MOLECULAR GENETICS

PROTEOMIC RESPONSE OF SUNFLOWER TO DROUGHT STRESS

***Mehdi GHAFFARI*, Mahmoud TOORCHI, Mostafa VALIZADEH, Bahram ALIZADEH**

ABSTRACT

Proteomics technique was used to identify of proteins involved in drought tolerance and sensitivity of sunflower inbred lines. Based on two years field evaluation under drought stress in flowering stage among 16 sunflower lines RGK 21 and BGK 329 were identified as more drought sensitive and tolerant lines respectively. In comparison of proteomic pattern using 2DE, 21 of 347 and 27 of 363 protein spots were affected significantly by drought stress in sensitive and tolerant lines respectively, among them 18 proteins were identified in sensitive and 24 in tolerant line by nano-LC MS/MS mass spectrometry. In sensitive and tolerant lines 81 and 52% of identified proteins were down-regulated respectively. Cytoplasm-chloroplast /metabolism-energy related proteins constituted the major group of identified proteins. The results indicated that preservation of relevant water statues by morpho-physiological changes, supporting natural cellular metabolism, and changes in energy and antioxidant defense related proteins were the main factors for adaptation and drought tolerance of sunflower.
Keywords; Sunflower, Proteomics, Drought, Root

INTRODUCTION

Sunflower as a main oilseed crop is a drought tolerant crop however its productivity is greatly affected by drought stress (Chimenti et al., 2002). There are different; morphological, physiological or molecular mechanisms at different stages for drought tolerance in plants (Farooq *et al*., 2009). At the molecular level drought tolerance is related to differential expression of some stress induced proteins as late embryogenesis abundant, aquaporins and heat shock proteins (Bartels and Sunkar, 2005). Generally it is believed that under drought stress photosynthesis related proteins are reduced as a result of stomata closure while energy and defense related proteins are increased to meet energy demands and to protection of sub-cellular structures (Dinakar *et al.* 2012).

Nowadays proteomics is an essential methodology for large-scale analysis of proteins in various fields of plant biology (Komatsu *et al.* 2007) which has been used for analysis of proteome changes against drought stress in different plants There are limited studies about response of sunflower to drought stress. Castillejo *et al.* (2008) and Fulda et al (2011) used this approach to identification of proteins involved in sunflower tolerance to drought stress. Linking proteome changes to physiological changes providing protein biomarkers for selection programs enable plant breeders to develop new varieties with enhanced drought tolerance. Considering the limited information about sunflower response to drought stress in protein level proteomics technique was used to compare changes of proteins induced by drought stress in roots of sensitive and tolerant sunflower inbred lines.

MATERIALS AND METHODS

Protein extraction from roots of sunflower was adapted from oervious work (Ghaffari et al., 2013) by some modifications. Protein contents were determined using the Bradford (1976) method with bovine serum albumin as the standard. After 2-DE electrophoresis the gels were

stained with coomassie brilliant blue for 1 h, and then destained with 35% methanol and 10% acetic acid for 12 h. 2-DE images were obtained using a GS-800 calibrated densitometer scanner and the position of individual proteins on gels was evaluated with PDQuest software. The isoelectric point and molecular mass of each protein was determined using 2-DE standard marker. Comparative analysis, were performed using PDQuest software. Student's t-test was used to assess the statistical significant of differences in protein abundance between control and drought treatment. The protein spots were excised from 2-DE gels and subjected to reduction and rehydration process using DigestPro. The desalted peptide solution was analyzed by nano-liquid column (LC)-MS/MS.

A nanospray LTQ XL Orbitrap MS was operated in data-dependent acquisition mode with the installed XCalibur software were used to obtaining MS/MS spectra. Full-scan mass spectra were acquired in the Orbitrap over a mass range of 150 - 2,000 m/z with a resolution of 15,000. The 3 most intense ions above an intensity threshold of 1,000 units were selected for collision-induced fragmentation in the linear ion trap at normalized collision energy of 35% after accumulation to a target value of 1,000 intensity units. The resulting peptide sequence data were used to search the NCBInr protein database using the MASCOT search engine. A homology search of the amino acid sequences of identified proteins was performed against the NCBI non-redundant sequence database using BLASTP to assign protein identities.

