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Chapter 6

FUNCTIONAL GENOMICS AND TRANSGENESIS APPLIED TO SUNFLOWER BREEDING

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ABSTRACT

The advances in genomics and post genomics in the last decade allowed the discovery and functional characterization of many genes simultaneously on a genome-wide scale. However, the sunflower genome was not systematically sequenced until the recent advent of next-generation sequencing technologies and is still in progress. In parallel, comprehensive EST datasets were developed and used to design oligo-nucleotide-based microarrays focusing both in genotyping and expression analysis purposes, which in turn, help to study the transcriptomics response to different growing conditions as water deficit, senescence or response to pathogens. In addition, first metabolomics analyses of tolerance to diseases started few years ago. This chapter reviews the functional genomics analysis of several important sunflower characters including the development and application of bioinformatic approaches to explore

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massive data derived from high throughput sequencing technologies to elucidate complex traits and identify candidate genes playing a key role in metabolic pathways with special emphasis in the generation of new breeding tools. In addition, functional strategies for candidate gene validation either by TILLING approach, and/or by overexpression in model and sunflower plants are also reviewed and discussed.

In vitro tissue culture techniques, like immature embryo rescue, were incorporated in sunflower breeding before the existence of the functional genomics disciplines allowing the development of four inbreeding generations per year and helping breeders to sort sterility or incompatibility barriers in wide interspecies crossings. Even so, sunflower was considered a recalcitrant species for genetic transformation due to difficulties in plant regeneration procedure. Sunflower transformation protocols have improved in the last years, but they are still time consuming and produce low numbers of transgenic regenerants per assay. For some applications, like complementation studies, these difficulties were solved by transient transformation and the stable transformation of lettuce, as model system. Still, the introduction of agronomical useful genes successfully delivered transgenic lines with herbicide tolerance, insect resistance, disease resistance and improved oil composition. They were developed by seed companies and nearly reached commercial stage. However, since pollen from cultivated sunflower can spread to adjacent wild populations, any intended release of transgenic sunflowers requires a previous analysis of the population biology of wild relatives to assess the potential added fitness or detrimental effects that agronomic traits might have on the ecosystem. This has impeded the intended release of commercial transgenic sunflowers until now. However, as a consequence of the appearance of the non-transgenic imidazolinone resistant sunflower and its global marketing, many of the concerns about the release of transgenic sunflowers are rapidly decreasing.

Keywords: Sunflower, transcriptomics, metabolomics, biotic and abiotic stresses, transgenesis, transient expression, *in vitro* culture

INTRODUCTION

During the last decade, sunflower crop production has been gradually driven to marginal areas due to the rapid change of agricultural practices in crops such as soybean and maize, which have greatly increased their cultivated areas as a consequence of favourable commodity prices and because farmers found more profitable to sow transgenic crops with resistance to herbicides and insects.

Difficulties in obtaining transgenic events (Davey and Jan, 2010) as well as the concerns raised about the environmental risk after the release of these events (Cantamutto and Poverene, 2007) prevented the appearance of genetically modified sunflowers on the global marketplace. This situation, together with global climate change and the increase in the frequency and severity of abiotic stresses that take place in the actual sunflower cultivated areas, has led researchers to redirect the objectives and widen the tools currently used in sunflower breeding (Sala et al., 2012). This adverse situation has been partially overcome in sunflower by contributing to yield maintenance through breeding for herbicide resistance and tolerance to biotic and abiotic stresses (Paniego et al., 2007, Sala et al., 2012). Different abiotic stresses such as nitrogen starvation, drought, cold, heat, salinity and high temperature can reduce crop yields and the genetic potential to increase the production is not reached,

indicating that the development of cultivars with an increased adaptation to environmental changing conditions should be undertaken (Boyer, 1982).

Sunflower is described as normally susceptible to low temperatures and salinity (Kratsch and Wise, 2000, Huang et al., 2005, Maas and Hoffman, 1977), but with a relative tolerance to drought stress due to its highly explorative root system (Connor and Jones, 1985, Sadras et al., 1991, Connor et al., 1985). However, available information on gene expression in response to abiotic stresses in sunflower is still limited to few works.

Biotic stress is considered one of the most important limiting factors affecting sunflower yield production worldwide. In the last ten years, several attempts have been made through conventional breeding and molecular biology studies to dissect the bases for fungal resistance to allow biotechnology assisted selection. Considering that sunflower is cultivated in vast marginal areas and therefore, the application of fungicides is not always feasible and unfriendly to the environment, breeding for generation of inbred lines with genetic tolerance to most common pathogens represents a main goal. Downy mildew (*Plasmopara halstedii*), *Sclerotinia* stalk and head rot (*Sclerotinia sclerotiorum*), black rust (*Puccinia helianthi*), *Verticillium* wilt (*Verticillium dahliae*), *Alternaria* leaf blight (*Alternaria helianthi*) among others, appear as the highest impact diseases, since they seriously reduce yield in many sunflower crop regions (Viranyi, 2008). Therefore, breeding for disease resistance has been, and will continue to be, essential to strengthen this crop. There exist several sources of resistance genes in sunflower germplasm, mainly present in the numerous wild sunflower species, landraces and old open pollinated varieties, some of which have been introgressed into cultivated elite inbred lines (Seiler, 2010, Gulya et al., 1999, Moreno, 2013) but many others certainly remain to be discovered, genetically characterized and incorporated in the high yielding cultivated genetic pool.

The advances in genomics in the last decade allowed the discovery and functional characterization of many genes in a simultaneous way by use of a genome-wide scale approach. Genomics tools can improve the rate of genetic gain in breeding programs by either extending the amount or nature of variation available for selection, or by accelerating the selection process to produce varieties more rapidly (Langridge and Fleury, 2011). In the case of sunflower, genomics approaches have already started both in the public and private sectors, which resulted in the generation of several tools that are likely to change some paradigms of sunflower breeding (Seiler and Jan, 2010, Kane et al., 2011, Fernandez et al., 2012a, Paniego et al., 2012). In this chapter we review and discuss different strategies that have been developed in the last decade from functional genomics and post genomics disciplines to contribute to the elucidation of gene regulation and identification of key metabolic pathways involved in the response to biotic and abiotic stresses in sunflower. The state of the art of strategies for gene function, studies *in silico* and *in planta*, induction of genetic diversity and gene transfer are discussed within the frame of their application in sunflower breeding.

FUNCTIONAL GENOMICS FOR ABIOTIC AND BIOTIC STRESS RESPONSES IN SUNFLOWER

cDNA Libraries, Differential Display and Candidate Gene Approaches

The first functional genomics studies applied to abiotic stress in sunflower came from cDNA libraries and differential display analysis.

Ouvrard et al. in 1996 assessed two drought contrasting sunflower lines and identified six sunflower drought induced genes (*sdi*) through subtractive hybridization. These genes were up-regulated by drought in the tolerant as well as in the sensitive line and included non-specific lipid transfer proteins (nsLTP), early light-induced proteins (ELIP), RAB proteins and ACC oxidases or dehydrins.

For three of these genes, putative ELIP, RAB -16B and the ACC oxidase protein, the level of transcripts accumulated in response to drought was higher in the tolerant genotype, indicating that these proteins might represent potential candidates for being involved in drought tolerance (Ouvrard et al., 1996).

Dehydrin proteins are usually expressed following any environmental stimulus involving dehydration, such as drought or cold stress and salinity (Close et al., 1993, Godoy et al., 1990, Neven et al., 1993). In sunflower, the increase of two dehydrins, *HaDhn1* and *HaDhn2* was correlated with the decrease of midday leaf water potential during progressive stress and mainly up-regulated in a drought tolerant line (Cellier et al., 1998). The influence of day/night cycle on the expression profile of these two dehydrin genes, showed an oscillation of *HaDhn1* in a diurnal way with a peak of messenger RNA (mRNA) at midday. In contrast the increase of *HaDhn2* transcript was independent of day/night periods (Cellier et al., 2000).

