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Functional Approaches to Study Leaf Senescence in Sunflower

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1. Introduction

Senescence is an age-dependent process at the cellular, tissue, organ or organism level, leading to death at the end of the life span (Noodén 1988). Annual plants as grain and oil crops undergo a visual process towards the end of the reproductive stage that is accompanied by nutrient remobilization from leaf to developing seeds (Buchanan-Wollaston et al. 2003). The final stage of this process is leaf death but this is actively delayed until all nutrients have been removed and recycle through the process of developmental senescence. It have been documented that 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 different crops (Ewing & Claverie 2000), including sunflower (Sadras et al. 2000; De la Vega et al. 2011). The potential yields of sunflower crop are far from the real ones in all Argentina productive regions. In Balcarce, for example, while the potential yields are estimated in 5,000 $kg.ha^{-1}$, those obtained by the best producers only reach 3,000 kg.ha-1, and the average in the region ranges in 1,800 $kg.ha^{-1}$ (Dosio & Aguirrezábal 2004). These differences could possibly be due to the inability of current hybrids to keep their green leaf area for long periods, which would allow greater use of the incident radiation during the grain filling period which plays an important role in determining the yield and oil concentration in sunflower (Dosio et al. 2000; Aguirrezábal et al. 2003).

Besides autonomous (internal) factors as age, reproductive stage and phytohormone levels, leaf senescence is hardly affected by environmental factors. Among these environmental factors, including extreme temperature, drought, shading, nutrient deficient and pathogen infection, the most limiting ones are water and nutrient availability (Gan & Amasino 1997; Sadras et al. 2000; Sadras et al. 2000; Dosio et al. 2003; Lim et al. 2003; Aguera et al. 2010).

During leaf senescence, critical and dramatic changes occurred in a highly regulated manner following a genetically programmed process of high complexity. Chlorophyll degradation, nutrient recycling and remobilization are preceded or paralleled by RNA and protein degradation. Even though leaf senescence has been widely recognized and accepted as a type of Programmed Cell Death (PCD) (Noodén & Leopold 1987), the onset and progression of senescence is accompanied by global changes in gene expression. Thus, deep extensive efforts have been achieved to reveal relevant molecular process by identifying and analysing

Senescence Associated Genes (SAGs) as prior tags to disclosure the core of this complex process (Kim et al. 2007). SAGs genes have been extensively studied in model plant species (Audic & Claverie 1997; Gepstein et al. 2003; Balazadeh et al. 2008; Hu et al. 2010) and in some agronomical relevant crops (Andersen et al. 2004; Conesa et al. 2005; Espinoza et al. 2007). Yet, although senescence and ageing might be considered synonyms, a distinct reference was previously discussed because the former comprises all those degenerative changes and cellular degradation occurring with little or non-reference to death, whereas the latter is considered the final developmental stage culminating in death (Nooden & Leopold 1988; Shahri 2011). In the last year, considering this limitation, many efforts are being achieved to disclosure and obtain genomic information for this oil crop (Kane et al. 2011) but complete sequence information are still no available.

Sunflower (*Helianthus annuus L.*) is one of the most relevant crops as source of edible oil and many efforts have been achieved to build up useful functional genomics tools for cultivated sunflower involving transcriptional and metabolic profiles (Fernandez et al. 2003; Cabello et al. 2006; Paniego et al. 2007; Fernandez et al. 2008; Peluffo et al. 2010). Although, molecular studies focused on the onset of the senescence process in sunflower leaf are scarce (Fernandez et al. 2003; Dezar et al. 2005; Manavella et al. 2006; Jobit et al. 2007; Paniego et al. 2007; Fernandez et al. 2008; Manavella et al. 2008; Peluffo et al. 2010; Fernandez et al. 2011). Thus, two different approaches are envisage for studying molecular events occurring during leaf senescence: the first strategy relays on the identification of sunflower SAGs based on a candidate gene approach while the second approach involves concerted gene expression studies based on high density oligonucleotide microarrays, whole transcriptome shotgun sequencing and microRNA detection by RNA-seq (Buermans et al. 2010; Dhahbi et al. 2011).

Leaf senescence is a complex and highly coordinated process (Noodén et al. 1997). Although symptoms have been explored, the involved processes and the 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, 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, concentration of CO_2, abiotic and biotic stresses caused by disease (Sadras et al. 2000) may affect the rate of senescence. A previous work (Pic et al. 2002) showed that the sequence 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, previous works assigned to sugar content in leaves an integrating role of environmental signals, regulating leaf senescence (Wingler et al. 2006). 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 (Sadras et al. 2000). Control of senescence by growth of reproductive structures was not observed in *Arabidopsis thaliana* (Noodén & Penny 2001). Moreover, determining the onset of senescence is complex because there is no a "symptom" indicating this moment. Visual parameters are often used to assess these processes, but both the variation in chlorophyll content and yellowing or necrosis of leaves, are detectable long after the signalling cascade of senescence process is activated.

