Integrating transcriptomic and metabolomic analysis to understand natural leaf senescence in sunflower

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Summary

Leaf senescence is a complex process, which has dramatic consequences on crop yield. In sunflower, gap between potential and actual yields reveals the economic impact of senescence. Indeed, sunflower plants are incapable of maintaining their green leaf area over sustained periods. This study characterizes the leaf senescence process in sunflower through a systems biology approach integrating transcriptomic and metabolomic analyses: plants being grown under both glasshouse and field conditions. Our results revealed a correspondence between profile changes detected at the molecular, biochemical and physiological level throughout the progression of leaf senescence measured at different plant developmental stages. Early metabolic changes were detected prior to anthesis and before the onset of the first senescence symptoms, with more pronounced changes observed when physiological and molecular variables were assessed under field conditions. During leaf development, photosynthetic activity and cell growth processes decreased, whereas sucrose, fatty acid, nucleotide and amino acid metabolisms increased. Pathways related to nutrient recycling processes were also up-regulated. Members of the NAC, AP2-EREBP, HB, bZIP and MYB transcription factor families showed high expression levels, and their expression level was highly correlated, suggesting their involvement in sunflower senescence. The results of this study thus contribute to the elucidation of the molecular mechanisms involved in the onset and progression of leaf senescence in sunflower leaves as well as to the identification of candidate genes involved in this process.

Introduction

Leaf senescence is a complex process, controlled by multiple genetic and environmental variables, which has strong impact on crop yield (Gregersen *et al.*, 2013). In sunflower, the fourth most important oil crop worldwide, the senescence process reduces the capacity of plants to maintain their green leaf area for longer periods, thus leading to economic losses (Aguirrezábal *et al.*, 2003; Dosio *et al.*, 2000). In different crops, including sunflower, a delay in leaf senescence thus has an important impact on yield, by maintaining photosynthetic leaf area especially during the reproductive stage (DelaVega *et al.*, 2011; Gregersen *et al.*, 2013; Kusaba *et al.*, 2013; Sadras *et al.*, 2000a,b). During leaf senescence, a massive degradation of photosynthetic proteins takes place. In addition, Martínez *et al.* (2008) and Carrión *et al.* (2013) demonstrated that the accumulation of small 'senescence-associated vacuoles' (SAVs)

with intense proteolytic activity in senescing leaves of soya bean and *Arabidopsis thaliana* is associated with chloroplast protein breakdown. This finding suggests autophagy is involved in the process. Considering the complexity and dynamic nature of these processes, embarking on an experimental systems biology approach is crucial in understanding the leaf senescence process (Guo, 2013). At the transcriptional level, the regulation of senescence has been well studied in model species such as *A. thaliana* (Gepstein *et al.*, 2003; Lim *et al.*, 2003; Lin and Wu, 2004). Furthermore, this process has been partially assessed in other species such as rice (Lee *et al.*, 2001), tobacco (Pageau *et al.*, 2006), pea (Pic *et al.*, 2002), wheat (Uauy *et al.*, 2006), cotton (Kong *et al.*, 2013), maize (Sekhon *et al.*, 2012; Zhang *et al.*, 2014), turnip (Gombert *et al.*, 2006), barley (Hollmann *et al.*, 2014; Jukanti *et al.*, 2008) and sunflower (Cabello *et al.*, 2006; Fernandez *et al.*, 2012a; Moschen *et al.*, 2014).

Transcription factors (TFs) are key proteins in the regulation of gene expression and signal transduction networks regulating different biological processes. Several TF families have been associated with leaf senescence in many species, particularly the NAC (Balazadeh et al., 2010, 2011; Guo and Gan, 2006; He et al., 2005; Hu et al., 2010; Kim et al., 2009, 2013b; Matallana-Ramirez et al., 2013; Nuruzzaman et al., 2010; Wang and Dane, 2013), MYB (Jaradat et al., 2013; Zhang et al., 2011), AP2 (Chen et al., 2012a,b; Koyama et al., 2013) and WRKY (Besseau et al., 2012; Miao et al., 2008; Rushton et al., 2012; Ulker et al., 2007; Zhou et al., 2011) families.

The advances in genome sequencing and gene expression profiling tools allowed transcriptome analyses during natural and induced senescence (Breeze et al., 2011; Buchanan-Wollaston et al., 2005; Guo and Gan, 2012; Guo et al., 2004). These transcriptomic analyses increased the understanding of senescence; senescence regulation at the metabolic level, however, remains limited.

Recently, Watanabe et al. (2013) performed a detailed spatio-temporal dissection of metabolic changes throughout senescence in A. thaliana. This studied covered approximately 260 metabolites, including different pigments, lipids, sugars, amino acids, organic acids, secondary metabolites and ionic nutrients.

An integrative study of different omics approaches for the study of leaf senescence was recently conducted in maize (Sekhon et al., 2012) and A. thaliana (Balazadeh et al., 2014).

The aim of the present study was to characterize transcriptional and metabolic pathways related to the triggering and progression of leaf senescence in sunflower, through the integration of transcriptomic, metabolomic and physiological profiles evaluated at different developmental stages. Moreover, considering the increasing concern about whether glasshouse experiments could reproduce the natural growth conditions of field experiments (Gregersen et al., 2013), we conducted the assays simultaneously under both conditions.

Results

Physiological measurement of leaf senescence

Plant development and flowering time (100 °Cd later) were delayed under glasshouse conditions compared to field conditions (Table 1). The plants grown in the glasshouse lacked the natural leaf senescence phenotype observed in field experiments. Thus, in glasshouse experiments, leaves remained green throughout plant development until an abrupt senescence concomitant with general plant senescence.

We also assessed the progression of leaf senescence throughout leaf development by measuring changes in chlorophyll content (glasshouse and field experiments) as well as nitrogen and soluble carbohydrates (field experiment). Under field conditions, maximum chlorophyll content reached 0.03 mg/cm^2, more than 200 °Cd ahead from flowering time. From 700 °Cd after emergence, chlorophyll began to decline up to undetectable

levels close to 900 °Cd (Figure 1a). The chlorophyll content under glasshouse conditions remained apparently unaltered along the evaluated time points (Figure 1a).

After an initial high value above 400 µg/cm², total soluble carbohydrates (TSC) decreased to values between 100 and 200 µg/cm² from 600 up to 850 °Cd just after flowering. Then, TSC dropped abruptly until leaf death around 900 °Cd (Figure 1b). Similarly, nitrogen content (%) started to decline at 600 °Cd after emergence (Figure 1c) with a similar rate to that of the chlorophyll degradation and before flowering time; at that time, the N content had already been reduced to 60% of its maximum content.

Transcriptomic analysis

Microarray data analysis allowed the detection of 10 173 and 7517 probes in the field and glasshouse experiments, respectively; these probes represent differentially expressed genes
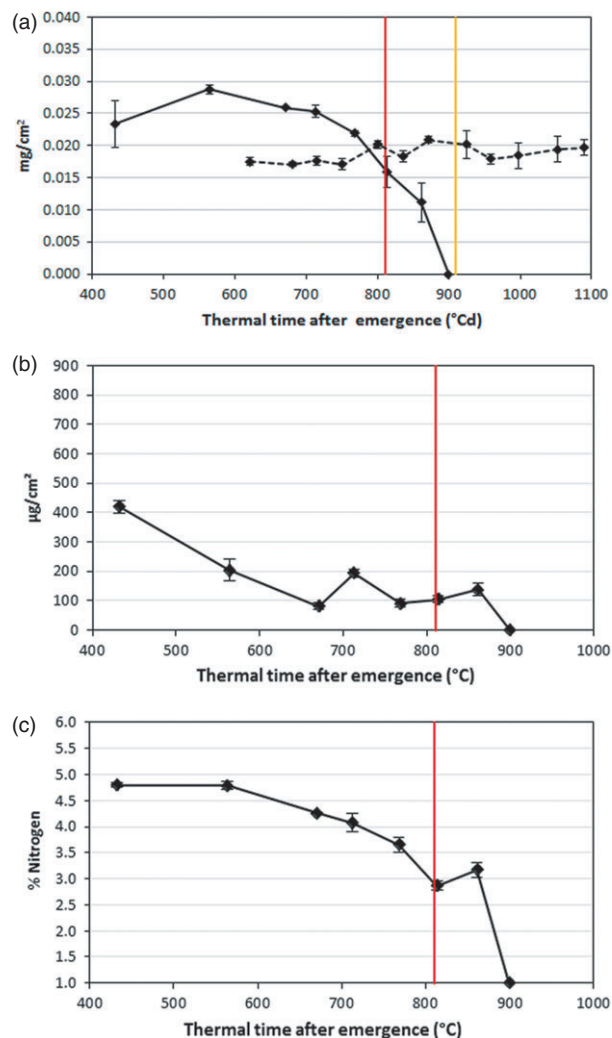

Figure 1 Physiological measurements during leaf senescence. (a) Chlorophyll content in mg/cm^2 under field (solid line) and glasshouse conditions (dotted line); (b) total soluble carbohydrates in µg/cm² under field conditions and (c) total nitrogen percentage under field conditions. The red line indicates anthesis time under field conditions and the orange line indicates anthesis time under glasshouse conditions. Error bars correspond to standard errors.