Changes in the dehydrin transcript levels were also studied in two sunflower mutants for ABA synthesis and accumulation during embryo and plantlet development and drought stress: *nd-1* (an albino, non-dormant and lethal mutant with a very low ABA content and no ABA accumulation in response to stress) and *w-1* (a wilty mutant, with reduced ABA accumulation). These results showed the existence of two regulation pathways of dehydrin transcript accumulation, an ABA-dependent manner and an ABA-independent one, which may have cumulative effects (Giordani et al., 1999).

Expression profiles of four water-stress associated genes, aquaporin, dehydrin, leafy cotyledon1-like protein and fructose-1,6 bisphosphatase were assessed in four RILs and parental lines showing contrasting responses to dehydration and rehydration (Kiani et al., 2007). The expression level of aquaporin and fructose-1,6 bisphosphatase genes in leaves was down regulated by water stress and was associated with relative water content. The level of dehydrin transcripts increased in leaves of all studied RILs in response to water stress. Transcript accumulations of dehydrin and leafy cotyledon1-like genes, likely involved in protective tolerance processes, were not directly correlated with plant water status. Down-regulation of fructose-1,6 bisphosphatase was observed under water stress. Net photosynthesis rate and the fructose-1,6 bisphosphatase gene expression levels were associated mainly after rehydration. This phenomenon indicates an association between physiological response to water stress and differential expression of water-stress related genes (Kiani et al., 2007).

Gene expression profiles between drought and salinity stressed plants in sunflower, two of the most important environmental stresses that alter plant water status and severely limit plant growth, development and crop productivity, were assessed through differential display polymerase chain reaction, (Liu and Baird, 2003). Five drought regulated cDNAs and 12 salinity regulated cDNAs were cloned and sequenced. Out of them, 13 genes were analysed in leaves of drought stressed plants, and in roots and shoots of drought and salinity stressed seed lines. These results showed that certain genes respond to both stresses, evidencing a cross-talk and complex regulation, while others are uniquely regulated either in terms of the stress stimulus or the plant tissue.

Sequence analysis of these cloned genes identified five with homology to known genes, guanylate kinase (signal transduction), lytB (antibiotic/drug resistance), selenium binding protein (heavy metal stress), polyprotein (reverse transcriptase), and AC-like transposable element (Liu and Baird, 2003). In addition, SOS2 and PMP3 genes, deeply involved in Na^+ efflux mechanisms in glycophytes, were reported in sunflower as expressed in root-cap as a way to exploit plant adaptation to saline environments. PMP3, a small hydrophobic peptide first described in yeast and involved in preventing Na^+ entry, was found in the root cap of a monocotyledonous halophyte but not identified in dicots (Inada et al., 2005), whereas SOS2 was found to be expressed gradually in seedlings of tolerant sunflower lines (Saadia et al., 2013). Recently Jabeen and Ahmad (2013) reported chitosan as an effective biostimulator to enhance safflower and sunflower seedlings growth and plant tolerance under salinity conditions. Chitosan is a natural biopolymer formed by alkaline deacetylation of chitin which has recently attracted additional attention because of its antioxidant activities, being previously reported in rice and other crops (Chibu and Shibayama, 2001, Ruan and Xue, 2002).

During 2003 it was reported for the first time in sunflower, the isolation and characterization of expressed sequence tags (ESTs) from organ-specific cDNA libraries constructed by suppressed subtractive hybridization (Diatchenko, 1996) as an alternative to identify differentially expressed sunflower transcripts. They presented the differential level of representation for functional EST groups based on Gene Ontology annotation (Ashburner et al., 2000), as well as a comprehensive description of the individual non-redundant sequences generated (Fernandez et al., 2003).

Within a large number of genes that are activated by stress conditions, the study of transcription factor genes (TFs) is essential to understand different stress mechanisms. Stress responses are regulated by multiple signalling pathways (Knight and Knight, 2001, Singh et al., 2002, Chen and Zhu, 2004); several abiotic stress pathways share common elements that are potential 'nodes' for cross-talk (Knight and Knight, 2001) and TFs can regulate various stress inducible genes controlling these gene networks.

HAHB4 transcription factor belongs to the sunflower subfamily I of HD-Zip proteins and is transcriptionally regulated by water availability, abscisic acid and ethylene signalling. The overexpression of HAHB4 cDNA in sunflower, together with knockdown by iRNA, showed that this TF helps to prevent the accumulation of gene transcripts related to photosynthesis (Manavella et al., 2006, Manavella et al., 2008a).

Moreover, *Arabidopsis* transgenic plants expressing this gene showed an up-regulation of *HAHB4* induced by different external factors such as drought, extreme temperatures, osmotic stress, and light conditions, evidencing a complex cross-talk network (Manavella et al., 2008b).

NAC transcription factors (TFs) represent a large family of the senescence-regulated genes in many plant species (Guo et al., 2004, Buchanan-Wollaston et al., 2005, Lin and Wu, 2004, Balazadeh et al., 2008, Gregersen and Holm, 2007). *ORE1*, a NAC-TF, was first reported to act downstream of ethylene and auxin signalling pathways. It is involved in salt stress response and lateral root development (He et al., 2005), and several of its downstream genes are also affected by salt stress leading to leaf senescence (Balazadeh et al., 2010). Leaf senescence is a complex and highly coordinated process, controlled by multiple variables, either from genetic and environmental origin that has a strong impact on crop yield (Noodén et al., 1997).

In sunflower, the senescence process takes place abruptly, coinciding with adverse environmental conditions and the incidence of foliar diseases (Dosio and Quiroz, 2010). Ha*ORE1* transcription factor, appears as a potential molecular functional marker, showing an early activation in relation to physiological and biochemical events, with an early expression pattern, followed by an increase of expression prior to anthesis and the first symptoms of senescence, when the critical period of grain filling has already begun, which makes this gene a potential indicator of the triggering of the process, (Fernandez et al., 2012b, Moschen et al., 2012). Senescence is tightly linked to nutritional stress and N starvation (Nasser, 2002, Cabello et al., 2006, Agüera et al., 2010) and CO_2 environmental level (Larios et al., 2004, de la Mata et al., 2012). Recently, different light intensity studies were performed in *H. annuus L.* where these results showed that high photon flux densities caused early senescence in primary leaves by altering not only the CO_2 fixation rate and sugar levels but also the activity of key enzymes of nitrogen metabolism (de la Mata et al., 2013).

Plants growing in adverse environmental conditions change their protein concentrations, by both transcriptional and/or post-transcriptional regulatory mechanisms. At a post-transcriptional level, an important aspect to understand plant signalling pathways related to different stresses is the study of microRNAs (miRNAs). MicroRNAs are noncoding, endogenous, small RNAs about 20–24 nucleotides long, conserved in plants and animals (Bonnet et al., 2004, Huang et al., 2010). They have an important role in post transcriptional gene regulation (Bartel, 2004, Carrington and Ambros, 2003). miRNAs negatively regulate gene expression either by inhibiting translation elongation or by triggering mRNA destruction (Aukerman and Sakai, 2003, Tang et al., 2003). In sunflower, precursors (pre-miRNAs), from known *Helianthus* expressed sequence tags (ESTs) were found by a systematic search approach (Barozai et al., 2012). The study resulted in 61 novel miRNAs belonging to 34 families from *Helianthus* ESTs database. Out of these 61 new miRNAs, 20 are from *H. tuberosus*, 17 miRNAs belong to *H. annuus*, 8 are from *H. ciliaris*, 5 from *H. exilis*, 4 from *H. argophyllus*, *H. petiolaris* each and 3 are from *H. paradoxus*. Targets of these miRNAs consist of growth and development related genes, transcription factors, signalling pathway kinases, stress resistant proteins and transport related proteins (Barozai et al., 2012).