Senescence studies are generally based on the accumulation of messenger RNA coding for enzymes involved in degradation of structures, however, this process has a high degree of interaction between endogenous and environmental signals, involving different genes whose expression is induced or inhibited in different stages of the process (Gan & Amasino 1997). On the other hand, there are relevant studies that inversely correlate senescence with a high level of nitrogen in soil. According to these evidences a high nutritional nitrogen performance along soil profile should lead to a delay leaf senescence in sunflower, avoiding the pronounced symptoms occurred for chlorophyll content (Aguera et al. 2010).

2. Candidate gene approach to identify SAGs in sunflower

Senescence Associated Genes (SAGs) refer to genes whose expression level is up-regulated during senescence, in contrast with Senescence Down-regulated Genes (SDGs). These genes could be classified into two classes depending on their expression patterns: Class I genes are those whose expression is only activated during senescence (senescence-specific) whereas class II are those that maintain a basal level of expression during early leaf development, but this level increases when senescence begins (Gan & Amasino 1997). The expression patterns of these genes may change in response to different conditions of plant growth. Many of these genes can be shared by different regulatory pathways whereas others may belong to a particular pathway. Thus, the inactivation or overexpression of many SAGs may not exhibits significant effect, suggesting a complex regulatory network in leaf senescence process. SAGs can be grouped into several categories based on their predictive function, including macromolecular degradation and recycling, amino acid transport, metabolism, detoxification, regulatory genes, among others (Gepstein et al. 2003).

The main objective in sunflower to open new insights into the early leaf senescence process focuses in the identification and characterization of genetic sequences and metabolic pathways involved in the onset and evolution of the leaf senescence process. This aim involved the analysis of transcriptional and metabolic profiles in leaves from plants growing under different conditions that may alter the senescence rate, concomitant with studies of physiological and biochemical aspects. The specific items involved in this work include:

1. Study of the evolution of leaf area, chlorophyll and sugar content in leaf of different ages in a traditional sunflower hybrid subjected to treatments that alter the senescence under both field and greenhouse conditions.
2. Identification in public sunflower databases of gene sequences orthologous to Senescence Associated Genes (SAG) or Senescence Down-regulated Genes (SDG).
3. Identification of new candidate genes through a sunflower microarray expression analysis.
4. Verification and quantification of the expression profiles of these genes under conditions that accelerate or delay the senescence process.
5. Study of metabolic changes that occurred during the senescence process.
6. Integration of metabolic and transcriptional profile analysis and physiological variables for the detection of useful biomarkers for application in sunflower breeding.

Following a candidate genes strategy, a preliminary assay to detect putative SAGs in sunflower was achieved by selecting few candidates previously described for *Arabidopsis thaliana*, due to the fact that this was the very first model plant for which a large-scale SAG

transcriptome was available (Gepstein et al. 2003). For this purpose six candidate SAGs were selected from this plant model (Moschen 2009) to search for orthologous genes in the sunflower EST database using the tblastx algorithm (Altschul et al. 1990), employing bioinformatics tools locally installed and developed. Sequences showing significant similarity parameters were selected and confirmed. Specific oligonucleotides were designed to amplify fragments of approximately 150 bp for further evaluation by quantitative PCR. In a previous study, we have reported the evaluation and identification of a panel of eight reference genes for their application to transcriptional analysis of the leaf senescence process, thus enabling the use of genuine reference genes in ongoing expression studies (Fernandez et al. 2011). Exploratory studies of senescence by qPCR comparing two treatments which affect the rate of leaf senescence were performed: water stress and head excision, relative to a control condition. Samples were taken from two leaves of different ages, leaf 15 and 25 in order to identify functional markers for this process. Two of the selected genes, a gamma vacuolar processing enzyme (AN At5g60360) (D3 gene) involved in the maturation and activation of vacuolar proteins and an aleurain protease AALP, (AN At1g18210) (D4 gene), belonging to the cystein-protease family are classified in the group of macromolecular degradation and recycling; the third gene, a calcium binding protein (AN At4g32940) (R2 gene) belongs to the group of regulatory genes (Gepstein et al. 2003). Furthermore two reference genes were evaluated against these conditions for relative expression studies, Elongation Factor 1-α (AN) and α-Tubuline, selected from a previous study of the performance of different reference genes against these experimental conditions in sunflower (Fernandez et al. 2011). Alfa tubuline (α-Tubuline) showed the most stable behavior; therefore, it was selected as internal control in further analysis of expression of these SAGs (Figure 1).