Table 1 Leaf sampling for transcriptomic and metabolomic analyses at three thermal time points

Condition	T-0	T-1	Flowering	T-2
Field (°CDAE)	564	768	810	861
Glasshouse (°CDAE)	621	871	910	1089

between the different time points throughout leaf development (T-1 vs. T-0 and T-2 vs. T-0). Values were considered significant with *P*-values lower than 0.05 and with a log2 fold change higher or lower than 2. Microarray validation through qPCR analysis yielded similar expression profiles in nine of the ten evaluated transcripts in comparison with the microarray data from the field experiment (Figure S1). Principal component analysis (PCA) of transcriptomic data showed a lower variability among biological replicates in the field experiment over the glasshouse condition. This result could, however, reflect the sample unit definition in each condition, as each biological replicate consisted of three randomly selected plants from each plot in the field experiment and one plant per plot in the glasshouse experiment (Figure 2a). Hereafter, elevated mRNA levels will be described as up-regulated and decreased mRNA levels as down-regulated.

Functional analysis

A *gene set analysis* allowed the detection of the differentially enriched functional categories represented on the microarray (Figure 3). This analysis revealed that most GO categories of *biological processes* and *molecular function* were down-regulated rather than up-regulated during senescence under both conditions (Figure 3). Furthermore, the list of differentially regulated functional categories was invariant between glasshouse and field conditions at the pre-anthesis time (T1-T0). Among the up-regulated genes, these GO categories contained genes mainly related to transcriptional regulation, TFs and ligase activity, especially involved in the carbon–nitrogen and carbon–oxygen link formation. Furthermore, among these genes, there were genes related to transferase activity, specifically nitrate and phosphate group transferases (Figure S2a and c). Among the down-regulated genes, we found high enrichment of oxido-reductase, transferase and isomerase catalytic activities, especially those related to glucosyltransferase activities involved in cell wall synthesis, as well as of genes related to carbon and sulphide metabolite groups (Figure S2b and d).

Senescence-associated genes (SAGs) analysis

To assess the behaviour of previously reported SAGs, we performed a BLAST search of approximately 1200 *A. thaliana* SAG sequences reported on the leaf senescence database (http://

psd.cbi.pku.edu.cn/) (Li *et al.*, 2014) against the sunflower database (*SUR v1.0*) (Fernandez *et al.*, 2012b). The output of this search was that 369 candidate unigenes had high sequence similarity. Of these candidates, 167 and 103 putative SAGs were differentially expressed during leaf senescence in the field (Figure 4a) and glasshouse (Figure 4b) experiments, respectively. Analysis of the most relevant molecular functions revealed that most of them were up-regulated and corresponded to genes associated either with nutrient recycling, lipid and/or carbohydrate metabolism, transcription regulation, protein degradation or redox regulation.

As mentioned before, a higher number of differentially expressed SAGs were detected under field conditions. For this reason, we selected the detected up- and down-regulated SAGs from this experiment for further analysis. The selected SAGs were those with a log2 fold change higher or lower than 2 (Table S2). Among this subset, the most significantly expressed categories were enzymes related to nutrient recycling such as glyoxylase I and lysine-ketoglutarate reductase enzymes. *SAG12*, a cysteine protease, which is normally used as a senescence marker gene (Lohman *et al.*, 1994), was also up-regulated during leaf senescence. Its senescence-associated up-regulation was previously demonstrated in several species including agronomic crops such as barley (Jukanti *et al.*, 2008; Parrott *et al.*, 2010) and wheat (Gregersen and Holm, 2007; Ruuska *et al.*, 2008). Furthermore, TFs previously reported as SAGs, such as representatives of the NAC, MYB, MBF1, HSF and AP2-EREBP families, also exhibited high expression levels during leaf senescence. Similarly, genes related to lipid and carbohydrate metabolisms and protein degradation displayed high expression levels throughout this process.

Analysis of transcription factors

We identified sunflower TFs by comparing approximately 23 000 sequences of TFs from *Arabidopsis lyrata*, *A. thaliana*, *Oryza sativa*, *Populus trichocarpa*, *Vitis vinifera* and *Zea mays*. These sequences are available at the plant TF database (http://plntfdb.bio.uni-potsdam.de/v3.0/) (Pérez-Rodríguez *et al.*, 2010) and were compared with SUR v1.0 database (Fernandez *et al.*, 2012b). A total of 687 candidate unigenes with high sequence similarity to TFs were identified. The expression analysis of these candidate TFs revealed that 140 and 123 were differentially
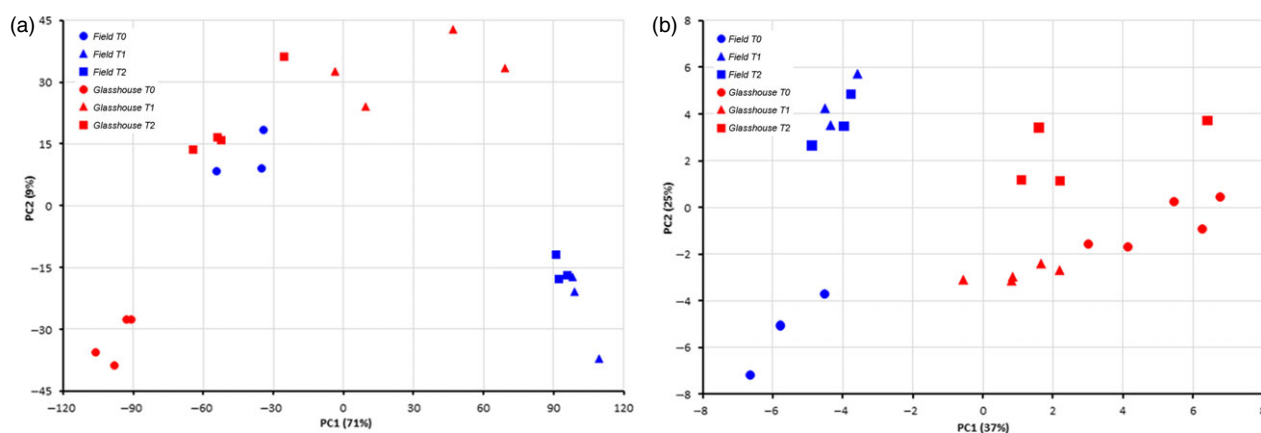