The sunflower HaWRKY6 is a particularly divergent WRKY transcription factor gene, unique to plants and identified as mediating varied stress responses (Ulker and Somssich, 2004, Eulgem and Somssich, 2007, Rushton et al., 2010). *HaWRKY6* exhibit a putative target site for the miR396. *Arabidopsis* plants expressing a miRNA396-resistant version of HaWRKY6, confirmed the dependency of HaWRKY6 silencing by miRNA (Giacomelli et al., 2012). Sunflower plants exposed to high temperatures or salicylic acid presented opposite expression of HaWRKY6 and miR396. Experiments using the wild type and miRNA-resistant versions of HaWRKY6 showed altered stress responses. These results showed a role

of the recently evolved miR396 in the regulation of HaWRKY6 during early responses to high temperature (Giacomelli et al., 2012). Candidate gene approaches based on sequence similarity to previously characterized genes has greatly contributed to identifying and characterizing orthologous genes in sunflower both for biotic and abiotic stress response. The isolation and characterization of transcription factors mentioned above is an example of the successful application of this strategy. Another example is the identification and functional characterization of members of the Germin-like Protein (GLP) genes in sunflower (Ehrenbolger, 2012). Over the last years, evidence has accumulated regarding the involvement of GLPs in defence against pathogens (Davidson, 2009).

In a previous work overexpression of the wheat oxalate oxidase (*gf2.8*), a member of the Germin family, showed enhanced tolerance to the oxalic acid producing pathogen *Sclerotinia sclerotiorum* (Hu et al., 2003). Germins are included within the Cupin Superfamily, along with the more diverse Germin-like proteins (GLPs), which are present in a wide range of species and are characterized by containing two highly conserved motifs. Although the biochemical properties of many of the GLPs described to date are still unknown, superoxide dismutase activity has been reported in different species such as barley, grape, and tobacco (Carter and Thornburg, 2000, Zimmermann, 2006, Godfrey, 2007). Given that most GLPs exhibit glycosylation and cell wall localization signals, they have been related to cell wall strengthening and papillae formation (Wei, 1998). In addition, they have been proposed to play a role in reactive oxygen species detoxification and to function as signalling molecules inducing a range of defence responses in a direct or indirect manner (e.g., Lane 1994; Zhou et al. 1998). The expression of a GLP from *Beta vulgaris* (BvGLP-1) in *Arabidopsis* has proved to elevate H_2O_2 content and confer significant resistance to *Verticillium longisporum* and *Rhizoctonia solani* (Knecht, 2010). Recently, Ehrenbolger et al. (2012) described the evolution, diversification, and function of sunflower GLPs (HaGLPs) with the aim of identifying new candidate genes for crop improvement. They identified nine putative subfamilies within HaGLPs and found that these genes are divergent in terms of their primary sequence, the size of their encoded proteins and the length of the introns. Expression studies showed that most HaGLPs were transcribed in major plant organs, albeit to varying degrees in different sunflower tissues (root, leaf, stem, flower, receptacle, and seed). Transgenic Arabidopsis lines overexpressing HaGLP1 showed enhanced tolerance to *Rhizoctonia solani*.

LARGE SCALE TRANSCRIPTOMICS ANALYSIS

The first breakthrough technology for transcriptome analysis was the development of microarrays. Microarrays have proven to be a powerful tool for the discovery of many stressed-induced genes involved in stress response and tolerance. Macro and microarray studies of stress responses in *Arabidopsis* and *Oryza sativa* allowed the identification of genes involving both functional and regulatory proteins (Seki et al., 2001, Sahi et al., 2006, Kanesaki et al., 2002).

Although analysis of gene expression using the microarray approach has already provided significant insights into the signalling processes involved in defence responses in model plants (Dowd et al., 2004, Maleck et al., 2000, Schenk et al., 2000, Scheideler et al.,

2002), there are few reports describing the global changes in transcription activities in response to pathogen infection in sunflower.

Microarrays can be classified into two groups based on the nature of the DNA fixed to solid support; oligonucleotide microarrays and cDNA microarrays (Aharoni and Vorst, 2002). For oligonucleotide (shortened as "oligo") microarrays, also called 'gene chips', synthetic oligos are arrayed on a glass substrate through direct synthesis or spotting. Oligo arrays have the advantage of greater gene density and more uniform hybridization because of similar sequence lengths, and more specific hybridization, which enables differentiation between duplicated or differentially spliced genes. The oligo approach also obviates concerns about potential mismatches between cDNA clones and EST sequences (Knight, 2001).

However, synthetic oligo arrays are expensive to produce and less sensitive than cDNA arrays, particularly in hybridizations involving distantly related species (Lee and Roy, 2004).

The first transcriptional studies for cultivated sunflower, involved the development of cDNA macro and microarrays to evaluate sunflower responses to biotic (Alignan et al., 2006) and abiotic stresses (Hewezi et al., 2006a, Roche et al., 2007, Fernandez et al., 2008).

Alignan et al. (2006) developed and used a 1000-element cDNA nylon microarray (also known as low density array). They chose genes putatively involved in primary metabolic pathways, signal transduction and response to biotic stress, identified on the basis of their homology to *A. thaliana* genes. The low density array allowed investigating transcriptional changes that occur during the activation of multi-genic resistance in partially resistant and susceptible sunflower lines inoculated with *P. macdonaldii*. They proposed a model in which negative regulation of a dual-specificity MAPK phosphatase could be implicated in sunflower defence mechanisms against the pathogen. The resulting activation of the MAP kinase cascade could subsequently trigger defence responses under the control of transcription factors belonging to MYB and WRKY families. It was also proposed that the activation of protein phosphatase 2A (PP2A), which is implicated in cell death inhibition, could limit pathogen development (Alignan et al., 2006).

Nylon microarrays containing >8000 putative unigenes were developed to assess two contrasting sunflower flowering genotypes under low temperature treatment (Hewezi et al., 2006a). A total of 108 cDNA clones were differentially expressed between plants grown at low temperature (15°C and 7°C) and their corresponding controls across the two genotypes. Almost 90% of the genes whose products are involved in low temperature tolerance such as LTPs, aquaporins, chaperones, and sucrose metabolism related genes were down-regulated. It was postulated that the down-regulation of genes having important functions under low temperature tolerance might be responsible for the sensitivity of sunflower plants to low temperature.

Drought tolerant and drought sensitive genotypes subjected to soil water deficit stress under field conditions were assessed through sunflower cDNA microarrays containing about 800 clones, representing high sequence similarity with known or predicted Arabidopsis genes (Roche et al., 2007). The tolerant genotype showed up-regulation of genes involved in cell detoxification (aldehyde deshydrogenase, amino transferases and tocopherol enzymes) and down-regulation of genes involved in the cell division control and cellular differentiation (proteins kinases, transcription factors and receptors), suggesting a specific adapted mechanism in response to water stress. Embryos and leaves showed different expression patterns. Genes related to amino acid and carbohydrate metabolisms, and signal transductions

were up-regulated in embryos and down-regulated in leaves, suggesting a differential response to water stress of vegetative and reproductive organs.