RESULTS AND DISCUSSIONS

Comparison of 2-DE gels revealed 347 reproducible protein spots in drought sensitive (RGK 21) and 363 in tolerant (BGK 329) line, among them 18 were affected by drought stress in sensitive and 24 in tolerant line significantly. Drought stress made lower reduction in abundance of identified proteins in BGK 329 which implies the more flexibility of the tolerant line to endurance of drought damages. This is also indicated as a general rule by Rossignol et al. (2006). Of the affected protein spots in RGK 21 the relative abundances of 4 were increased and 14 decreased. Of the protein spots in BGK 329, relative abundances of 10 were increased, 11 decreased, 2 protein spots turned up as new spots and one spot disappeared. According to the direction of changes all identified proteins categorized in 6 groups.

Classification of all differentially changed protein spots under drought stress based on cellular function revealed more impressibility of metabolism followed by energy related proteins in both sensitive and tolerant lines. Relative abundance of all metabolism related proteins were decreased in sensitive line but 36% of these proteins were increased in tolerant line. Relative abundance of disease/defense related proteins were decreased in sensitive while increased in tolerant line. According to the sub-cellular localization, cytoplasmic followed by chloroplastic proteins were primary drought responsive proteins in both lines. There was more reduction while more increase in relative abundance of cytoplasmic proteins in sensitive and tolerant lines respectively, however chloroplastic proteins were down expressed considerably in both lines.

Among the identified proteins, 15 protein spots were commonly affected by drought stress in both lines; relative abundance of 4 of them were increased and 6 decreased . Relative abundance of the 5 remaining protein spots i.e. sucrose synthase, bifunctional polymyxin resistance protein ArnA-like, ATP synthase subunit beta, glyceraldehyde-3-phosphate dehydrogenase, putative cytosolic NADP-malic enzyme (protein spots 1, 9, 14, 32 and 33) were expressed differentially; decreased in sensitive while increased in tolerant line. There were 3 proteins specific to RGK 21 and 9 to BGK 329. The abundances of RGK 21 specific proteins were decreased while 3 of BGK 329 specific proteins; carbonic anhydrase, Cu/Zn superoxide dismutase, methionine synthase (spots 7, 13, 29) were increased.

Two groups of proteins had same changes in two lines. Oxygen-evolving enhancer (OEE) and ferredoxin-NADP reductase (FNR) as major photosynthetic proteins were suppressed in both lines**.** Impairment to the OEE as a result of drought stress has been reported by Vander Willigen et al. (2003). OEE has a key role in providing of reducing power to the electron transport chain (ETC) in chloroplast by splitting of H_2O. It seems that OEE act as a primary drought sensor and changes of its abundance can shift normal ETC mode to stress tolerance mode. Involvement of FNR in ROS scavenging and NADPH/NADP+ homeostasis (Rodriguez et al. 2007) expresses this protein as a common intermediate in response to drought stress. **I**nduction of tubulin alpha-3 chain in both lines as a cytoskeleton-related protein which is also involves in cell division can enhance cellular structure to repel drought injuries.

Reduction of enolase (spot 16) assumed as a sign of metabolic impairment to the glycolytic pathway in sensitive line. Down turning of enolase can limit malate supply for mitochondria which is an explanation for reduction of ATP synthase and energetic depletion in sensitive line. Drought induced accumulation of carbonic anhydrase (spot 7), Cu/Zn superoxide dismutase and methionine synthase was assumed as a BGK 329 specific response to endurance of drought injuries. The same results reported in other plants previously (Merewitz et al. 2011). Carbonic anhydrase has a key role in CO_2 enrichment before Calvin cycle. The energetic cost of this adaptation is more ATP which is provided by increased abundance of ATP synthase in the tolerant line.