Figure 2 Principal component analyses. (a) Transcript analysis. (b) Metabolite analysis. The blue and red dots correspond to the biological replicates of field and glasshouse experiments, respectively, during leaf development.
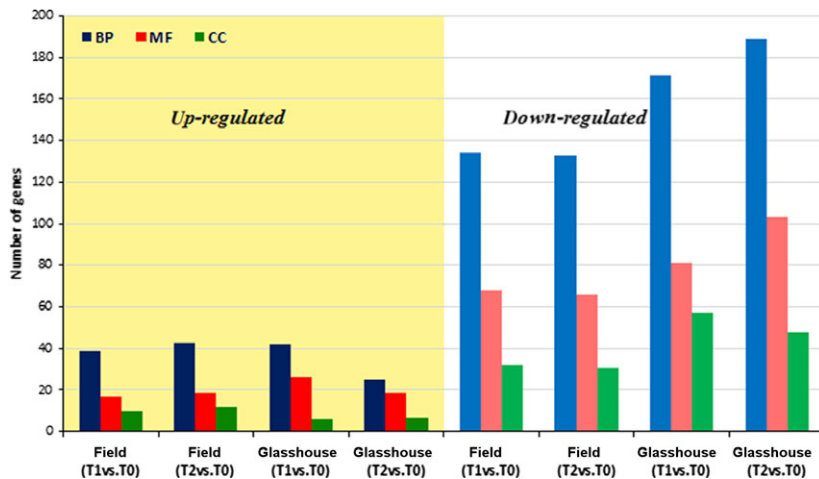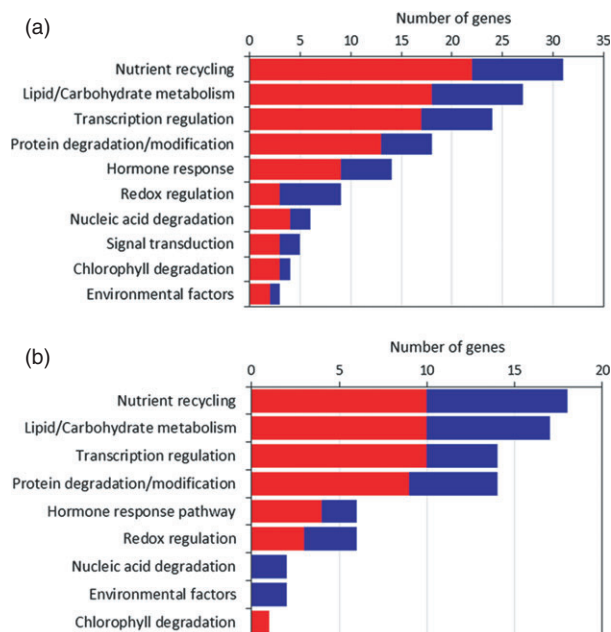
(a)

(b)

Figure 4 Differentially expressed SAGs assessed by microarray analysis. Differentially expressed genes during leaf development (T0 vs. T1 and/or T2 vs. T0) in the (a) field and (b) glasshouse experiments. The red and blue bars indicate the number of up-regulated and down-regulated genes, respectively.

expressed along leaf senescence under field (Figure 5a) and glasshouse conditions, respectively (Figure 5b). The most represented families correspond to the AP2-EREBP, MYB, NAC, bZIP and HB TF families. Several members of these TF families have been previously reported to be associated with leaf senescence as well as with different biotic and abiotic stress responses in model species. Furthermore, most of these TF families showed a high ratio of up-/down-regulated genes throughout the senescence process.

Similarly to the results of the SAG analysis, we detected several putative TFs with higher expression levels during leaf senescence in the field experimental condition. Moreover, when we selected the top up- and down-regulated TFs with a log2 fold change higher or lower than 2, we observed that the most represented TF

families not only exhibited a large number of differentially expressed genes, but also showed higher expression levels in relation to other gene families (Table S3).

Spearman correlation analysis was performed to assess possible co-expression and coregulation patterns during natural leaf senescence. This analysis was performed between the differentially expressed TFs of the field experiment (Figure 6). It revealed many positive correlations mainly among the up-regulated genes. The NAC TF family showed a large number of inter-relationships with other candidate TFs including those of the NAC, AP2-EREBP, MYB, MYB-related and ARF families. Among the NAC TF family, we found high sequence identity with genes associated with leaf senescence and different stresses in *A. thaliana*: *NAC19*, *NAC02*, *NAC47*, *NAC55* and *NAC81*.

Metabolic analysis

Primary metabolite analysis

By GC-TOF-MS analysis, we detected around 60 primary metabolites during leaf development in both conditions, including different amino acids, organic acids, sugars and sugar alcohols (Figure 7).

The metabolic profiles of field- and glasshouse-grown materials showed similar patterns. In both experiments, carbohydrate levels along with metabolites of the tricarboxylic acid (TCA) cycle decreased during leaf senescence. By contrast, many other metabolites involved in the amino acid metabolism increased their level, in particular aromatic amino acids such as tryptophan, tyrosine and phenylalanine, and branched-chain amino acids such as isoleucine and valine. We also observed high levels of asparagine and glutamine, amino acids involved in the nutrient recycling process.

As in the transcriptomic analysis, these changes were considerably stronger in the field experiment, with higher fold changes during the progress of leaf development. The different growth conditions could explain the observed differences. For instance, in the field experiment, plants receive higher light intensity, which could enhance the differences in metabolite levels.

Similarly to the transcriptomic results, PCA of metabolite data revealed a closer clustering among biological replicates of the field experiment compared to the glasshouse condition (Figure 2b). With these results in mind, we focused the integrated
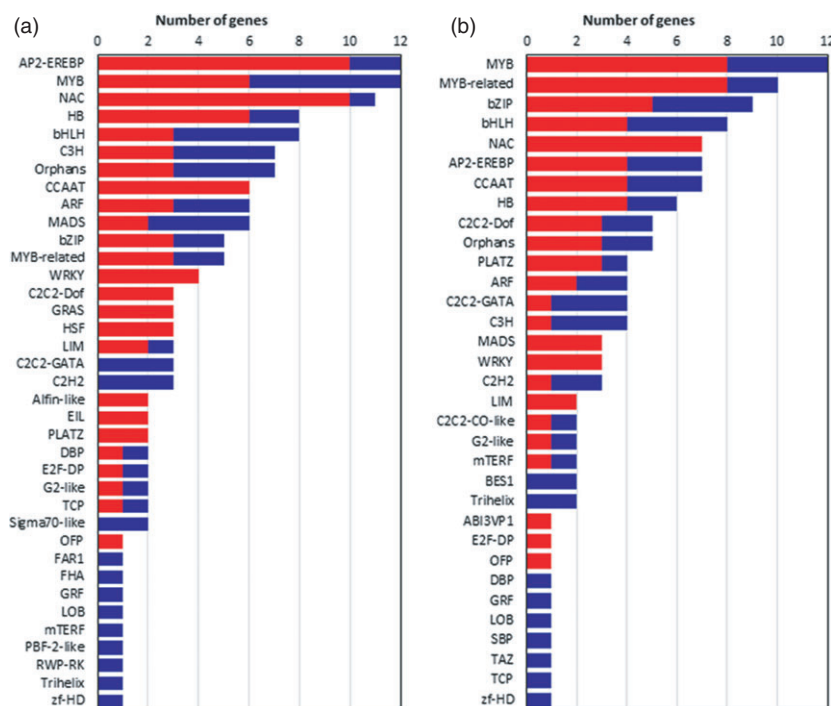
Figure 5 Differentially expressed putative transcription factors derived from the microarray analysis. Differentially expressed genes during leaf development (T0 vs. T1 and/or T2 vs. T0) in the (a) field and (b) glasshouse experiments. The red and blue bars indicate the number of up-regulated and down-regulated genes, respectively.

data analysis on the field experiment to assess leaf senescence under natural environmental conditions.

Ionic nutrient analysis

Four anionic nutrients (chloride, nitrate, sulphate and phosphate) and five cationic nutrients (sodium, ammonium, potassium, magnesium and calcium) were detected and quantified by ion chromatography in samples from both field and glasshouse experiments (Figure 8). Among the anionic nutrients, nitrate levels increased during leaf development under both conditions, whereas sulphate content decreased throughout leaf senescence. Phosphate contents slightly decreased under field conditions, whereas remained apparently unaltered under glasshouse conditions. In contrast, cationic nutrients did not display well-defined patterns during leaf senescence.

Data integration

With the aim of characterizing the progression of leaf senescence in sunflower, we performed a transcriptomic and metabolic data integration using MapMan (Thimm *et al.*, 2004). Field (Figure 9) and glasshouse (Figure S3) experiments showed similar up- and down-regulated patterns, with a higher fold change observed in the field experiment. For this reason, we focused our analysis in the field experiment.

Data integration allowed the detection of the most important biological processes during leaf senescence in sunflower plants. Under the evaluated conditions, we observed a decrease in the expression levels of genes and in metabolite contents related to photosynthesis and photorespiration as well as to the ascorbate–glutathione cycle involved in antioxidant defence mechanisms. Similarly, glycolysis-associated genes were also down-regulated, and as expected, the metabolites involved in these pathways were showed lower levels. On the other hand, sucrose and starch synthesis genes had low expression levels throughout leaf senescence with the exception of sucrose

degradation genes whose expression increased through the process. In addition, we found high expression of genes related to lipid degradation pathways, the glyoxylate cycle, nucleotide and amino acid degradation, as well as genes associated with nutrient recycling.