Fluorescence cDNA microarray assays based on sunflower organ-specific unigenes was first developed to study gene expression in early responses to chilling and salinity (Fernandez et al., 2008). The aim of this work was to detect candidate genes associated to regulatory and stress response pathways common to both stresses and to identify those genes exclusively expressed in response to each kind of stress condition. A total of 80 candidate genes for either salinity and/or chilling stresses were detected. Fifty of them were up or down regulated under both stresses, evidencing a cross-talk of regulatory mechanisms in the responses to chilling and salinity. Only 15 and 12 sequences were up regulated or down regulated specifically in one stress but not in the other, respectively.

Interestingly, 39 genes detected in this study correspond to genes with unknown predicted function. Microarray profiling revealed dynamic changes in transcript abundance, including transcription factors, defence/stress related proteins, and effectors of homeostasis, all of which highlight the complexity of both stress responses.

The development of genomics projects during the last decade generated an exponential increase in the number of sequences available in public databases involving complete genome sequencing for some species and Expressed sequences (ESTs), in the case of species with large genomes, such as sunflower.

The availability of large number of EST sequences in public databases for cultivated sunflower and wild *Helianthus* species, jointly with the development in the last years of high-scale transcriptional analysis techniques (through development of oligonucleotide microarrays) allowed the implementation of expression studies across different stages of development in many species, organs and/or growth conditions, including biotic and abiotic stress. In the case of sunflower different oligonucleotide based microarray platforms were developed. Affymetrix and NimbleGen technologies have been applied to the development of chips for the *Helianthus* genus and weeds of the *Asteraceae* family, respectively (Lai et al., 2012, Bazin et al., 2011). The Affymetrix GeneChip was designed based on wild and cultivated sunflower raw ESTs available in public databases (Bazin et al., 2011). The NimbleGen platform comprises one 4-plex microarray developed from the assembly of Sanger ESTs from several *H. annuus L.* cultivars deposited in GenBank up to the year 2007, and one 12-plex array based on the 454 Titanium platform transcriptome assembly from one weedy *H. annuus L.* genotype (Lai et al., 2012). Using the same public *Helianthus* EST data set, plus 454 sequences from the HA89 inbred line transcriptome, a *Helianthus* gene reference assembly was built to conduct SNP discovery and to design an Illumina Infinium BeadChip for genotyping (Bachlava et al., 2012). More recently, an oligonucleotide based chip on Agilent technology has been developed for stress-induced gene expression studies (Fernandez et al., 2012c). The use of a longer probe format represents an advantage of the Agilent oligonucleotide microarrays over other technologies, because the longer oligonucleotides provide higher hybridization stability for sequence mismatches; being thus more suitable for the analysis of highly polymorphic regions (Hardiman, 2004). This chip consisted of approximately 41,000 unigenes representative of the sequences available for sunflower within public ESTs and non-redundant sequences (Fernandez et al., 2012c). A repository of the sunflower unigene collection, plus the probes associated to each unigene and functional annotation information is available at the URL atgc-sur.inta.gob.ar (Figure 1). The microarray was experimentally validated for a pilot senescence experiment and the performance of

different statistical and bioinformatics strategies for data analysis were explored to identify candidate genes and key metabolic pathways associated to the outbreak of the leaf senescence process and response to biotic stress in sunflower (Fernandez et al., 2012c, Fernandez et al., 2012a).

The advent of next-generation sequencing methods with reduced costs and higher throughput has encouraged the generation of more comprehensive and in-depth studies for a wider range of organisms and transcriptomes. RNA-Seq technology allows the generation of valuable information for species with high economic interest but limited genomics resources.

Recently, the interaction between sunflower and *Plasmopara halstedii* (downy mildew) has been studied using the 454 FLX pyrosequencing of cDNAs from *H. annuus* seedlings infected by the oomycete.

As a result, sequences expressed by either organism were identified in the frame of their interaction. This study represents a substantial improvement of existing knowledge regarding *P. halstedii* sequences that are expressed during the interaction of this species with sunflower because twenty putative cytoplasmic effectors were characterized, including RXLR and CRN effectors that are responsible for crinkling and necrosis phenotypes in the leaves of the infected plants. Additionally, 22 SNPs were detected, providing new information on pathogen polymorphisms (As-sadi et al., 2011).

OTHER POST GENOMICS FUNCTIONAL STRATEGIES: METABOLOMICS

Multiparallel analyses of mRNA, metabolite and protein profiles are central to today's functional genomics initiatives. The use of metabolite profiling as a tool for a comparative display of gene function has the potential to provide deeper insight into complex regulatory processes (Fiehn et al., 2000).

Studies of soluble metabolite exchange between sunflower and *Sclerotinia* have been performed by Jobic et al. (2007). This investigation reported a metabolic study of the necrotrophic interaction between *S. sclerotiorum* and cotyledonary leaves of sunflower based on NMR-spectroscopy (nuclear magnetic resonance) (Roberts and Jardetzky, 1981, Shachar-Hill, 1996, Ratcliffe and Shachar-Hill, 2001). In order to analyse metabolic processes that promote fungal development in plant tissues, they established the profiles of soluble metabolites for each partner and followed the quantitative modifications of the metabolites during the course of infection. The results indicated a progressive exhaustion of plant carbohydrate stores in favour of the accumulation of glycerol of fungal origin. Expression of fungal hexose transporters in plants was also described (Jobic et al., 2007).

Other metabolic studies adopted a gas chromatography–mass spectrometry (GC–MS)-based metabolite profiling approach capable of identifying a broad spectrum of metabolites including major and minor sugars and sugar alcohols, organic acids, amino acids, fatty acids and few soluble secondary metabolites in the sunflower capitulum (Peluffo et al., 2010). As plant–host interactions represent one of the most complex systems at the biochemical level, the aim of this work was to develop a metabolomics approach to shed light onto the metabolism of sunflower florets by a comparative analysis of two well characterized