Enhancement of ROS scavenging capacity by induction of Cu/Zn-SOD constituted one of the major aspects of drought tolerance in BGK 329. Induction of this protein by drought was also reported by Salekdeh et al. (2002). Increased demand for more methyl groups for lignification justifies accumulation of methionine synthase in tolerant line too. Involvement of this protein in osmotic adjustment (Yan et al. 2005), imply critical role of osmo-protection and lignification in drought tolerance of BGK 329.

Differential changes in abundances of 5 proteins (spots 1, 9, 14, 32 and 33) can be a cause for different response of the lines to drought stress. Drought induced accumulation of sucrose synthase has been also reported in Fern-Ally (Wang et al. 2010). We suggest that drought induced accumulation of sucrose synthase in tolerant line was an adaptive mechanism to energy conservation and maintaining cell structure integrity by invigoration of osmotic adjustment.

Induction of mitochondrial ATP synthase (spot 14) in tolerant line assumed as a compensatory mechanism to meet energy demand and to alleviation of drought effects within mitochondrion. Kotapalli et al. (2009) also observed similar results with peanut. Regarding the role of NADP-malic enzyme in breaking down of malate which results in reduction of turgor in guard cells and so on in stomata closure (Laporte et al, 2002) induction of this protein in tolerant line assumed as an adaptive mechanism for water conservation under drought condition. The results of this study indicated that sunflower genotypes shut down photosynthesis by down expression of ETC related proteins as a protective mechanism to avoid generation of ROS during drought condition. Proteomic changes related to ROS scavenging, energy conservation, cell structure integrity and water conservation constituted the major aspects of drought tolerance in sunflower.

LITERATURE

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72**, 248-254.

Castillejo MA, Maldonado AM, Ogueta S, Jorrin JV (2008) Proteomic analysis of responses to drought stress in sunflower (*Helianthus annuus* L.) leaves by 2DE gel electrophoresis and mass spectrometry. *The Open Proteomics Journal **1***, 59-71.

Chimenti CA, Pearson J, Hall AJ (2002) Osmotic adjustment and yield maintenance under drought in sunflower. Field Crops Research **75**, 235-246.

Dinakar C., Djilianov D. and Bartels D. 2012. Photosynthesis in desiccation tolerant plants: energy metabolism and antioxidative stress defense. Plant Sci., 182: 29-41.

Farooq M., Wahid A., Kobayashi N., Fujita D. and Basra S. M. A. 2009. Plant drought stress: effects, mechanisms and management. Agron. Sustain. Dev., 29: 153-188.

Fulda S, Mikkat S, Stegmann H, Horn R (2011) Physiology and proteomics of drought stress acclimation in sunflower. Plant Biology **13**, 632-642.

Ghaffari M, Toorchi M, Valizadeh M, Komatsu S (2013) Differential response of root proteome to drought stress in drought sensitive and tolerant sunflower inbred lines. Functional plat Biology **40(6)**, 609-617.

Komatsu S, Konishi H, Hashimoto M (2007) The proteomics of plant cell membranes. Journal of Experimental Botany **58 (1)**, 103-112.

Kottapalli KR, Rakwak R, Shibata J, Burow G, Tissue D, Burke J, Puppala N, Burow M, Payton P (2009) Physiology and proteomics of the water-deficit stress response in three contrasting peanut genotypes. Plant, Cell and Environment **32**, 380-407.

Laporte MM, Shen B, Tarczynski MC (2002) Engineering for drought avoidance: Expression of maize NADPmalic enzyme in tobacco results in altered stomatal function. Journal of Experimental Botany **53**, 699-705.

Merewitz EB, Gianfagna T, Huang B (2011) Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expressing an ipt gene for cytokinin synthesis. Journal of Experimental Botany **62**, 5311-5333.

Rodriguez RE, Lodeyro A, Poli Ho ZM, Peisker M, Palatnik JF, Tognetti VB, Tschiersch H, Hajirezaei MR, Valle EM, Carrillo N (2007) Transgenic tobacco plants overexpressing chloroplastic FNR display normal rates of photosynthesis and increased tolerance to oxidative stress. Plant Physiology **143**, 639-649.