At the pre-anthesis stage, prior to the appearance of first visual symptoms of leaf senescence, we detected high expression levels of genes and contents of metabolites related to nutrient recycling. Beta-oxidation of fatty acids and the glyoxylate cycle-related genes showed particularly high expression levels whilst the expression of malate synthase and isocitrate lyase enzymes was also considerably higher. Furthermore, genes related to amino acid and nucleic acid degradation were up-regulated during leaf development. For instance, glutamate dehydrogenase was up-regulated and thus could be anticipated to lead to an enhanced production of ammonia. Ammonia in turn is substrate of the glutamine synthase enzyme, which was also up-regulated, and thus could be anticipated to generate glutamine as a transport amino acid. The asparagine synthetase gene was also up-regulated at an early stage during senescence, thus potentially increasing asparagine production, another important process of mobilization during nutrient recycling.

Discussion

Transcriptomic analysis of sunflower leaf senescence

Different genes are up- or down-regulated during senescence, and these genes might be involved in multiple signalling pathways interconnected in a network that controls leaf senescence. In the present study, a transcription profile analysis of leaf senescence in sunflower plants grown under field or glasshouse conditions was performed using Agilent microarray technology (Fernandez *et al.*, 2012b).

A functional enrichment analysis via *gene set analysis* methodology (Montaner and Dopazo, 2010) demonstrated a decrease

(a)

(b)
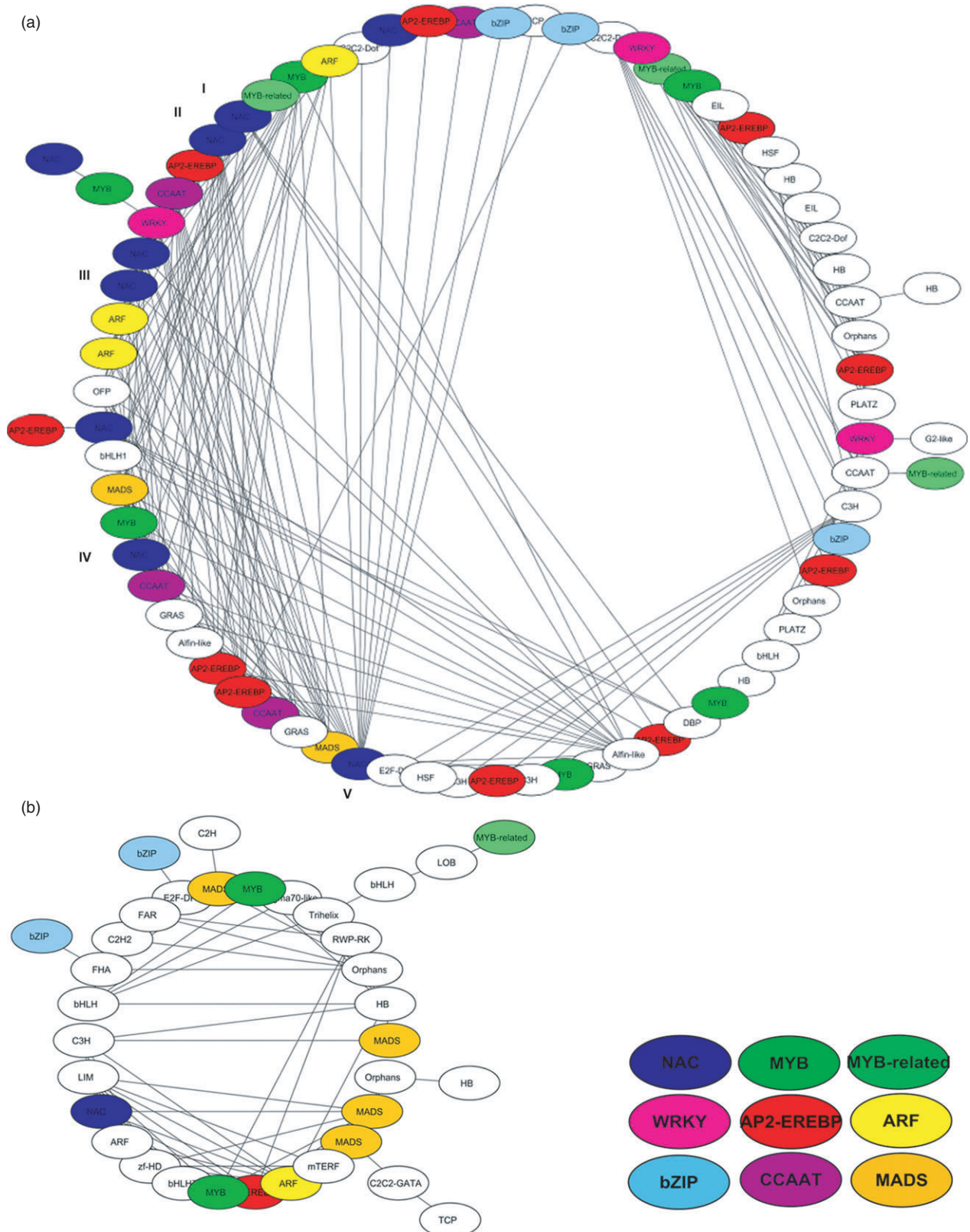
Figure 6 Transcription factor correlation analysis. Spearman correlations between differentially expressed TFs during leaf senescence progress under natural conditions, the field experiment (T0 vs. T1 and/or T2 vs. T0). (a) Up-regulated and (b) down-regulated TFs during leaf senescence. Correlation *P*-value <0.01. Roman numerals indicate high sequence similarity to *Arabidopsis thaliana* genes: I: *NAC19*, II: *NAC02*, III: *NAC47*, IV: *NAC55* and V: *NAC81*.
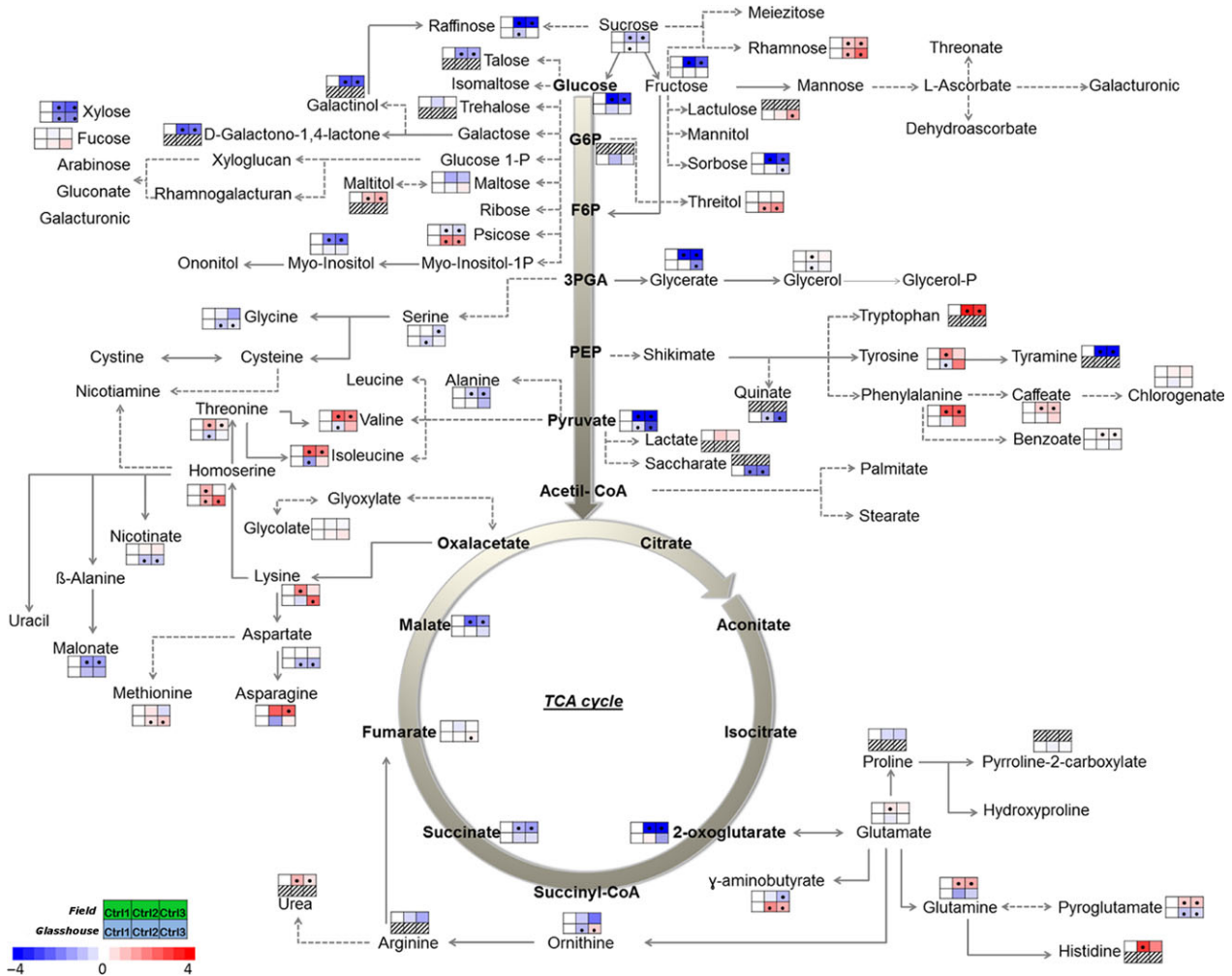
Figure 7 Metabolic profile analysed by GC-TOF-MS. Each graph represents the fold change considering the time 0 as a control; columns represent the three evaluated times for each metabolite. The first row corresponds to the field experiment, whereas the second row corresponds to the glasshouse experiment. The points within each graph indicate significant differences (*P*-value <0.05).