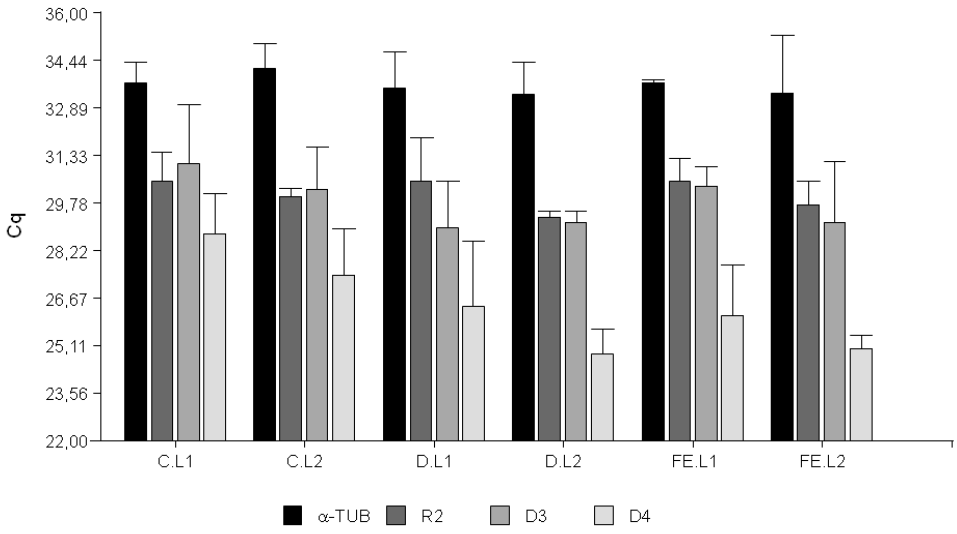

Fig. 1. Average Cq of analyzed SAGs genes normalizing against a-TUB as RG. Error bars show standard deviation (Fernandez et al. 2011).

The three selected genes did not show significant differences between the evaluated conditions at the sampling times tested (63 days post-emergence) (Table 1). It is worth noting that the target genes showed high expression levels even in controls plants with values close to the water stressed samples. Thus, these genes were probably induced by internal plant factors at an early time point, prior to the tested time in that assay. On the other hand, sampling for the incidence of head excision assessment on senescence could be consistent with an early stage of bud development in which there would be no evident differences between the two conditions (Zavaleta-Mancera et al. 1999a; Zavaleta-Mancera et al. 1999b; Thomas & Donnisson 2000).

		SAGs genes (Gepstein et al. 2003)						RGs genes (Fernandez et al. 2011)			
		R2 (AN At4g32940)		D3 (AN At5g60360)		D4 (AN At1g18210)		α-TUB (AN AF401481.1)		EF-1α (AN CAA37212.1)	
Treatment	Samples	Cq	CV	Cq	CV	Cq	CV	Cq	CV	Cq	CV
C.L1	3	30.49	2.5	31.06	5.0	28.74	3.9	33.69	1.7	30.08	2.6
C.L2	3	30.00	0.8	30.19	3.8	27.42	4.5	34.20	1.9	25.57	7.2
FE.L1	3	30.46	3.9	28.96	4.3	26.42	6.6	33.52	2.9	27.16	1.7
FE.L2	3	29.28	0.6	29.13	1.1	24.84	2.7	33.32	2.6	26.73	12.9
D.L1	3	30.45	2.1	30.31	1.7	26.10	5.1	33.67	0.2	27.80	6.7
D.L2	3	29.75	2.0	29.12	5.5	24.98	1.5	33.38	4.5	30.07	5.7

Table 1. Average Cq and CV value for R2, D3 and D4 genes and the two best ranked RGs for three biological replicates per treatment (Fernandez et al. 2011).

As a result from these analyses, the adjustment of the sampling time and frequency turns out as a highly critical point in studying gene expression profiling of candidate genes, according to the treatments on evaluation. Earlier samplings are necessary to detect the trigger moment of different candidate genes for leaf senescence process in sunflower. Considering Table 1, it is worth mentioning that relative quantification of a putative SAG would be overestimated if EF-1α (AN CAA37212.1) would have been used as a single reference gene, which reinforces the importance of normalizing against two or more experimentally validated RG when quantifying transcripts (Fernandez et al. 2011). In order to reach a wider search of new candidate genes, an additional set of new published genes were considered and their predicted functionality was evaluated with the aim to give new insights into this process. For a preliminary detection of potential SAGs, classical macromolecular degradation SAGs were discarded of our analysis because they are probably not associated with early leaf senescence, but with induced changes later in the time course of the process. In this sense, Chlorophyll-Binding Proteins (CBP) were first isolated in soybean (Guiamet et al. 1991) whereas SAGs N4 and SAG12 were detected by differential screen of *Arabidopsis* leaf senescence cDNA libraries (Gan & Amasino 1995; Park