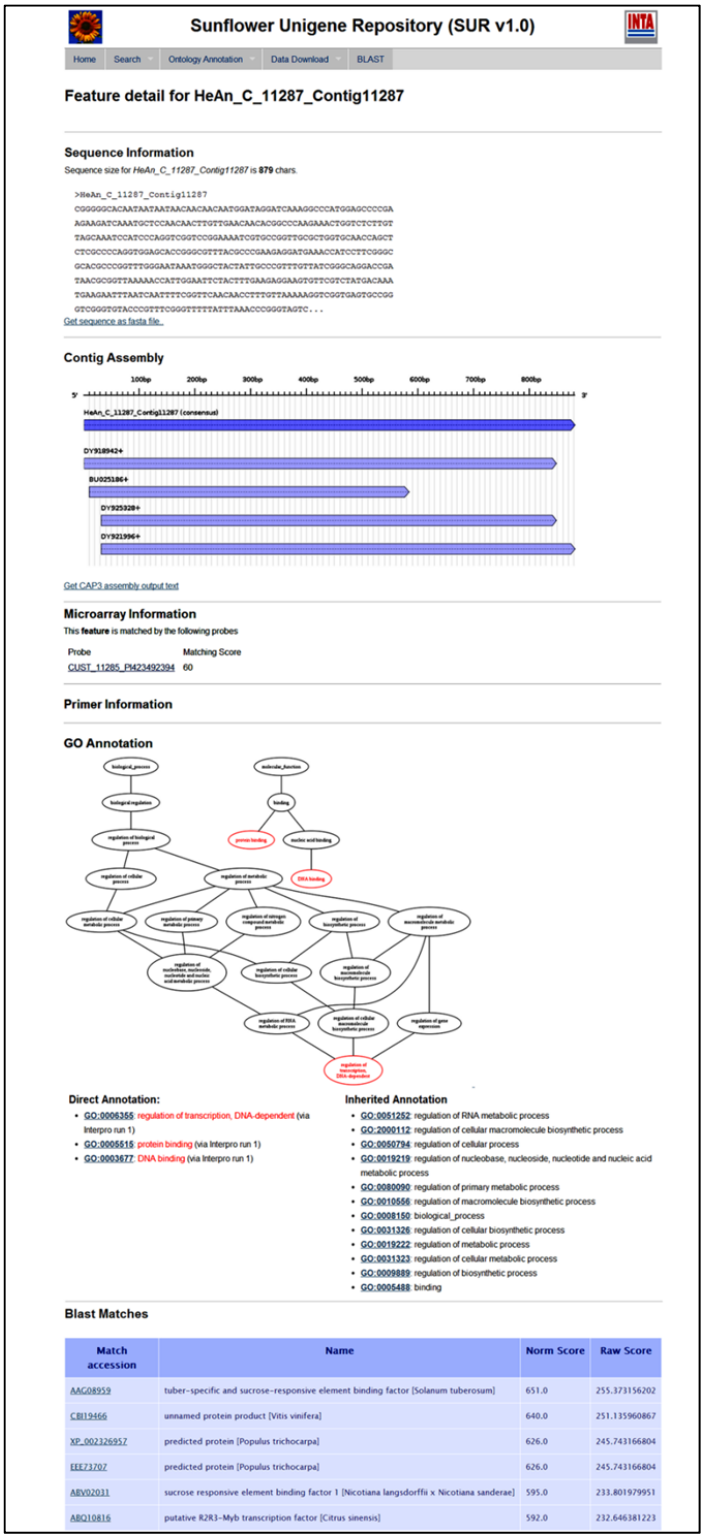

Figure 1. Sunflower Unigene Repository (SUR) for curated sunflower unigenes. (http://atgc-sur.inta.gob.ar).

genotypes with contrasting tolerance levels to *S. sclerotiorum*. The metabolite data obtained in the work of Peluffo et al. (2010), showed that, under field conditions, primary metabolism is differentially synchronized in the different genotypes.

As a result, the analysis allowed detecting differential metabolic regulation between susceptible and resistant genotypes in the sunflower-*Sclerotinia* system. Primary metabolic events in floret cells are differentially synchronized in genotypes with contrasting behaviours tested under field conditions. GC-MS approach has proven to be one of the key tools in metabolomics *per se* and by extension in plant functional genomics (Sumner et al., 2003, Fernie, 2007).

Thus, NMR and GC–MS approaches have been proposed as complementary technologies for metabolic profiling, especially in the context of biomarker discovery (Fernie et al., 2004, Meyer et al., 2007).

REVERSE GENETICS BY TILLING STRATEGY

Non-conventional sunflower breeding has advanced through a variety of approaches including Molecular Marker Assisted Selection (see chapter *Genetics and Genomics Applied to Sunflower Breeding* in this book), genetic engineering (Lucas, 2000), *in vitro* techniques (somaclonal variation, protoplast fusion and *in vitro* embryo rescue) and conventional mutagenesis (Encheva, 2008) to improve yield, oil quality and disease-, salt- and pest-resistance. TILLING (Targeting Induced Local Lesions in Genomes) is one of the latest additions. It is a molecular biology-based method that allows directed identification of mutations in a specific previously characterized gene. TILLING was first reported in 2000, using the model plant *Arabidopsis thaliana* and has been adapted since then as a method in other plant species (McCallum et al., 2000a, McCallum et al., 2000b, Prina et al., 2010).

TILLING provides a reverse genetic technique that is suitable for most plants (McCallum et al., 2000b) in order to select new genetic variability for useful alleles to more precisely design new traits. Thanks to the generation of random point mutations at high density by this technique, allelic series of missense mutations can be discovered; as well as short insertion/ deletions (INDELs) (Greene et al., 2003). An advantage is that even working with only a small population, multiple alleles of a specific gene may be obtained regardless of the gene size (Till, 2007).

Due to the complexity and cost of releasing transgenic traits, TILLING became the method of choice when the desired trait is related to the silencing of a given gene. It significantly reduces costs and time for product development when compared to transgenic crop plants.

The first sunflower TILLING population was developed by Sabetta et al. (2011). They were able to generate point mutations like nucleotide substitutions by conventional ethyl methanesulfonate (EMS) mutagenesis and optimized the procedure for an efficient sunflower SNP detection system.

As result, a sunflower TILLING population of 3,651 independent mutant lines was developed, providing an important tool for the identification of interesting phenotypes; 37 of the mutant lines showed a single altered trait, while 138 lines displayed multiple mutant traits. This mutagenic strategy was successfully applied to assay 1,152 sunflower M2 lines. Four

genes have been subjected to the reverse genetics screening: the *kasII* and *kasIII* genes (codifying the isoforms II and III of the β-keto-acyl-ACP-synthetase); the fad2-1 gene, encoding the enzyme responsible for the converting reaction of oleic acid to linoleic acid; the AY490791 gene, involved in *P. halstedii* resistance. The authors focused on some key enzymes of the fatty acid pathway, because of the interest in increasing the nutritional value of sunflower oil by the reduction of the ratio of saturated to unsaturated fatty acids. Moreover, *P. halstedii* is one of the most dangerous pathogens that affect sunflower cultivation in the Mediterranean area (Radwan et al., 2005). Therefore the availability of a stable and effective system, as genetic resistance, for the pest-control results of prime importance (Sabetta et al., 2011).

This reverse genetics tool has also been used in sunflower by Kumar and co-workers with the objective of screening mutations in two genes, *FatA* and *SAD*, which are involved in the accumulation of short and medium chain fatty acids. As a result they obtained 26 induced mutations using an EMS mutant population under controlled conditions.

They focused in the production of more stable oils for biolubrication and the increase of healthy substitutes for food industry (Kumar et al., 2013).

Currently, the shortage of well characterized candidate genes underlying agronomic important traits represents one of the main drawbacks in sunflower molecular breeding. In this context, functional tools which allow concerted transcriptional studies, such as high density oligonucleotide microarrays, TILLING populations and RNA-seq, strongly support the discovery and characterization of novel genes.

IN VITRO CULTURE AND GENETIC TRANSFORMATION

In vitro tissue culture techniques, as immature embryo rescue, were incorporated in sunflower breeding before the existence of the functional genomics and post genomics disciplines allowing the development of four to six generations per year and therefore, notably accelerating inbreeding speed. Embryo rescue and protoplast fusion also helped to sort sterility or incompatibility barriers in wide interspecies crossings. Examples of protoplast fusion techniques applied to rescue of wide crossings are those between *H. annuus* and *H. petiolaris* or with *H. debilis* (Alibert et al., 1994). The usefulness of these techniques is currently valuable as they were recently applied in the transference of architectural traits from *H. mollis* Lam. to *H. annuus* L. (Breton et al., 2012) and for the development of drought and broomrape resistant sunflower germplasm utilizing *H. argophyllus* and *H. maximiliani* (Petcu and Pâcureanu, 2011).

Transgenic breeding has clearly demonstrated its ability to make significant improvements to agriculture, being maize and soybean the best examples of the advantages that have been achieved using transgenics. The establishment of an efficient and reproducible *in vitro* regeneration system is the first, and maybe the most important, step to develop a plant transformation protocol.