in mitochondrial activity and cell growth during senescence (Figure S2b and d). In addition, an enrichment of genes related to transcriptional regulation and nutrient recycling was detected (Figure S2a and c). Similar results were obtained in *A. thaliana,* in which the transcriptional profile was assessed throughout senescence (Breeze *et al.*, 2011). This process was further described in other nonmodel plants such as barley (Parrott *et al.*, 2007) and wheat (Gregersen and Holm, 2007).

Genes related to recycling processes, such as kinases, phosphatases, proteases and hydrolases, were not only found to be differentially expressed throughout senescence, but most of them were specifically up-regulated during this process (Table S2). In this analysis, we identified differentially expressed TFs mainly from the NAC, MYB, AP2-EREBP, HB and bZIP families (Figure 5). Members of these TF families have been widely studied and reported as candidate genes activating the leaf senescence programme in different species (Balazadeh *et al.*, 2008; Breeze *et al.*, 2008). Genes with high sequence similarity to *ANAC055, ANAC019* and *ANAC029* from *A. thaliana* were highly expressed during sunflower senescence (AT3G15500, AT1G52890 and AT1G69490, respectively) (Table S3). In addition, two genes with high similarity to *ANAC055* and *ANAC019,* which are involved in

jasmonic acid and/or ethylene signalling pathways particularly in response to pathogen attack (Bu *et al.*, 2008), were also detected as highly correlated. ANAC019 was further postulated as a triggering factor of senescence with a role in the flavonoid and anthocyanin biosynthesis; ANAC055, on the other hand, could accelerate senescence and seems to be involved in the chitin response (Hickman *et al.*, 2013). Additionally, ANAC029, also called NAC-LIKE ACTIVATED BY AP3/PI (AtNAP), has previously been reported as a senescence activator (Guo and Gan, 2006).

Two MYB TFs, which displayed high expression levels, exhibit high sequence identity to the *A. thaliana* *MYB44* and *MYB73* (AT5G67300 and AT4G37260, respectively) (Table S3). *MYB44* is expressed during plant development especially in senescent leaves and behaves as a negative regulator of ABA, stress, wounding and senescence, through an homoeostatic function, by maintaining the growth (Jaradat *et al.*, 2013). This TF may be induced by ethylene (Liu *et al.*, 2010). *MYB73* is induced by salt stress and is a negative regulator of salt overly sensitive genes (*SOS*) (Kim *et al.*, 2013a).

Regarding AP2-EREBP TF family, three members with high sequence similarity to *ERF104, ERF3* and *ERF72* (AT5G61600, AT1G50640 and AT3G16770, respectively) showed high
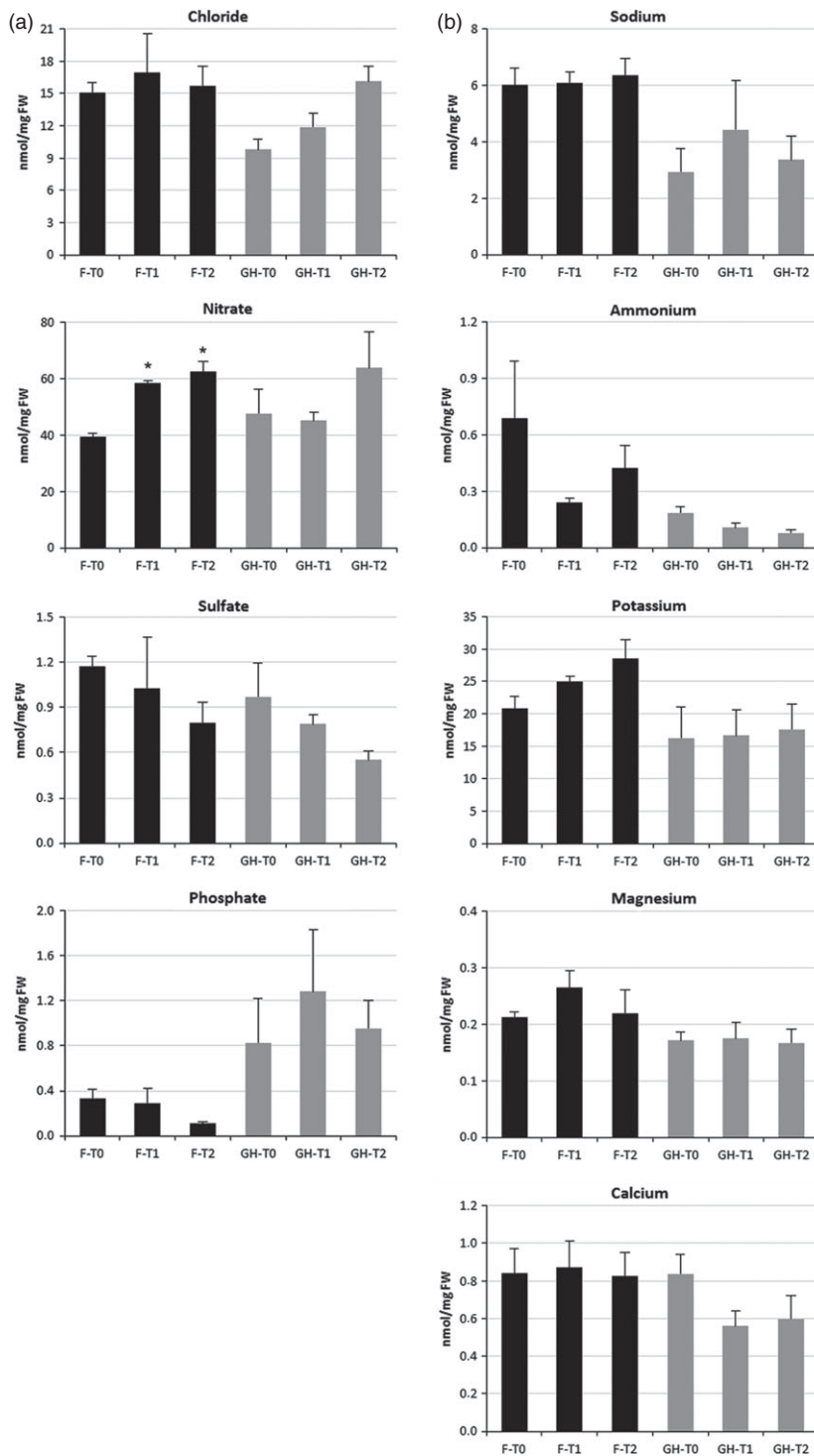
Figure 8 Ion chromatography analysis. (a) anion and (b) cation analysis. Ionic nutrients in nanomole per milligram fresh weight. F, field; GH, glasshouse. T0, T1 and T2 correspond to the three leaf samplings. The asterisk indicates significant differences (*P*-value <0.05).

expression levels during senescence in sunflower (Table S3). These TFs have not yet been directly associated with senescence process; however, this family is part of a regulatory network involved in hormonal regulation by sugars and redox signalling in response to several abiotic stresses (Dietz *et al.*, 2010).