et al. 1998). They encode an apparent cysteine proteinase and their expression is highly senescence specific (Lohman et al. 1994; Gan & Amasino 1995; Martinez et al. 2007) mainly localized in small senescence associated vacuoles (Saeed et al. 2003; Otegui et al. 2005). However, neither SAG12 nor SEN4 match any full sequence in sunflower with a high identity score level. For this reason, a second set of candidate SAGs (OsNAC5, WRKY6, ORS1 YUCCA6, among others) (Ülker & Somssich 2004; Balazadeh et al. 2011; Kim et al. 2011; Song et al. 2011) was compared against *Helianthus annuus* unigene collection but a low score level to *Helianthus annuus* sequences was detected. Therefore, other candidate genes were added to be functionally tested for early leaf senescence in sunflower. The special case of transcription factors (TFs) as crucial regulators of gene expression by binding to distinct cis-elements, generally located in the 5′ upstream regulatory regions of target genes, were specially considered to detect early senescence leaf makers (Balazadeh et al. 2008). NAC transcription factors related to senescence have been recently identified in model species and they play a relevant role in the regulation of development of leaf senescence related to programmed cell death (Olsen et al. 2005; Kim et al. 2009; Balazadeh et al. 2010; Hu et al. 2010; Nuruzzaman et al. 2010; Balazadeh et al. 2011). A single one NAC gene (AtNAP), also called NAC2 or ANAC029 (Guo & Gan 2006), has been the main one identified to control leaf senescence, although approximately 20 NAC genes in *Arabidopsis* shown high expression in senescing leaves (Guo et al. 2004; Lin & Wu 2004). ROS reagents acting as senescence stimulus were also reported within a narrow cross talk involving hormones and TFs both in natural and stress-related senescence (Rivero et al. 2007; Khanna-Chopra 2011), indicating that elevated ROS levels might be detected as a potential signal of senescence induction. Under this assumption *ORE1*, a NAC transcription factor that has been extensively studied in recent years, has been described as strongly related to leaf senescence, probably coevolving genes with ORS1 (Ooka et al. 2003). This TF can be considered a new further positive regulator of senescence in conjunction with AtNAP (Balazadeh et al. 2011), controlling leaf senescence in *Brassicacea*e. In *Arabidopsis*, *ORE1* mutants show a delay in leaf senescence whereas overexpression through an inductive promoter, accelerates senescence in relation to wild type plants (Balazadeh et al. 2010) and the forest tree *Populus trichocarpa* in which approximately 2,900 TFs were reported (Hu et al. 2010) and will be soon tested for sunflower candidate SAG detection. Microarray studies showed that 46% of up regulated genes in *Arabidopsis ORE1* overexpression lines, are known as senescence-associated genes, including many genes previously reported as senescence regulated, suggesting an important role in the development of the senescence process (Balazadeh et al. 2010). In wheat, it was reported that NAC TFs not only accelerate senescence but also improve nutrient remobilization by increasing protein, iron and zinc content (Uauy et al. 2006). ORE1 expression is under control of the ethylene signaling pathway and is subjected to regulation by miRNA164, being negatively regulated. When the leaf is young, miR164 transcripts remain at high levels regulating the expression of ORE1 but during the leaf aging process, its expression gradually decreases, thus increasing the expression of ORE1 (Kim et al. 2009).

In sunflower, a sequence similar ORE1 has been detected in the *Helianthus annuus* unigene collection developed at INTA (ATGC Sunflower Database: http://bioinformatica.inta.gov.ar/ATGC) with a Blast score of 96 and E-value of e-10 (Altschul et al. 1990). Expression profiles studies at different sunflower developmental stages showed a significant increase of putative ORE1 transcripts in samples close to anthesis stage, prior to the start of the first symptoms of senescence, when the critical period

of grain filling has already begun (Figure 2). These results are consistent with those observed in *Arabidopsis*, and turn this gene a potential functional marker of the progress of senescence, representing an important tool for future implications in the sunflower crop improvement (Moschen et al. 2010). In order to confirm *in-situ* the functionality of this putative ORE1 gene in sunflower, a comparative bioinformatics analysis has been performed using the Blastx algorithm (Altschul et al. 1990), searching for proteins in the database at the National Center for Biotechnology Information NCBI (http://www.ncbi.nlm.nih.gov/), using as query the nucleotide sequence of putative sunflower ORE1. These results showed a high similarity with ORE *Arabidopsis* protein (GI 15241819) suggesting a possible role of this gene as NAC transcription factor. Moreover, searches for functional protein domains in Pfam (http://pfam.sanger.ac.uk/) revealed that main protein domain in sunflower ORE1-like gene sequence corresponds to the family of NAM transcription factors (No Apical Meristem) (pfam02365), as well as the *Arabidopsis ORE1 sequence* pfam02365. Figure 3 shows *Arabidopsis* alignments and putative sunflower ORE1 proteins against Pfam NAC domain. Others relevant *in-silico* candidates for a putative sunflower SAG are: RAV1 gene, a transcription factor whose expression is closely associated with leaf maturation and senescence (Woo et al. 2010), which has been detected with a high score level and statistically low E-value, and CAT2, a member of a small gene H_2O_2 detoxifying enzyme family, widely characterized in *Arabidopsis* (Gergoff et al. 2010; Smykowski et al. 2010), although not yet tested in sunflower.