Sunflower plant regeneration was obtained by direct or indirect organogenesis (depending on the induction and passage through an undifferentiated callus stage), somatic embryogenesis, and vegetative multiplication. Indirect organogenesis and embryogenesis through callus stages were soon discarded because of seldom regenerated viable shoots or

embryos (Greco et al., 1984, Paterson and Everett, 1985, Wilcox McCann et al., 1988). While somatic embryogenesis was mainly obtained from immature embryos, the most successful approach seems to be direct organogenesis, which was reported to occur from different explant sources. Thus, several tissues have been used as a source of explants in an important number of publications, plant regeneration have been essayed from cotyledons (Power, 1987; Knittel et al., 1991; Pugliesi et al., 1993; Ceriani et al., 1992; Chraibi et al., 1992; Fiore et al., 1997; Deglene et al., 1997; Alibert et al., 1999; Baker et al., 1999; Flores Berrios et al., 1999a; Flores Berrios et al., 1999b; Dhaka and Kothari, 2002; Molinier et al., 2002; Mayor et al., 2003; Lewi et al., 2006; Radonic et al., 2006; Radonic et al., 2008; Dağüstü et al., 2008; Pradeep et al., 2012; Sujatha et al., 2012b), immature embryos (Power, 1987; Finer, 1987; Freyssinet and Freyssinet, 1988; Wilcox McCann et al., 1988; Jeannin and Hahne, 1991; Bronner et al., 1994; Jeannin et al., 1995; Witrzens et al., 1988; Espinasse et al., 1989; Lucas et al., 2000; Sujatha and Prabakaran, 2001; Hewezi et al., 2002), shoot-tips (Knittel et al., 1994; Laparra et al., 1995; Grayburn and Vick, 1995; Burrus et al., 1996; Gürel and Kazan, 1999; Mohamed et al., 2004), protoplasts (Burrus et al., 1991; Fischer et al., 1992; Krasnyanski et al., 1992; Krasnyanski and Menczel, 1993; Laparra et al., 1995; Moyne et al., 1988; Müller et al., 2001), hypocotyls (Everett et al., 1987; Pelissier et al., 1990; Escandón and Hahne, 1991; Müller et al., 2001; Dağüstü et al., 2008), leaves (Konov et al., 1998; Yordanov et al., 2002) and two days old seedlings (Rao and Rohini, 1999).

Regarding production of transgenic plants, different approaches were achieved. Polyethylene glycol (PEG)-induced uptake of vectors was used for the transformation of protoplasts (Moyne et al., 1988), but because of the labour required to isolate and culture protoplasts, it was necessary to develop other transformation procedures. Some attempts were made to transform sunflower *via* microprojectile bombardment with DNA coated particles (Hunold et al., 1995; Laparra et al., 1995) or electroporation (Kirches et al., 1991) but no transgenic plants were obtained. A combination of microprojectile bombardment with uncoated particles and *Agrobacterium tumefaciens* was also used obtaining transgenic plants (Bidney et al., 1992; Malone-Schoneberg et al., 1994; Knittel et al., 1994; Lucas et al., 2000; Müller et al., 2001; Mohamed et al., 2006). Despite of these attempts, most published transformation systems for sunflower are based on *Agrobacterium*-mediated transformation (Everett et al., 1987; Schrammeijer et al., 1990; Escandón and Hahne, 1991; Grayburn and Vick, 1995; Burrus et al., 1996; Alibert et al., 1999; Müller et al., 2001; Molinier et al., 2002; Mohamed et al., 2004; Ikeda et al., 2005; Lewi et al., 2006; Radonic et al., 2006; Dağüstü et al., 2008; Radonic et al., 2008; Neskorodov et al., 2010; Pradeep et al., 2012; Sujatha et al., 2012b) and these protocols are modifications of the following scheme: Imbibition of seeds, excision of embryonic axes, co-culture with *Agrobacterium tumefaciens*, induction of shoots, recovery of transformed shoots, selection, shoot elongation, transfer to greenhouse and acclimatization.

All these published protocols suffer from overall low transformation efficiencies. The critical steps in plant transformation procedure are the transfer of the T-DNA into as many cells as possible with good regeneration potential, and the regeneration of the transformed cells into shoots. However, the induction efficiency of adventitious shoots in sunflower is low due to the fact that the regeneration process is in almost every case by direct organogenesis, without a callus phase that might serve to amplify the response. This regeneration event is often of multicellular origin, so regenerated shoots and plants are frequently chimeric (Schrammeijer et al., 1990) and the transgenic sectors may or may not lead to the recovery of

transgenic progeny (Burrus et al., 1996). Some efforts were made to increase the regeneration potential in the meristematic area, like bombardment with coated particles with a construct containing the *ipt* gene, which codes for an isopentenyl transferase involved in the biosynthesis of cytokinins in plants, to induce transient expression of this growth regulator and consequently an increase in the number of shoots per explant (Molinier et al., 2002). Furthermore, to stimulate shoot regeneration from hypocotyl explants, Koopmann and Kutschera (2005) reported a novel system by immersing the explants in a suspension of *Metylobacterium* prior to culture on a shoot-regeneration medium.

Other important reported improvement in plant regeneration was the addition of silver nitrate in all regeneration media to diminish hyperhydricity of primordia and shoots (Mayor et al., 2003). Besides, rooting was improved by transferring the regenerated shoots to a medium containing ancymidol as gibberellin inhibitor (Fiore et al., 1997) and naphthalene-acetic acid as the only auxin (Baker et al., 1999).

More recently, Sujatha et al. (2012a) used 2-isopentenyladenine in the shoot induction and elongation media to prevent precocious flowering.

Some of these transformation protocols rely on one of several treatments in order to stimulate *Agrobacterium* virulence genes, such as adding phenolic compounds as acetosyringone to the co-culture medium (Mohamed et al., 2004), tissue wounding by either bombardment with naked particles (Bidney et al., 1992; Müller et al., 2001; Sawahel and Hagran, 2006), or glass beads (Grayburn and Vick, 1995), or by macerating with enzymes (Alibert et al., 1999; Weber et al., 2003), sonication (Weber et al., 2003), vacuum (Hewezi et al., 2003), DMSO treatment (Sujatha et al., 2012b), explant dehydration for several minutes before co-culture (Hewezi et al., 2002) or combination of some of these treatments (Weber et al., 2003; Radonic et al., 2006; Radonic et al., 2008; Sujatha et al., 2012b).

Efficient selection of transgenic plants is an important aspect of any transformation protocol. Kanamycin is the most widely used selection agent for sunflower transformation, although it is known to be detrimental to organogenic potential (Everett et al., 1987; Müller et al., 2001).

It has been used in different concentrations (from 1 to 200 mg/L) and with different levels of success, ranging from the inability of regenerating shoots to a 79% of escapes (Knittel et al., 1994; Hunold et al., 1995; Gürel and Kazan, 1999; Rao and Rohini, 1999; Lucas et al., 2000; Hewezi et al., 2002; Rousselin et al., 2002; Ikeda et al., 2005; Lewi et al., 2006; Radonic et al., 2006; Sawahel and Hagran, 2006; Hewezi et al., 2006b, Radonic et al., 2008; Pradeep et al., 2012; Sujatha et al., 2012b; Escandón and Hahne, 1991; Malone-Schoneberg et al., 1994; Grayburn and Vick, 1995; Burrus et al., 1996).

Most of these works used shoot bleaching as selection criteria, except Radonic et al. (2006, 2008) that showed that *in vitro* root development in presence of kanamycin was the most effective parameter to discriminate between transformed and non-transformed shoots with no escapes (Figure 2).

In some cases where kanamycin selection could not be applied, GFP (Müller et al., 2001; Weber et al., 2003; Mohamed et al., 2006) and GUS (Mohamed et al., 2004) were used as vitals marker to select transgenic shoots. Escandón and Hahne (1991) tried selection in phosphinotricin as they found that kanamycin was not suitable for selection because it reduced regeneration potential in sunflower. More recently, Neskorodov et al. (2010) demonstrated that *bar* is an efficient selective marker for sunflower genetic transformation as false transformants did not regenerate.

TRANSIENT TRANSFORMATION AND USE OF LETTUCE AS MODEL SYSTEM FOR SUNFLOWER FUNCTIONAL GENOMICS STUDIES

From all the transformation attempts mentioned in the previous paragraphs there are two remarkable conclusions: neither there is a rapid protocol nor it is possible to obtain a large number of transgenic regenerants per assay.