Rossignol M, Peltier JB, Mock HP, Matros A, Maldonado AM, Jorrin JV (2006) Plant proteome analysis: A 2004–2006 update. Proteomics **6**, 5529-5548.

Salekdeh GH, Siopongco J, Wade LJ, Ghareyazie B (2002) Bennett J Proteomic analysis of rice leaves during drought stress and recovery. Proteomics **2**, 1131-1145.

Yan S, Tang Z, Su W, Sun W (2005) Proteomic analysis of salt stress-responsive proteins in rice root. Proteomics **5**, 235-244.

APPROACHES FOR IMPROVEMENT OF RESISTANCE TO POWDERY MILDEW IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

Sujatha Mulpuri *, Kadırvel Palchamy, Rao Sankaraneni Chander, Varaprasad Kodeboyina Sivannarayana

ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad 500030, India

**mulpuri.sujata@icar.gov.in*

ABSTRACT

Powdery mildew disease caused by *Golovinomyces cichoracearum* (DC) V.P. Heluta var. *Cichoracearum* has become a serious problem in sunflower cultivation in India since the last decade. Initially, the disease was confined to *rabi* crop (October-March) at flowering and post-flowering stages but in the recent past, the pathogen attack is witnessed during all seasons and all stages of crop growth necessitating resistance deployment strategies. A screening method and scoring scale were developed for reliable identification of genotypes resistant to the disease. Screening of germplasm, breeding lines and wild *Helianthus* species resulted in identification of two interspecific derivatives namely HIR-1734-2 (EC-633077) and RES-834-3 (EC-633089), two exotic lines namely PI 642072 (EC-595333) and USDA-25 (EC-537925) and ten *Helianthus* species namely *H. argophyllus, H. agrestis, H. debilis, H. praecox, H. angustifolius, H. atrorubens, H. rigidus, H. salicifolius, H. pauciflorus* and *H. resinosus* tolerant to the disease. Based on consistent reaction in different accessions and across seasons, four accessions namely RES-834-3, PI 642072, *H. debilis* and *H. praecox* along with the highly susceptible line PS 2023 were studied extensively for host-pathogen interactions, biochemical profiling of defense related enzymes and transcript profiling in control and post-infected samples which indicated different mechanisms of tolerance. Development of mapping populations (RILs, BC_1F_1) involving the resistant donors are in various stages towards mapping of genes conferring resistance to powdery mildew in sunflower.

Keywords: Sunflower, powdery mildew, *Golovinomyces cichoracearum*, differential transcripts, host-pathogen relationships

INTRODUCTION

In India, powdery mildew disease was sporadically observed before 2006, but during the year 2006-07 it was reported in high intensity (80%) on *rabi* crop (October-March) in some areas around Bengaluru and Raichur which increased over the years (Anonymous, 2007). Polycyclic nature and short life cycle of the pathogen under conditions of high humidity resulted in rapid spread of the disease to all the sunflower growing states (South, Central and North India) and seasons (rainy, spring and summer) in India (Sujatha et al., 2015). The disease begins during the post-flowering stage as minute discoloured specks on leaves from which powdery mass radiates on all the sides. All the aerial parts of the host are covered with white powdery mass containing mycelia and conidia of the fungus. At present, the disease is seen regularly in all sunflower growing areas of the country in moderate to severe form. A field experiment on yield loss assessment of powdery mildew in sunflower was conducted and the results revealed that, at 30% and 64% of disease severity levels the seed yields were reduced by 20.5% and 52.6%, respectively (Anonymous, 2014) necessitating research for development of appropriate

management strategies. Yield reduction is mainly due to the reduced photosynthetic activity, physiological changes and increased rate of senescence.