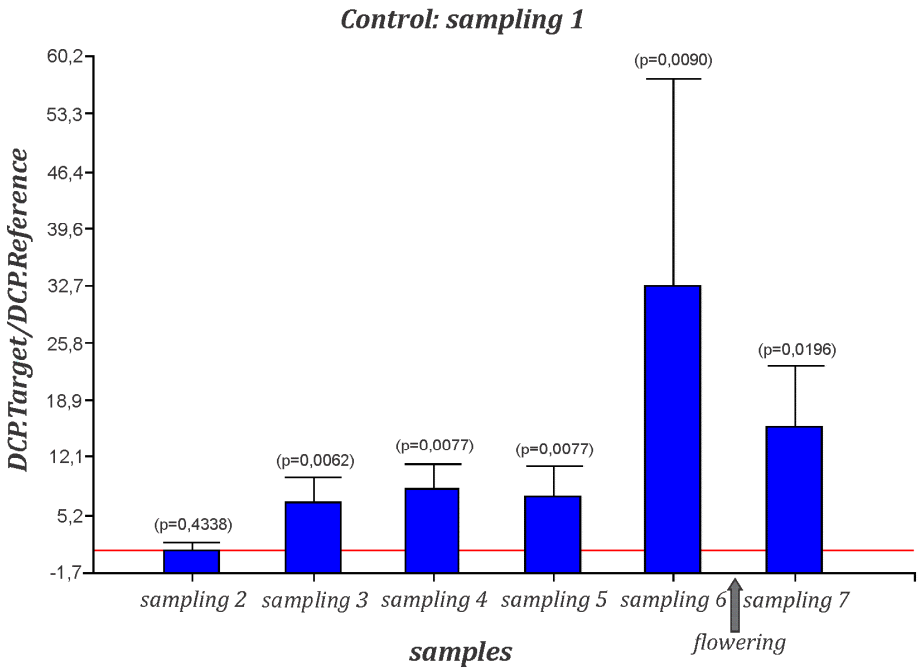

Fig. 2. Differential expression of putative sunflower *ORE1* gene in subsequent samplings, taking as control condition sampling number 1 and referred to α-TUB expression level (Moschen et al. 2010).

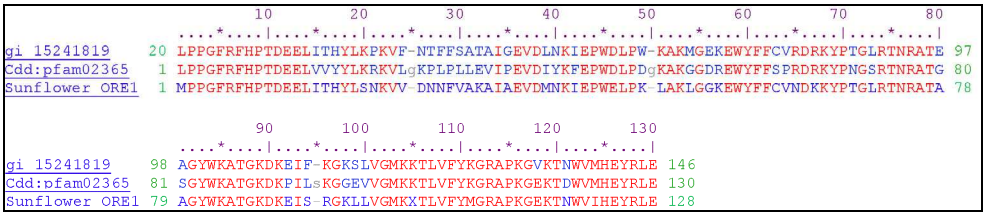

Fig. 3. *Arabidopsis* NAM domain and putative *sunflower* ORE1 protein alignment (pfam02365) (http://www.ncbi.nlm.nih.gov/cdd/).

As mentioned above, the execution of the senescence process consists of multiple interconnecting pathways which regulate and/or modulate this series of orderly steps; therefore different transcription factors play an important role as regulators of these pathways. Recently, a list of transcription factors that regulate leaf senescence in *Arabidopsis* has been published (Balazadeh et al. 2008). The search for tentative orthologous genes in the *Helianthus annuus* unigene collection, using Blast algorithm, led to the identification of 42 genes with a significant score value to transcription factors like NAC, MYB, WRKY, ARP among others, some of these genes are being studied their expression patterns by qPCR.

3. Concerted gene expression studies to elucidate sunflower senescence process

Although microarray technology started a new era of high-throughput transcriptomic analysis approximately ten years ago, starting with 8,000 printed genes by Affymetrix in *Arabidopsis thaliana* (Zhu & Wang 2000) and later on scaling up to 45,000 printed genes in rice (Jung et al. 2008) and 90,000 in *Brassica* (Trick et al. 2009), next generation sequencing (NGS) technologies are nowadays opening a new era of even deeper understanding of genomics and transcriptomics in different species . However, for the foreseeable future both technologies will coexist each focusing on different tasks, or by complementing biological and value information (Fenart et al. 2010) or by designing dedicated oligonucleotide arrays to support functional studies on a specified pathway/developmental stage (Kusnierczyk et al. 2008; Cosio & Dunand 2010; Ott et al. 2010). One obvious application of microarray technology is the transcriptional profiling in species that have neither their own genome sequenced nor a reference genome from a closely related species. For some of these species a commercial microarray based on an existing own-design are available (Agilent, Affimetrix, Nimblegen, etc) (Close et al. 2004; Li et al. 2008; Martinez-Godoy et al. 2008; Mascarrell-Creus et al. 2009; Trick et al. 2009; Booman et al. 2010; Curtiss et al. 2011). Sunflower is a species that fits into this framework, even though a genome sequence initiative is in progress (Kane et al. 2011), there is no reference genome available. In this case, the only source of functional information is limited to ESTs databases, which in the case of cultivated sunflower is rather extensive, more than 133,000 ESTs are publicly available (http://ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) covering libraries prepared from several lines and cultivars (Table2). However, it should also be noted that ESTs libraries tend to be significantly contaminated with vector sequences and chimeras, and have relatively low quality DNA information derived from the library sequencing strategy which prioritizes obtaining a large number of single pass sequences, being necessary to standardize a set of bioinformatics routines in order to clean and decontaminate public raw sequences (Figure 4).