On the other hand, the availability of an Agrobacterium-mediated transient transformation system represents a powerful tool to study both biological processes and the role played by selected candidate genes derived from genomics approaches on agriculture important traits.

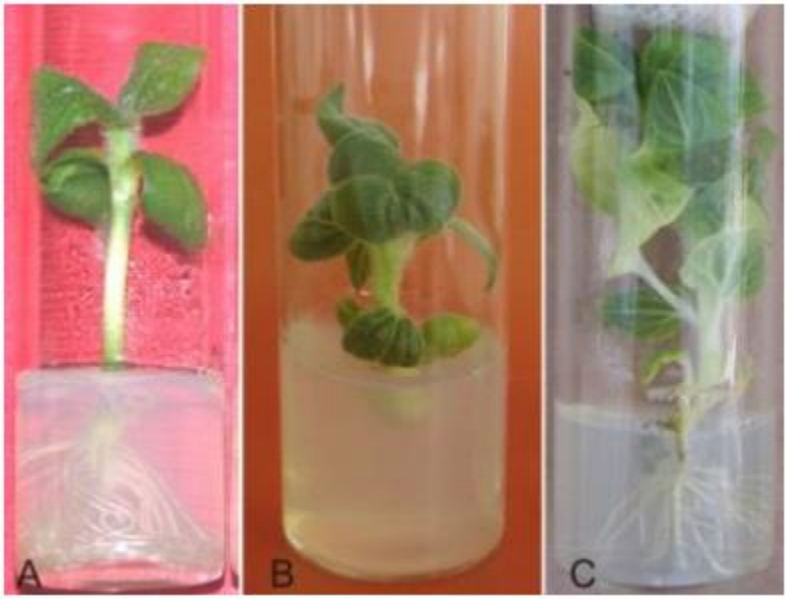

Figure 2. Kanamycin selection of transgenic plants using rooting as selection criterion. Non-transformed plants develop a normal root in media without kanamycin (A). In kanamycin containing media, non-transformed plants do not root (B) whereas transformed plants develop a normal root (C).

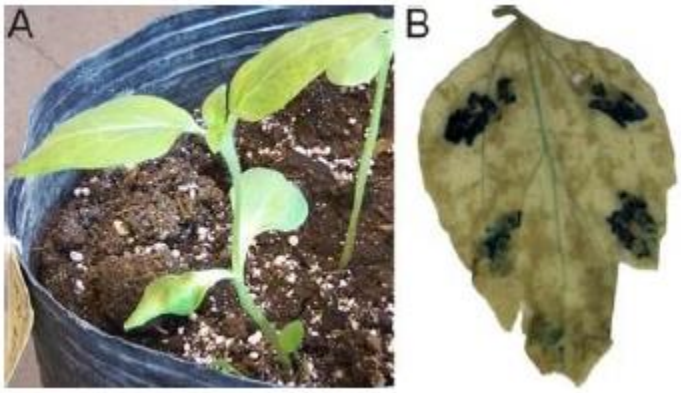

Figure 3. Standard agroinfiltration system. Sunflower greenhouse plant in V2 stage used to perform agroinfiltration (A). GUS staining of a leaf showing expression in different agroinfiltration areas (B).

Agroinfiltration of sunflower is not as easy as in Nicotiana benthamiana or other species, and it was first reported by Manavella and Chan (2009) who developed a system where leaf discs in vitro cultured for 3 days could be transiently transformed.

More recently, a standard agroinfiltration system was developed by establishing basically the Agrobacterium strain and the developmental stages of the greenhouse grown plants used (Radonic, 2010) (Figure 3).

However, transient expression systems only allow studies for up to 3 or 4 days and when a follow up experiment for a longer time is required, for example to assess the effect of a gene or a promoter on developmental stages, the stable transformation of plants is completely necessary.

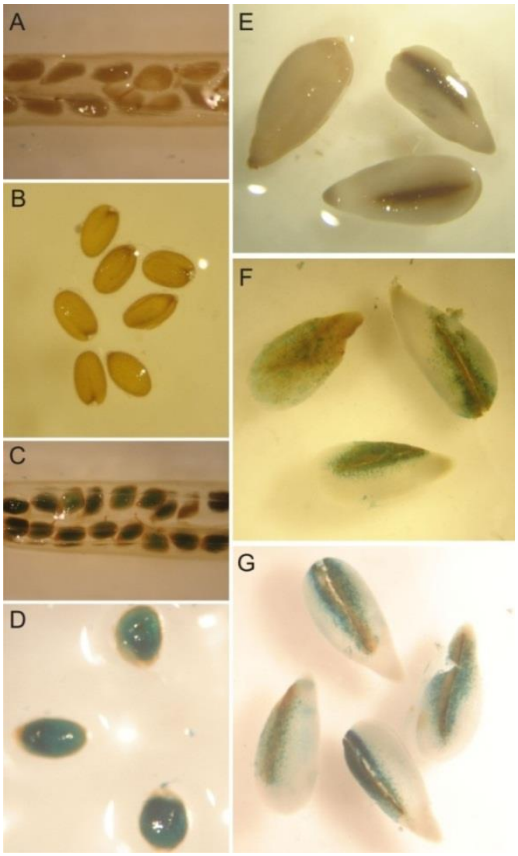

Figure 4. Use of lettuce as a model system for the *Asteraceae* family, in this case, to evaluate tissue specificity of sunflower *HaAP10* promoter. GUS staining results from non-transformed (A, B) and transformed (C, D) *Arabidopsis* seeds showing expression only in transformed seeds. The same result was obtained for non-transformed (E) and transformed (F, G) lettuce seeds.

In this context, in a meeting of the Compositae Genome Project (http://compge-nomics. ucdavis.edu/cwp) it was concluded that lettuce is the best model system as both species belong to the Asteraceae family. Lettuce is friendly to in vitro culture producing a high number of regenerants that later adapt easily to greenhouse conditions. This system was used to evaluate tissue specificity of the sunflower HaAP10 promoter transiently directing GUS expression only in lettuce seeds (Zavallo et al., 2011), in the same way as it was shown in Arabidopsis seeds (Zavallo et al., 2010) (Figure 4).

MOST IMPORTANT AGRONOMICAL TRAITS ENDORSED BY GENETIC ENGINEERING OF SUNFLOWER

The majority of the transgenic plants obtained in the previously mentioned publications were developed to establish the transformation methodology with selective markers and reporter genes, like kanamycin resistance gene *nptII*, phosphinotricin resistance gene *bar*, glucuronidase gene *uidA* and green fluorescent protein gene *egfp*. Nevertheless, there are some studies centred upon the introduction of agronomically useful genes into sunflower.

Three agronomically important transgenic traits were incorporated in sunflower, basically by seed companies: herbicide tolerance, insect resistance and disease (fungal) resistance.

Regarding insect resistance, transgenic expression of the *Cry1F* (a *Bacillus thuringiensis* or "Bt" gene) in sunflower confers significant control of *Rachiplusia nu* and *Spilosoma virginica*, two important insect pests that impact on sunflower production in various producing countries. A feeding assay was performed using transgenic leaf discs of *Cry1F* Bt plants and both, larvae at seedling and preflowering stages, clearly exhibited enhanced insect resistance compared to the control. Also, two field bioassays were carried out in Argentina where two *Cry1F*-transgenic sunflower events exhibited complete resistance to *R. nu* (Pozzi et al., 2000). Another Bt gene, *Cry1Ac*, when introduced in a transgenic sunflower line also showed insect resistance. When this transgenic event was crossed with a wild *H. annuus* accession, the hybrid progeny showed significant enhanced resistance to *Lepidopteran* damage, which was not a surprising result. However, the transgenic × wild sunflower hybrid produced 55% more seeds than the control hybrids lacking the *Cry1Ac* gene (Snow et al., 2003). This study confirmed the effectiveness of Bt genes in controlling insect pests in sunflower, and at the same time raised a concern about "gene flow" to wild species that has the potential to enhance the fitness of weeds in the environment.