SCREENING AND IDENTIFICATION OF RELIABLE SOURCES OF RESISTANCE TO POWDERY MILDEW

It is reported that three genera namely *Golovinomyces cichoracearum* f.sp. *helianthi* (syn *Erysiphe cichoracearum* DC ex Meret; *Oidium asteris punicei* Peck), *Leveillula taurica* (=*Leveillula compositarum*) and *Podosphaera xanthii* Castagne Braun & Shishkoff (=*Sphaerotheca fuliginea* auct p.p.) are the causative agents of powdery mildew in sunflower; of which, *G. cichoracearum* is of the most common occurrence in all the continents (Saliman et al., 1982; Gulya et al., 1991; Chen et al., 2008). Classical identification methods based on microscopical analysis and spore trapping are labour intensive and require considerable experience in differentiating the morphologies of the powdery mildews (Grote et al., 2002). Hence, morphological characteristics supported by molecular analysis of the powdery mildew isolates collected from different geographical locations in India using the powdery mildew specific ITS universal primer pair (Bardin et al., 1999) and also primers that are specific to the ITS regions of *P. xanthii*, *G. cichoracearum* and *L. taurica* indicated that disease infection is caused by *G. cichoracearum* (Prathap Reddy et al., 2013). Reliable sources of resistance are not available in the released cultivars and the parental lines of hybrids. Hence, wild *Helianthus* species, backcross inbred lines, interspecific derivatives, core germplasm set, inbred lines and few exotic accessions were screened under field conditions by simulating the conditions followed by rescreening under artificial inoculation conditions (Fig. 1). Sources of resistance were identified in five annual wild species namely *H. argophyllus, H. agrestis, H. debilis, H. niveus, H. praecox* and six perennials namely *H. angustifolius, H. atrorubens, H. rigidus, H. salcifolius, H. pauciflorus* and *H. resinosus*, two interspecific derivatives (HIR-1734-2/EC-633077, RES-834-3/ EC-633089) and two exotic lines accessions (PI 642072/EC-595333/TX16R, USDA-25/EC-537925). The species, *H. strumosus* was highly susceptible and harboured the pathogen throughout the year. Seven different methods described earlier (Karuna, 2010) were tested; of which, dusting of spores from infected leaves on to the healthy leaves of the test plants proved to be the most convenient and effective method of infection. Artificial screening showed low infestation of powdery mildew on ID-25 (RES-834-3) and other accessions (TX16R, EC-537925) with negligible conidial count (2500 conidia/cm^2) when compared to 1,30,000 conidia/cm^2 in the control (Prathap Reddy et al., 2013). Based on the differential response of the accessions derived from diverse genetic backgrounds, a 0-9 scale for obtaining reliable estimate of the disease has been devised based on the percentage of leaf area as well as the spread of the disease on the plant on different leaves (Prathap Reddy et al., 2013; Sujatha et al., 2015). Crosses were effected with the resistant and susceptible lines and plant-pathogen interaction studies in lines with contrasting reaction were done to understand the mechanism of resistance in different sources.