Microarrays using ESTs and full length gene sequences allowed SAGs identification during leaf senescence at the genome-wide scale in *Arabidopsis* and other plants (Lim et al. 2007). In parallel, other high-throughput system has been assayed in other species: cDNA macro and microarray were developed for sunflower to study sunflower seed development (Hewezi et al. 2006) and the response to biotic (Alignan et al. 2006), and abiotic stresses (Hewezi et al. 2006; Roche et al. 2007; Fernandez et al. 2008). This last work reported for the first time, a concerted study on gene expression in early responses to chilling and salinity using a fluorescence microarray assay based on organ-specific unigenes in sunflower. These two strategies, although useful, are limited to the analysis of a limited set of genes. Currently, the shortage of candidate genes underlying agronomically important traits represents one of the main drawbacks in sunflower molecular breeding. In this context, functional tools which allow concerted transcriptional studies, as high density oligonucleotide microarray, strongly support the discovery and characterization of novel genes. Oligonucleotide-based chips not only allow the analysis for a whole transcriptome but they are also considered more accurate than cDNA-based chips due to the reduction of manipulation steps (Larkin et al. 2005; Lai et al. 2006). The possibility to implement this technology on any custom array system like Agilent, Nimblegen, and others, has the potential to create a very useful tool for gene discovery in orphan crops (Nazar et al. 2010; Ophir et al. 2010). In addition, the use of longer probe format represents a major advantage of Agilent oligonucleotide microarrays over others technologies based on a higher stability in the presence of sequence mismatches, being consequently, more suitable for the analysis of highly polymorphic regions (Hardiman 2004).

In general, the analysis of complex biological processes based on a gene by gene approach seldom leads to limited or erroneous conclusions requiring an alternative approach based on systemic association studies. Under this assumption, new insights into molecular senescence events might be cleared up by high-resolution microarray data, for example, considering different points of leaf development (Breeze et al. 2011) or predicting putative SAGs by tissue and functional categories (Thomas et al. 2009). In our lab, a public and proprietary datasets of *H. annuus L.* ESTs have been used to create a comprehensive sunflower unigene collection. This dataset comprises 34 cDNAs libraries available from different cultivars, various tissues and anatomical parts, from plants grown at different physiological conditions.

Figure 4 describes the routines applied for the *H. annuus L* unigene collection design.

A Digital Gene Expression Profile (Audic & Claverie 1997) was assayed with the EST public data in order to detect any bias that would be pseudo-enriching the gene index by full representation of one library over another considering full public ESTs derived from public collections (Table 2). This analysis ("digi-Northern") detected that ESTs were equally represented among differential cDNA libraries, showing that the *H. annuus* unigene collection generated would be fully represented by different transcripts, lacking of a potential enrichment or overestimation among organ-specific ESTs libraries. This unigene collection was used to design the first custom sunflower oligonucleotide-based microarray based on Agilent technology as a main goal for functional genomics approaches, generated within the frame of a collaborative project involving Argentinean research sunflower groups (Sunflower PAE Consortium), Facultad de Agronomía (UBA) and the Bioinformatics facility at the Principe Felipe Institute, Valencia , España. A Chado-based database (Mungall et al.

2007) and a visualization tool call ATGC (Clavijo et al., unpublished) was developed to integrate and browse sunflower transcriptome information. Figure 5 shows the output of the ATGC interface for one functional annotated sunflower unigene.

