2* (marker of senescence gene; SDG in sunflower). Relative transcript levels are shown as the ratio (log_2 scale) between premature senescence genotype gene expression, in relation to its contrasting delayed senescence genotype gene expression. Time 1 (black bars): 5 days post-anthesis, time 2 (white bars): 15 days post-anthesis. In all figures, standard errors are shown by error bars, and asterisks indicate significant differences according to LSD Fisher test ($P \leqslant 0.05$).

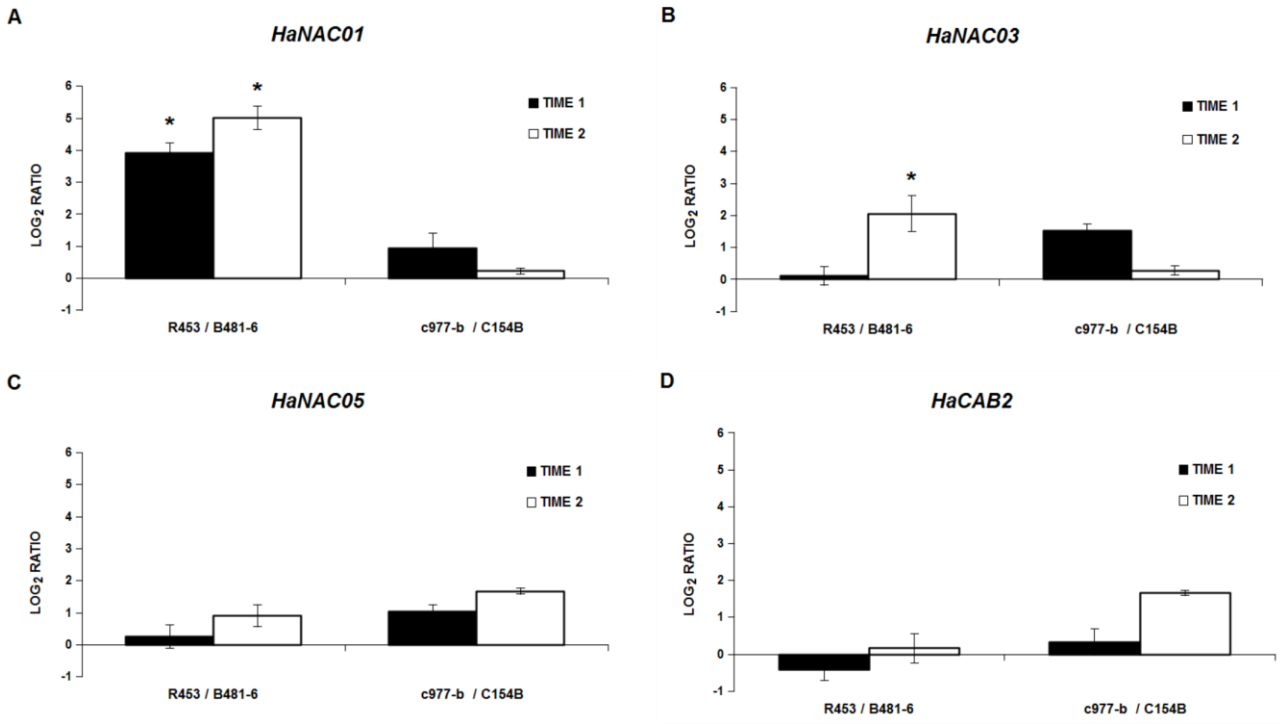

Table 1: Sunflower inbred lines used at the present study and their origin.

Line	**Origin**
2021	INTA Sunflower Breeding Program
2091	
B481-6	
R453	
c977-b	INTA Manfredi Sunflower Germplasm Collection
C154B	
C818R	
C829B	
B10	INTA Manfredi Breeding Site
B473-2	

Table 2: Average anthesis time and maximum leaf number (MLN) of each genotype, compared to its contrasting genotype. °CdAE: thermal time after emergence. DAE: Days after emergence. Each value is accompanied by its standard error. Asterisks indicate significant differences in MLN between contrasting genotypes according to LSD Fisher test ($P \leqslant 0.05$).

Grown under	Premature senescence				Delayed senescence			
	Genotype	Average anthesis time		MLN	Genotype	Average anthesis time		MLN
Field conditions	c977-b	46.0 DAE ± 0.3	650.0 °CdAE ± 0.3	26.3 ± 0.9	C154B	46.0 DAE ± 0.0	650.0 °CdAE ± 0.0	29.2 ± 1.2
	R453	48.0 DAE ± 1.0	688.0 °CdAE ± 1.0	22.2 ± 0.7*	B481-6	51.7 DAE ± 0.3	738.0 °CdAE ± 0.3	19.3 ± 0.7*
	2091	49.0 DAE ± 2.7	705.0 °CdAE ± 2.7	31.0 ± 1.3*	2021	48.3 DAE ± 1.2	688.0 °CdAE ± 1.2	27.8 ± 0.5*
	B473-2	48.0 DAE ± 1.7	688.0 °CdAE ± 1.7	29.8 ± 0.3*	C829B	48.7 DAE ± 1.3	669.0 °CdAE ± 1.3	23.2 ± 0.7*
	C818	48.0 DAE ± 1.2	688.0 °CdAE ± 1.2	23.3 ± 0.5*	B10	51.7 DAE ± 0.3	738.0 °CdAE ± 0.3	29.5 ± 0.8*
Greenhouse conditions	c977-b	70 DAE	n/d	22.0 ± 1.4*	C154B	67 DAE	n/d	24.3 ± 1.9*
	R453	62 DAE	n/d	16.3 ± 0.7	B481-6	63 DAE	n/d	15.7 ± 0.7

Table 3: Average seed number (SN), seeds fresh weight (FW) and yield of each capitulum, compared between contrasting genotypes. Each value is accompanied by its standard error and an asterisk when differences between the compared genotypes were significant according to LSD Fisher test ($P \leqslant 0.05$).

Genotype	SN	FW (g)	Yield (g)
c977-b	365.67 ± 26.18*****	12.95 ± 1.66*****	34.85 ± 3.47
C154B	97.25 ± 9.66*****	2.36 ± 0.93*****	24.99 ±10.2
R453	493.60 ± 59.18*	11.24 ± 1.59*	22.54 ± 1.30*
B481-6	837.33 ± 62.02*****	30.78 ± 3.07*****	36.63 ± 2.76*
2091	626.83 ± 128.57	23.84 ± 5.05	37.59 ± 2.93
2021	957.67 ± 107.62	31.94 ± 3.35	33.77 ± 1.63
B473-2	574.83 ± 62.69*****	26.46 ± 2.62*****	46.56 ± 2.45
C829B	318.67 ± 85.13*	9.76 ± 2.05*	34.55 ± 5.53
C818	422.17 ± 49.82	11.55 ± 1.45	27.38 ± 0.93*
B10	349.83 ± 33.81	13.90 ± 1.61	41.01 ± 4.81*