HAHB4 (AF339748) is a sunflower HD-Zip TF family that has been extensively studied in last years in response to biotic and abiotic stress (Dezar *et al.*, 2005a,b; Gago *et al.*, 2002; Manavella *et al.*, 2006, 2008a,b). HAHB4 displayed high levels during leaf

senescence in sunflower (Table S3). This TF is positively regulated by ethylene and, once induced, it itself negatively regulates ethylene biosynthesis- and signalling-related genes (Manavella *et al.*, 2006). HAHB4 is also involved in response to wounding and abiotic stress, positively regulating ethylene and jasmonic acid production and negatively controlling ethylene sensitivity and abscisic acid accumulation (Manavella *et al.*, 2008b).

High levels of correlation between these TFs families were detected (Figure 6), especially among members of the NAC family in agreement with previous results described for *A. thaliana*
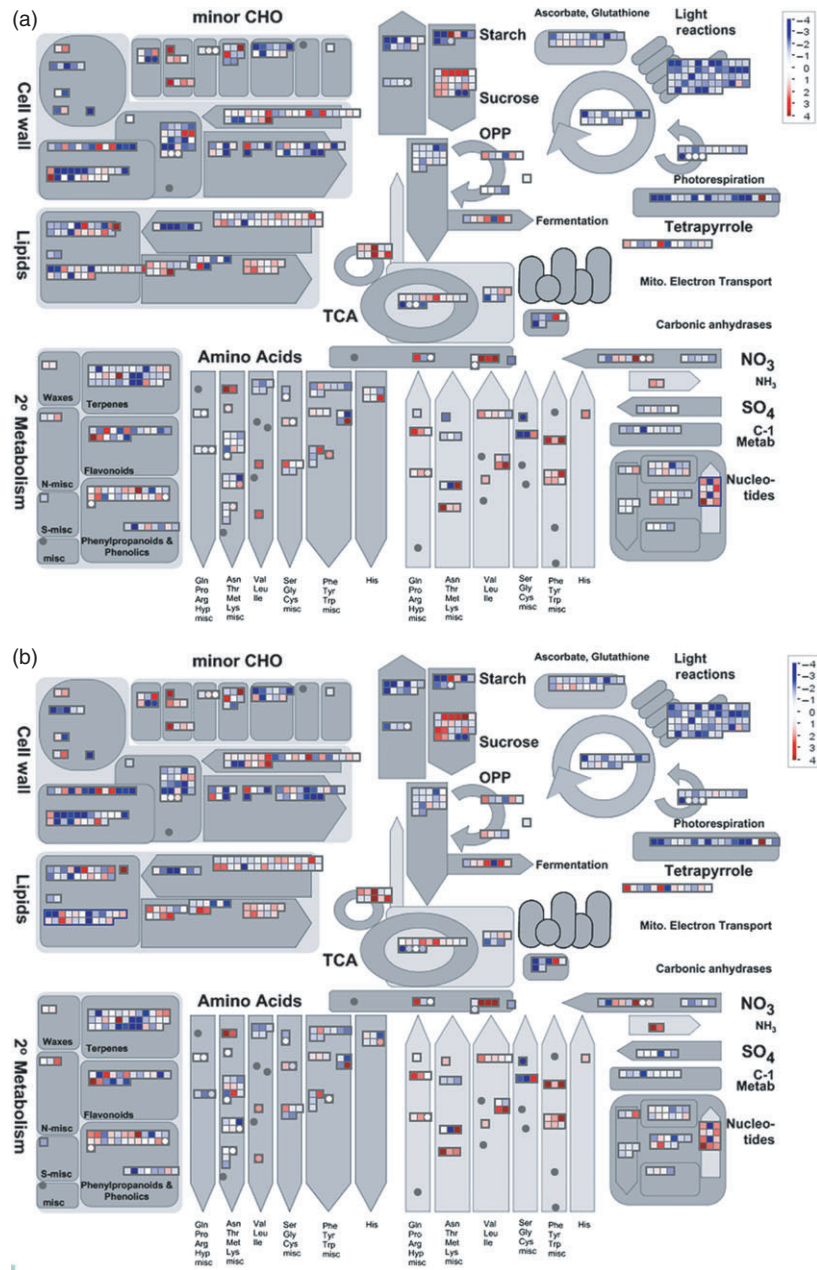
Figure 9 Metabolism overview in the field experiment. (a) Pre-anthesis time T1 vs. T0 and (b) postanthesis time T2 vs. T0. Genes and metabolites are represented by squares and circles, respectively. Colour intensity corresponds to the expression ratio at logarithmic scale (red: up-regulated, blue: down-regulated).

(Balazadeh *et al.*, 2008). In this analysis, we also found a transcript with high sequence identity to *ANAC02* or *ATAF1*. This transcript plays an important role in the adaptation processes to different environmental stresses and in plant development regulating ABA synthesis (Jensen *et al.*, 2013). It also responds to different pathogens (Wang *et al.*, 2009) and is involved in ethylene signalling pathways (Shan *et al.*, 2012). Furthermore, ATAF1 might be associated with an upstream regulation of the signalling pathway *ORE1* and *EIN2* (Balazadeh *et al.*, 2013), thus activating their expression and inhibiting the expression of *Golden2-like (GLKs)* genes, which are important for chloroplast development and maintenance (Rauf *et al.*, 2013). ORE1 also acts as an antagonist of GLK protein and in this way adds more complexity to this regulation pathway (Rauf *et al.*, 2013). In *A. thaliana*, the ORE1 TF can induce leaf senescence (Balazadeh *et al.*, 2010). In addition, the micro-RNA *miR164* may suppress

ORE1 transcript levels; *miR164* and *ORE1* may be regulated in a loop that would also involve *EIN2*, where EIN2 would promote the expression of *ORE1* and would inhibit *miR164* (Kim *et al.*, 2009). In a previous work conducted in sunflower, the expression profiles of candidate genes *Ha-EIN2* and *Ha-NAC01* (with high sequence identity to *ORE1*) were evaluated together with *miR164* levels (Moschen *et al.*, 2014). In that study, the candidate genes and *miR164* displayed high expression levels prior to anthesis and ahead of the first senescence symptoms. This finding was in line with the increase in the nutrient remobilization rate. In addition, Kim *et al.* (2014) demonstrated that *ANAC019*, *ANAC047* and *ANAC055* together with *ORE1* are preferentially under the control of EIN2 during leaf ageing. These three NAC TFs are within the highly correlated group. Therefore, this TF family is a putative regulator of leaf senescence in sunflower.

Transcriptomic analysis plays an important role in the study of leaf senescence. These results suggest a complex regulatory network underlying the senescence process; thus, the identification of regulatory networks based on expression profiling is an important starting point for the detection of new key genes involved in the triggering of the senescence process.

Metabolic analysis of sunflower leaf senescence

Senescence development in plants is a physiologically coordinated process that is critical for nutrient recycling from older leaves to growing organs. Metabolite remobilization from mature and senescent leaves to the different sinks, particularly into seed development, affects their quality and quantity and is one of the most important aspects in crop improvement (Gregersen et al., 2013). In this study, we performed a metabolic profile analysis of leaf senescence in sunflower in the same leaves assessed through transcriptomic profile.

Carbon metabolism

The role of sugars in senescence has been widely discussed in recent years. Sugars are central elements of the source–sink relationships (Balibrea Lara et al., 2004; Roitsch and González, 2004) and have been reported as growth (Smeekens et al., 2010) and photosynthetic rate regulators (Wingler et al., 1998). However, the effect of sugars on senescence is controversial and differs between different species (Nooden, 1988; Wingler et al., 2009; Yoshida, 2003). In A. thaliana, for example, sugar accumulation occurs during leaf development, whereas exogenous application of sugars induces an early senescence (Diaz et al., 2005; van Doorn, 2008; Masclaux et al., 2000; Masclaux-Daubresse et al., 2005; Quirino et al., 2001; Watanabe et al., 2013; Wingler et al., 2006, 2012). In sunflower, on the other hand, sugar content decreases during leaf development in field and glasshouse experimental conditions (Figure 7). This finding is in line with previous studies, in which the photosynthetic rate decreased together with sugar levels in a mature leaf (Quirino et al., 2000). Furthermore, sunflower is a plant with a strong demand for nutrient, especially sugars as substrate for oil synthesis, during the grain-filling phase. Likewise, low levels of sugars may increase production and/or ethylene sensitivity, which acts as senescence enhancer (Grbic and Bleecker, 1995; Hoeberichts et al., 2007). Several studies have described the role of sugars in the senescence programme. However, the results are variable and depend on the species, treatments, leaf and leaf section assessed; therefore, general conclusions concerning their effect are ambiguous (van Doorn, 2008).