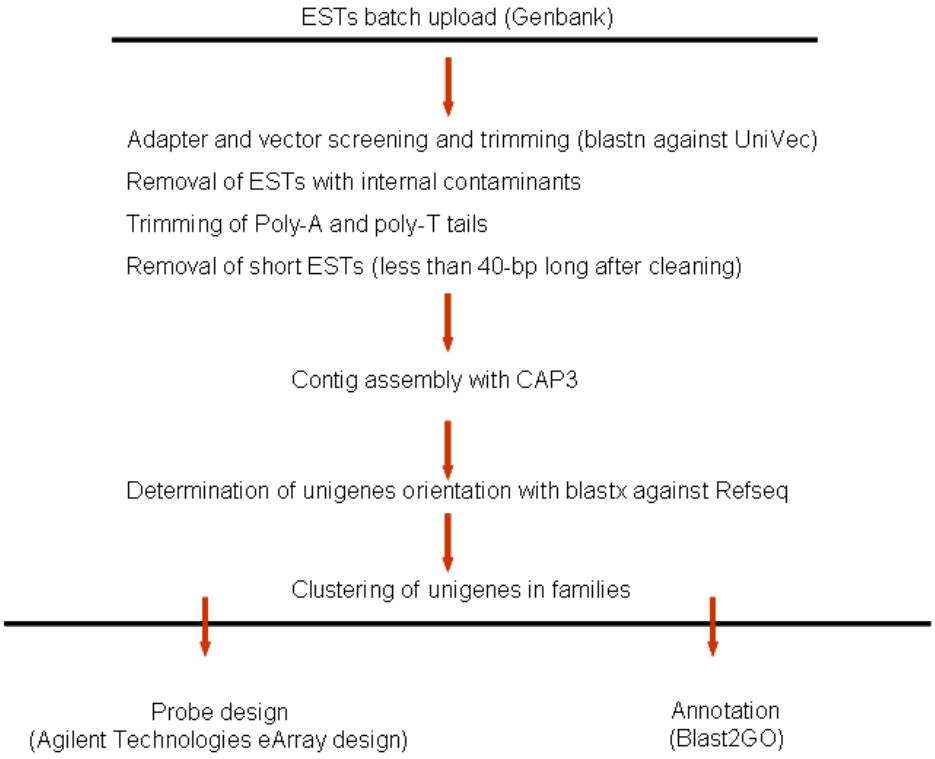

Fig. 4. Bioinformatics routines applied to design *Helianthus annuus* unigene collection (http://bionformatica.inta.gov.ar/ ATGC/).

Sunflower gene expression chip probes were designed using eArray® web application (Agilent Technologies). For this instance, two probe sets were designed: one including non-control specific probes for the sequences of sunflower unigene collection and a second control probe set consisting in 74 probes derived from 80 differentially expressed sunflower genes identified in a previously work (Fernandez et al. 2008). The latest group was used as 'Replicate Controls' with 10 replicates each. To utilize the full capacity of the microarray, probes were randomly selected to be represented in duplicate in the final design, which also included Agilent Technologies' standard panel of quality control and spike-in probes. This design was then used to manufacture microarrays using Agilent SurePrint™ Technology in the 4 x 44 format. Agilent's microarrays include the Spike-In Kit that consists of a set of 10 positive control transcripts optimized to anneal to complementary probes on the microarray, minimizing self-hybridization or cross-hybridization. This work contemplates the microarray validation through diverse differential expression analysis in order to analyze early senescence in sunflower through a classical approach and a pipeline-based

methodology. Differential gene expression was also carried out using the limma package (Smyth 2004). Multiple testing adjustments of p-values was done according to Benjamini and Hochberg methodology (Benjamini & Hochberg 1995). Gene set analysis was carried out according to the Gene Ontology terms using FatiScan (Al-Shahrour et al. 2007) integrated in Babelomics suite (Al-Shahrour et al. 2005).

Library ID	Developmental stage
HaSSH	Molecular characterization of phosphorus-responsive genes in sunflower
CCF (STU)	EST sequences from several different strains/cultivars
QH-RHA 280/QH_ABCDI sunflower RHA801	shoots/hulls/flowers environmental stress/chemical induction
CHA(XYZ) common wild sunflower	girasol silvestre (wild sunflower)
HaHeaS	heart-shaped embryo vs cotyledonary embryo
HaHeaR	heart-shaped embryo
HaCotR	cotyledonary embryo
HaGlbR	globular embryo
HaDevS1	4 days after self-pollination embryo
HaDevS2	7 days after self-pollination embryo
HaDevR1	leaves
HaDevR2	terminal bud
HaDevR3	stem
HaDevR6	embryo
HaDevR5	4 days after self-pollination embryo
HaDevR8	15 days after self-pollination embryo
HaDis	unknown/cotyledons/ (Genoplante)
HaSemS4	hypocotyl
HaDpsR1	hypocotyl
HaDplR2	hypocotyl 1-5 days
HaDplR	protoplast
HaERF	embryo
HaERS	embryo
HaR	INTA: organ-specific cDNA libraries (root)
HaT	INTA: organ-specific cDNA libraries (stem)
HaEF	INTA: organ-specific cDNA libraries (early flower)
HaF	INTA: organ-specific cDNA libraries (flower)
HaH	INTA: organ-specific cDNA libraries (leaf)

Table 2. Public cDNA libraries deposited in GenBank for which *H. annuus* unigene collection was designed.