For fungal diseases like downy mildew and rust that can be controlled by single dominant resistance genes, breeders have been very successful in identifying new resistance alleles in wild species and integrating them into elite germplasm (Gulya et al., 1997). However, for complex fungal diseases like *Sclerotinia* head rot which is the most damaging disease, the success of conventional breeding was only partial and many breeders feel that the only possible solution is to develop transgenic resistant plants. This approach was carried out by introducing the wheat germin gf2.8 *OXO* gene (Lane et al., 1991) to generate the so-called *OXO*-transgenic sunflower plants (Lu et al., 2000, Scelonge et al., 2000). Bioassays of these plants were carried out at second generation (T2) and at more advanced progeny generations indicating the stable inheritance of the transgene. Lesion sizes in the transgenic leaves were inversely related to the endogenous levels of OXO activity and *Sclerotinia* spread to the capitulum tissue only in control plants while the lesions were confined to the main stem on transgenic plants, proving that OXO can confer significant *Sclerotinia* resistance in transgenic sunflower (Hu et al., 2003). Moreover hybrids from the crossing of transgenic lines with natural *Sclerotinia* rot-tolerant lines that carried the *OXO* transgene were more resistant to *Sclerotinia* than the corresponding non-transgenic isogenic hybrids, so the combination of *OXO* with natural tolerance genetic background provides a greater level of resistance than the one observed in the commercial hybrids (Bazzalo et al., 2000). This was the first example of a successful transgene-mediated fungal resistance mechanism in plants. However, the OXO enzyme has been found to persist in simulated gastric fluid digestion studies, raising concerns

about whether the protein may become a human allergen (Jensen-Jarolim et al., 2002). Regarding the environmental concerns, Burke and Rieseberg (2003) suggest that genetically modified wild plants will not be a worse weed because they do not produce more seeds than the other wild plants, so the *OXO* transgene will diffuse neutrally after its escape in the absence of selection pressure for *Sclerotinia* tolerance. Chapman and Burke (2006) studied the escape of transgenes from crop × wild hybridization and showed that natural selection is the main factor governing the spread of favourable transgene alleles, and not the gene flow.

Genetically transformed crops with herbicide resistance genes give farmers greater flexibility for the control of weeds. Among the herbicides used in combination with herbicide tolerant transgenic crops, phosphinothricin and glyphosate have demonstrated effective control of weeds, low toxicity and low impact on the environment. In the case of sunflower, this is also the case for a non-transgenic trait that was introgressed from a wild accession. An imidazolinone (IMI) genetic resistance was identified in a wild population of *H. annuus* in Kansas by Miller and Al-Khatib (2002).

The basis of this resistance is the expression of two natural alleles containing mutations in the gene that encodes for ALS (acetolactate synthase) that produce a conformational change in the structure of the enzyme which avoids herbicide binding. IMI resistance was incorporated into elite germplasm through conventional breeding and is currently sold worldwide under the Clearfield™ trademark owned by BASF.

Although this resistance to IMI herbicides was incorporated through conventional breeding methods, some concerns have raised on gene flow of the IMI-resistance trait to wild species. Massinga et al. (2003, 2005) created hybrids of domesticated sunflower with both common sunflower and prairie sunflower with and without the IMI-resistance trait, and measured the growth of IMI-R and IMI-S hybrids under non-competitive conditions, concluding that hybrids of both sunflower species were equally competitive. These results show the safety on environmental impact of IMI technology and besides that the ALS mutant genes can be used for engineering sunflower to produce transgenic sunflower for this or other herbicide resistance genes.

However, this door is not completely open yet, since transgenic tolerance to glyphosate and phosphinotricin have already been introduced in sunflower showing efficacy in field trials, but they did not reach the market because of the concerns regarding the environmental consequences of the transfer of these genes to sexually compatible weedy relatives.

Other transgenic approaches are directed to modify oil quality. Sunflower oil is one of the four major sources of edible oils worldwide and is well known as healthy oil. Besides it is very stable against lipid peroxidation and is used for salad dressings, cooking and frying. High oleic acid sunflower, Pervenets lines, were originally developed by mutagenesis (Soldatov, 1976). The cross of a Pervenets female with the inbred line HA89 created the first mid oleic or NuSun™ sunflower hybrid (Miller et al., 1987). The existence and the rapid adoption of these lines encourage the improving intentions for oil profile by transgenesis.

The coding sequence of D9-stearoyl-(acyl carrier protein) desaturase from *Ricinus communis* was introduced into sunflower, under the control of seed-specific promoter and terminator sequences of the late embryogenesis abundant gene from sunflower, *Hads10*. Fatty acid composition of the seed oil was followed over five generations under greenhouse and in open field conditions.

Some of the transgenic lines produced oil with a significantly reduced stearic acid content compared with non-transformed plants under greenhouse and field conditions (Rousselin et

al., 2002). Dağüstü et al. (2008) introduced into sunflower the *Erwinia uredovora* phytoene desaturase (*crtl*) and hydroxymethylglutaryl-CoA (*Hmgr-CoA*) genes, which have the potential to increase oil quality.

In summary, current scenarios in sunflower transgenesis shows contrasting facts. As mentioned previously, Snow and colleagues (2003) showed some environmental concerns regarding Bt transgenic sunflower release, while Burke and Rieseberg reported lack of such risk after gene flow to weeds for the *OXO* event.

Besides, as detailed by Cantamutto and Poverene (2007) each new release should be analysed separately as it must be taken into account whether the release would take place in the centre of origin (North America) or in other geographical areas (South America, Europe or Africa) and also that it must be considered the adaptive nature of the introduced character. For example, it is foreseen that the adaptive impact of a change in oil quality will give no selective advantage to weeds, compared to the case of an herbicide tolerance under the selective pressure of the agroecosystem.

However, even in this extreme example of adaptive advantage it may be argued that it rapidly disappears in the absence of the selective agent (the herbicide) in natural environment outside the agroecosystem.

Moreover, it would be interesting for environmental sustainability to count with the variety of plant tolerant to different herbicides in order to alternate their sowing with other herbicide tolerant species, like soybean or even with IMI sunflowers, thereby preventing the emergence of resistant weeds to one of these herbicides or helping to control them.

As a consequence of the appearance of Clearfield sunflower and its global marketing since 2010, many of the concerns about the release of transgenic sunflowers are rapidly decreasing. In addition, the constant efforts in improving sunflower transformation protocols will strength the emergence of some transgenic varieties of this oilseed in the near future.

CONCLUSION

The advances and emergence of new technologies in functional genomics, post genomics and transformation were described in this chapter.

Although sunflower is still an orphan crop, remains as a crop without reference genome available; the development of knowledge at transcriptomics, metablomics and proteomics level along with statistical and bioinformatics procedures for the analyses and data mining of the massive information derived from high-throughput processing methodologies greatly contributes to support plant breeding.

The application of these strategies allows not only the elucidation of complex agronomical pathways but also the identification of candidate robust genes playing key roles in determining these traits.

In addition, the possibility to analyse those candidate sequences as genes or promoters in a transient or stable expression system both in sunflower plants as in model species like *Arabidopsis* or lettuce assures their functional validation.

These genomics approaches are nowadays being used both in the public and private sectors, and will impact positively on sunflower breeding bringing this crop to a competitive place in the international marketplace.

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