Amino acid metabolism

Aromatic amino acids, which are derivatives of shikimate pathway, such as tryptophan, tyrosine and phenylalanine showed high levels throughout senescence (Figure 7). These amino acids have an important role as precursors of the secondary metabolite synthesis such as flavonoids. Specifically, flavonoids act as cellular protectants during senescence (Pichersky et al., 2006; Radwanski and Last, 1995; Watanabe et al., 2013). Particularly, tyrosine is a precursor of vitamin E or α-tocopherol biosynthesis, which acts as a powerful antioxidant in tissues (Almeida et al., 2011; Collakova and DellaPenna, 2003; Falk and Munné-Bosch, 2010; Quadrana et al., 2013). Indeed, vitamin E accumulation occurs in senescent leaf tissue (Abbasi et al., 2009; Molina-Torres and Martinez, 1991; Rise et al., 1989).

Furthermore, branched amino acids such as isoleucine and valine also displayed high levels throughout leaf development. These amino acids have a role in respiration as alternative substrates under stress and increase their level under dark-induced senescence (Araújo et al., 2010, 2011). Glutamine and asparagine levels also increased through leaf senescence. These amino acids are involved in nitrogen and carbon transport between the different organs and are the most abundant amino acids in the xylem and phloem, which indicates that they are actively involved in nutrient remobilization (Lea and Miflin, 1980; Urquhart and Joy, 1981).

GABA highly increased during senescence under glasshouse conditions (Figure 7). GABA is a product of glutamic acid decarboxylation, and its accumulation is associated with several stresses (Kinnersley and Turano, 2000; Obata and Fernie, 2012; Roberts, 2007) and also to senescence leaves of legumes and tobacco (Lahdesmaki, 1968; Masclaux et al., 2000).

Different roles have been proposed for GABA accumulation. For instance, it could be an amplifier of stress signals through the increase of ethylene synthesis; GABA could also be involved in temporary storage of nitrogen and could be an anaplerotic compound by providing TCA cycle intermediates during stress response (Kinnersley and Turano, 2000).

Ionic nutrients

Among the different ionic nutrients detected in this analysis, nitrate represents one of the most relevant groups during senescence. The concentration of this element increased during senescence in both evaluated conditions (Figure 8). This fining differs from a study in A. thaliana. In A. thaliana, the nitrate content decreased during senescence in the whole plant with an accumulation gradient from the tip to the base of the leaves (Watanabe et al., 2013). However, an increase in nitrate content has been detected in older leaves of tobacco plants, whereas the opposite effect was observed in young leaves (Masclaux et al., 2000). This increase could be related to the role of nitrates as osmoprotectants in senescent vacuoles (McIntyre, 1997), which would explain the elevated cell turgor observed in senescent cells (Brugière et al., 2000).

The sulphate content decreased progressively during leaf development in sunflower and this decrease negatively correlates with the increase in nitrate content (Blom-Zandstra and Lampe, 1983; Diaz et al., 2005). However, the mechanisms by which sulphates are remobilized and exported during leaf senescence have not yet been clearly defined. Oilseed species such as canola (Brassica napus L.) are particularly sulphur demanding and highly sensitive to the lack of this compound with considerable consequences for seed yield and quality (Dubousset et al., 2009; Janzen and Bettany, 1983; Scherer, 2001).

Altogether, these results suggest high nutrient mobility throughout the leaf senescence process in sunflower plants. Effective nutrient remobilization from leaf to seed during senescence is very important for weight increase and seed quality, especially in agronomically important crops such as sunflower. The transition between sink and source that takes place in leaves is important and critical for a proper development of the plant (Watanabe et al., 2013).

Integrative analysis of sunflower leaf senescence

Throughout the study of complex cellular processes such as leaf senescence, the experimental systems biology approach attempts to assess the dynamics and integration of the

different molecular components associated with physiological responses.

In annual crops, senescence is typically induced in the whole plant after flowering. Foliar nutrients are remobilized and translocated into the fruit, and this leads ultimately to cell death. Therefore, this is a process tightly controlled by the development of reproductive structures (Davies and Gan, 2012; Gregersen et al., 2013). This highlights the strong pressure of the source–sink relationships that control the onset of senescence in monocarpic plant species.

In the present study, we focused leaf sampling at early stages of plant development in both field and glasshouse experiments, to find candidate genes involved in triggering of the senescence process. In natural field growth conditions, chlorophyll and nitrogen content sharply decreased after flowering, whereas in glasshouse condition, chlorophyll content remains relative stable throughout the sampling period likely due to radiation limitation (Figure 1). In rice, it was reported that low irradiance strongly retarded the decline in chlorophyll content which remained almost constant until late senescence when an abrupt decreased took place (Hidema et al., 1991). The relationship between productivity and senescence is complex. Productivity would benefit from a delay in senescence, and this delay has to be coordinated with the early stages of development, particularly at the beginning of the reproductive phase growth (Gregersen et al., 2013).

Studies at transcriptional and metabolic levels in sunflower leaves allowed us to detect significant changes in the metabolism at an early stage of development (T1 vs. T0), prior to anthesis and before the onset of the first senescence symptoms assessed by physiological measurements especially in the field experiment (Figure 9a and Figure S3a). In the postanthesis period (T2 vs. T0), the detected changes were similar to those observed at an early stage. However, at postanthesis, we detected the highest values of gene expression and metabolite levels associated with different cell metabolism (Figure 9 and Figure S3a).

The genes and metabolites related to the photosynthetic processes were down-regulated during leaf development. A decrease in photosynthetic activity directly impacts sugar metabolism. In this study, we detected a decrease in sugar levels during senescence, in contrast to what happens in A. thaliana, where sugar accumulation occurs (Watanabe et al., 2013). The genes related to sucrose degradation, such as different cell wall, vacuolar and neutral invertases and fructokinases, displayed high levels of expression.

Lipid metabolism-associated genes were highly expressed, especially those related to the beta-oxidation process. The beta-oxidation process culminates in acetyl-CoA production, which is substrate of both the TCA cycle and the enzyme malate synthase. Acetyl-CoA also displayed high expression levels. Glyoxylate cycle enzymes such as isocitrate lyase and malate synthase increased their activity at early stages of senescence in barley (Gut and Matile, 1988) and cucumber (Graham et al., 1992). These findings indicate a role of carbon recycling during senescence. However, the glyoxylate cycle in A. thaliana does not play a major role in nutrient recycling, and the malate synthase expression is specifically restricted to young seedlings and root tips of older plants; in senescent leaves, both malate synthase and isocitrate lyase were undetectable (Charlton et al., 2005).

A critical component associated with the senescence process is the protein degradation (Roberts et al., 2012). Genes associated with protein processing, ubiquitination and degradation such as

kinases, phosphatases, cysteine protease (SAG12), F-box protein, and heat-shock proteins (Hsp70 and Hsp90) also displayed high expression levels during sunflower senescence. Proteases also show high expression levels in senescent leaves in different species (Desclos et al., 2009; Hollmann et al., 2014; Schiltz et al., 2004) and many of them are reported as SAGs. Throughout protein renovation and degradation, ammonium release occurs. Indeed, ammonium release is accelerated during senescence (Gregersen, 2011). This ammonium group could be channelled back into amino acid structures by the action of glutamine synthetase, which plays a central role in nitrogen remobilization (Bernard and Habash, 2009; Buchanan-Wollaston et al., 2003; Martin et al., 2006; Tabuchi et al., 2007). In support of this theory, enzymes involved in recycling, such as asparagine synthetase and glutamine synthase transcripts, as well as the associated metabolites, asparagine and glutamine, showed high levels during sunflower leaf development. This finding indicates a strong recycling activity at early stages of leaf development, prior to the appearance of the first senescence symptoms.