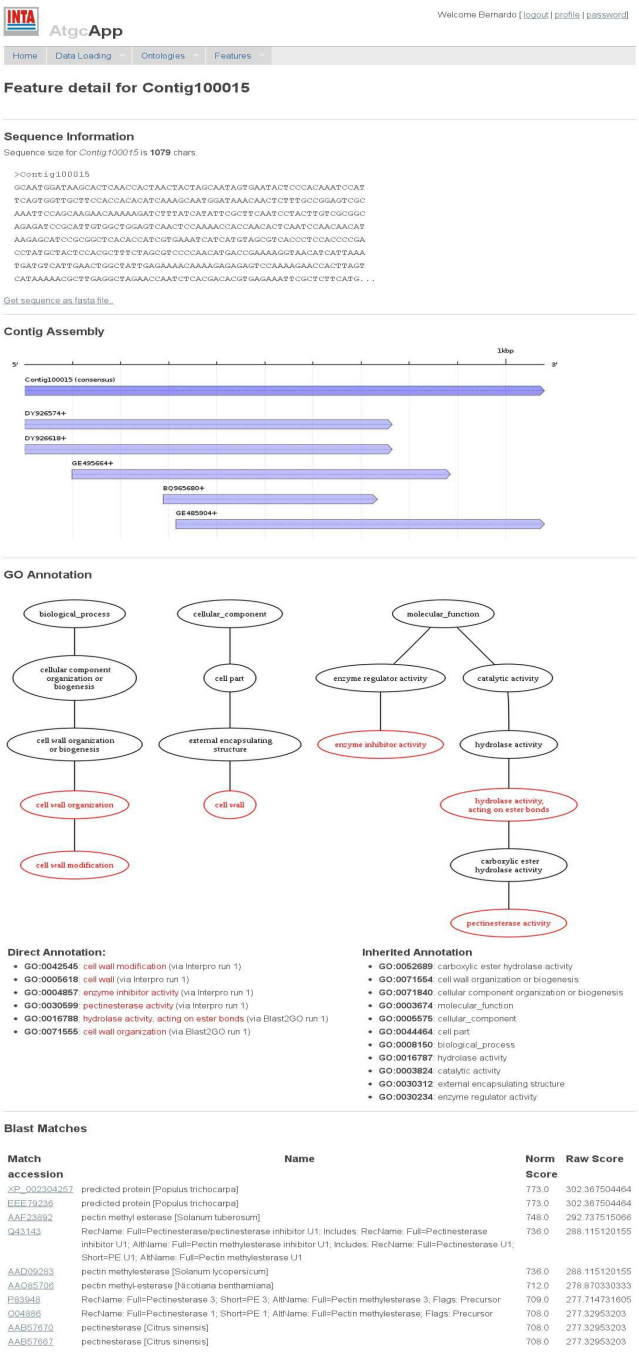

Fig. 5. ATGC view for an annotated sunflower unigene.

4. Conclusions and perspectives

Knowing the time of onset the the cascade of events that trigger senescence could determine the causes of this process and generate molecular tools to facilitate future interventions on it, useful for application in assisted breeding of this crop with major growing oil impact in the world.

The sunflower chip, designed within a PAE Consortium made up of six laboratories and one private company working in different areas of research and development, was validated by means of the analysis of global changes in gene expression profiles in response to water deficit as a physiological event which induces senescence, taken as a model experiment, for which reference genes have also been previously identified (Fernandez et al. 2011). This high-throughput transcriptome tool will allow the discovery, identification and analysis of a new set of putative SAGs for sunflower which would bring novel insights for this process. The integrated analysis of transcriptional and metabolic profiles will allow the identification of concerted regulation of distinct metabolic pathways facilitating the discovery of robust candidate genes and key metabolic pathways involved in the outbreak of the early senescence process in sunflower leaves. We expect that the integration of the information generated by this project will allow the construction of the quantitative predictive model of senescence in sunflower, under field and greenhouse conditions, which is required to interpret the regulation of the underlying complex biological processes. There will also be practical applications in directed gene discovery for other important agronomic traits involving plant responses to biotic and abiotic stresses. Finally, this project will have impact based in the establishment of microarray technologies and metabolic analysis, as well as on the knowledge of appropriated statistical and bioinformatics procedures supporting functional genomics ranging from the transcriptome to the metabolome.

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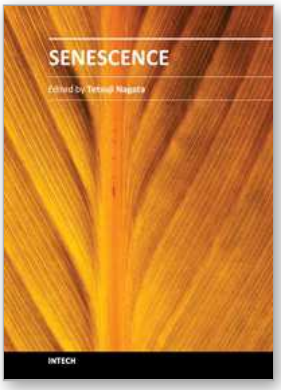

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The book "Senescence" is aimed to describe all the phenomena related to aging and senescence of all forms of life on Earth, i.e. plants, animals and the human beings. The book contains 36 carefully reviewed chapters written by different authors, aiming to describe the aging and senescent changes of living creatures, i.e. plants and animals.

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