In summary, in sunflower many biological processes were up- and down-regulated at an early stage of plant development, prior to anthesis and before the onset of the first visual leaf senescence symptoms. We identified candidate genes associated with the senescence process, especially NAC TFs that could act as triggers for senescence. Furthermore, we demonstrated the importance of conducting experiments under field conditions that reproduce their natural growing environments. Even though the use of larger pots in glasshouse help to simulate field conditions, the radiation limitations are difficult to overcome. This limitation leads to a marked delay in leaf senescence and, thus, to a temporal shift of the gene expression pattern and metabolomic profiles in glasshouse compared to field experiments. Therefore, a better characterization of the molecular processes that underlie leaf senescence in sunflower was assessed in field experiments.

This is the first study that uses an integrated approach related to the senescence process in sunflower. Thus this study provides an important starting point for future analysis and opens new insights to explore alternative strategies and possibilities.

Understanding the onset of the process will in turn impact on the development of different senescence management strategies and could help controlling the grain-filling process. This could eventually improve crop yields, which represent an important challenge for the future of agriculture attending to the increase in both, world population and climate risks that affect productivity.

Experimental procedures

Plant material and experimental conditions

The sunflower leaf senescence assays were conducted under field and glasshouse conditions. The field experiment was carried out at the INTA Balcarce Experimental Station (37°45′ S, 58°18′ W) as previously described (Moschen et al., 2014). The glasshouse experiment was performed at the Biotechnology Institute, INTA Castelar (34°36′ S, 58°40′ W). Both experiments were sowed simultaneously during the 2010/11 growing season to maintain similar photoperiod. The sunflower hybrid VDH 487 (Advanta Seeds, Balcarce, Argentina) was sown at a 7.2 plants/m² with three and five biological replicates (plots) in the field and glasshouse experiments, respectively; each biological replicate consisted of three randomly selected plants from each plot in the field experiment. In the glasshouse experiment, one plant per plot was evaluated.

Under glasshouse conditions, 20 kg pots containing a mixture of soil, sand and peat (5 : 3 : 1) as substrate were used (one plant per pot). In both experiments, temperature, diseases, weeds and insects were adequately controlled. Soil fertility assured maximum yields under nonlimiting water conditions.

Transcriptomic and metabolic profiles were performed using the leaf 10 (numbered from the bottom to the top of the plant) at three different development stages in both experiments, labelled as T-0 (young leaf), T-1 (pre-anthesis leaf) and T-2 (postanthesis leaf, with senescence symptoms) (Table 1). Time was expressed on a thermal time basis by daily integration of air temperature with a threshold temperature of 6 °C and considering plant emergence as thermal time origin (°CDAE: °C days after emergence) (Kiniry et al., 1992).

Physiological measurements

The physiological measurements were assessed as previously described (Moschen et al., 2014) including chlorophyll, TSC and nitrogen determinations.

Transcriptomic analysis

RNA isolation, quantification and quality controls

The sampled leaves were immediately frozen in liquid nitrogen upon collection and saved at −80 °C until processing. High-quality total RNA was isolated from 100 mg of frozen tissue using TriPure, according to the manufacturer's instructions (Roche, Buenos Aires, Argentina). The genomic DNA was eliminated after treatment with DNase I for 20 min at room temperature using DNase I (Invitrogen, Buenos Aires, Argentina). The RNA concentration was measured using a Nanodrop ND-1000 spectropho-tometer (NanoDrop Technologies, Wilmington, DE). The purity and integrity of total RNA was determined by 260/280 nm ratio and by NanoBioanalyzer RNA-6000 analysis (Agilent Technologies, Palo Alto, CA).

Microarray analysis

The transcriptomic profile was performed using a previously developed custom sunflower microarray, Agilent 4x44K (Fernandez et al., 2012b). Data preprocessing was performed using the *Limma* library (Smyth, 2005) from the R-package (R Core Team, 2012). The background correction was performed using the *rma* algorithm from the *backgroundCorrect* function. Between-array normalization was performed using the *quantile* method from the *normalizeBetweenArray* function. Finally, fold changes were transformed to log2 scale and the information from technical replicates was incorporated by calculating the median parameter.

Differential gene expression analysis

Statistical analysis was performed using in-house routines *to fit*, *gene by gene*, *a linear mixed-effects model*. The Sunflower Custom Oligo Microarray includes four arrays per chip; therefore, the chip effect (incomplete block) was included as a random effect. The set of routines mentioned above were based on the lme function of the nlme library of R (Pinheiro et al., 2012) implemented in InfoStat statistical software (Di Rienzo et al., 2013). The differential gene expression analysis was carried out using the limma package. Gene set analysis was conducted according to the gene ontology terms (Ashburner et al., 2000) using FatiScan (Al-Shahrour et al., 2007) integrated in the Babelomics suite (Al-Shahrour et al., 2005). The correction of *P*-values in a multiple test context was performed using the

Benjamini–Hochberg method, and the respective adjusted *P*-values (Benjamini and Hochberg, 1995) were obtained.

Microarray validation: qPCR analysis

The expression of 10 genes was evaluated in relation to elongation factor-1α (EF-1α), which has been previously selected as a reference gene for expression senescence experiments (Fernandez et al., 2011) (Figure S1). Specific primer pairs were designed using Primer3 software (Rozen and Skaletsky, 2000) with default parameters (Table S1).

For each sample, 500 ng DNase-treated RNA was reverse-transcribed using Superscript III first strand synthesis system (Invitrogen) and random hexamer primers according to the manufacturer's instructions (Moschen et al., 2014).

Metabolic analysis

GC-TOF-MS analysis

The metabolite extraction was performed promoting the extraction of lipophilic and polar compounds according to recently published protocols (Roessner-Tunali et al., 2003) and further adaptations to sunflower tissue samples (Peluffo et al., 2010). The samples were derivatized and injected (1 μL) into the GC-TOF-MS system (LECO Corporation, St. Joseph, MI). Chromatography was performed on a 30 m SPB-50 column with 0.25 mm inner diameter and 0.25 lm film thickness (Supelco, Belfonte, CA). The injection temperature was 230 °C, the interface was set to 250 °C, and the ion source was adjusted to 200 °C. The carrier gas was He at a constant flow ratio of 1 mL/m. The chromatograms and spectra were evaluated using the ChromaTOF (LECO Corporation) and TagFinder (Luedemann et al., 2008). The ion spectra were compared to the Golm metabolome database (http://gmd.mpimp-golm.mpg.de/). Metabolite levels were normalized to fresh weight and the internal control ribitol. Changes in metabolite levels along leaf development were calculated as the fold change relative to the first sampled time (T-0) as control.

Ion chromatography

Ionic nutrients were assessed by ion chromatography. A total of 500 μL of 4 °C ULC water (*ultra liquid chromatography*) was added into each metabolite sample extraction. The samples were shaken in vortex 10 s followed by centrifugation 30 min at 16000 × g at 4 °C. A total of 480 μL of supernatant was transferred into glass vials. Serial dilutions of $MgCl_2$, KNO_3, KH_2PO_4, $MgSO_4$, NH_4SO_4 and $CaCl_2$: 100, 50, 25, 12.5, 6.25 and 3.125 μM were used as standard solutions. An ionic nutrient analysis was carried out using Ion Chromatograph Dionex IC3000 (Thermo Scientific, Fitchburg, WI).

Transcriptomic and metabolic integration

MapMan analysis

MapMan software (Thimm et al., 2004) was used to integrate transcriptomic and metabolic profiles. For mapping generation, fasta sequences of *Helianthus annuus* were downloaded from Sunflower Unigene Repository (SUR v1.0) (Fernandez et al., 2012b). Annotation was processed through Mercator annotation pipeline (Lohse et al., 2014).

The log2 ratio of fold change (T1/T0 and T2/T0) was calculated for each experimental condition. The expression cut-off was log2 fold change higher or lower than 2 and with a *P*-value lower than 0.05. The resulting data table was used for MapMan analysis.

Acknowledgements

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Microarray validation.

Figure S2 Significant GO distribution for molecular function at pre-anthesis time (T1 vs. T0).

Figure S3 Metabolism overview under glasshouse conditions.

Table S1 Primer sequences for qPCR validation analysis.

Table S2 Up- and down-regulated SAGs with a log2 fold change higher or lower than 2 under field conditions.

Table S3 Putative transcription factors up- and down-regulated with a log2 fold change higher or lower than 2 under field conditions.