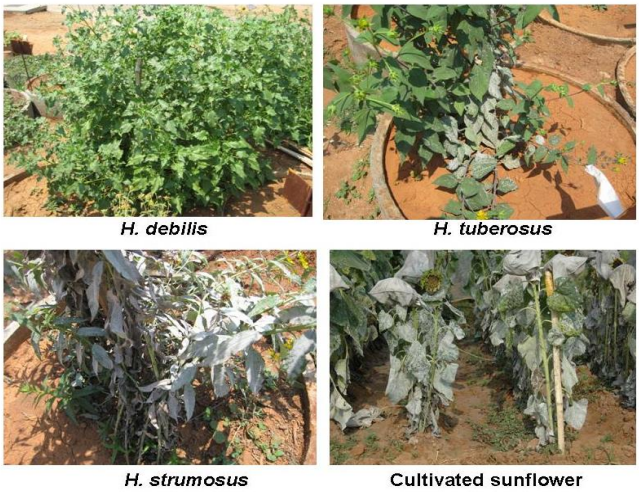


Fig. 1. Reaction of wild sunflowers to powdery mildew

HOST-PATHOGEN INTERACTIONS AND DIFFERENTIAL EXPRESSION OF GENES

The infection process of *G. cichoracearum* was studied in sunflower which included immune/resistant (*H. debilis, H. praecox*), tolerant (RES-834-3, TX16R) and susceptible (Morden, PS 2023A) genotypes both in controlled environment and field conditions. Inoculation was done by dusting the conidia on leaf blades of plants using camel hair brush. At 8, 12, 16, 20, 24, 36, 48, 72 and 96 hours following inoculation, leaves were sampled, cleared and stained. Powdery mildew infection in susceptible (2023B) line was within 8 hrs while spread and infection was slow in TX16R. There was no conidial germination and hyphal growth even after 4 days in *H. debilis* and *H. praecox.* Biochemical analysis of the accumulation of reactive oxygen intermediates (ROIs) such as superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2) was done using ROI-specific dyes such as nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB). This study provided a distinct accumulation pattern during host-pathogen interaction and it was observed that the level accumulation of ROIs was higher in resistant than susceptible genotypes. It is presumed that a higher inducible level of ROIs during infection in resistant lines is responsible for the arrest of the pathogen.

Following preliminary light microscopic and biochemical analysis, the host-pathogen interactions were studied by transcriptome profiling. Leaves of the resistant species- *H. debilis*, *H. praecox*, *H. niveus*, tolerant genotypes- TX16R, USDA-25 and the susceptible genotype - PS 2023B were dusted with the powdery mildew conidia from infected leaves of the susceptible accession (PS 2023B). Infected leaves were fixed at 0 (no infection), 24, 48 and 72 hours post infection (hpi) and subjected to transcriptome profiling. Libraries were prepared using TruSeq RNA library prep kit (Illumina) and were sequenced (PE-2x100) on HiSeq to obtain 80 million reads per sample. Following filtration of organelle genome and non-coding RNA sequences, the cleaned reads were aligned to the reference genome of *H. annuus* cv. Ha-412-HO with a gene model downloaded from Genomics of Sunflower database using Tophat2 tool. Results showed that in each of the donors, the mechanism of resistance varied as evident for the upregulation and downregulation of genes following infection. Maximum number of genes upregulated in response to the pathogen infection was observed in TX16R and *H. praecox* (Table 1).

Table 1: Total up and down regulated genes in transcript level [P value <= 0.01 and FPKM >= 1] found using Cuffdiff analysis

Samples	Up Regulated	Down Regulated
2023_B_Control vs 2023_B_Pool (24,48,72 hpi)	779	335
TX16R_Control vs TX16R_Pool (24,48,72 hpi)	4,464	211
ID25_Control vs ID25_Pool (24,48,72 hpi)	441	723
*H. niveus*_1452_Control vs *H. niveus*_1452_Pool (24,48,72 hpi)	909	263
*H. praecox*_1823_Control vs *H. praecox*_1823_Pool (24,48,72 hpi)	3,818	186
*H. debilis*_Control vs *H. debilis*_Pool (24,48,72 hpi)	308	468
2023_B_Control vs TX16R_Pool (24,48,72 hpi)	677	803
2023_B_Control vs ID25_Pool (24,48,72 hpi)	892	435
2023_B_Control vs *H. niveus*_1452_Pool (24,48,72 hpi)	1,252	797
2023_B_Control vs *H. praecox*_1823_Pool (24,48,72 hpi)	3,824	204
2023_B_Control vs *H. debilis*_Pool (24,48,72 hpi)	677	803

Analysis was done to check the genes which are commonly upregulated and downregulated in the susceptible versus resistant donors, among the resistant lines, and the tolerant lines, which are presented in Fig. 2 and 3, respectively. Only two genes were commonly upregulated in the susceptible and resistant genotypes while no genes were commonly downregulated between the two groups. The tolerant genotypes (TX16R and ID-25) had 14 and 19 genes in common that were upregulated and downregulated, respectively. Venn diagrams showed more common genes between *H. praecox* and *H. niveus* than those between *H. debilis* and *H. niveus.*

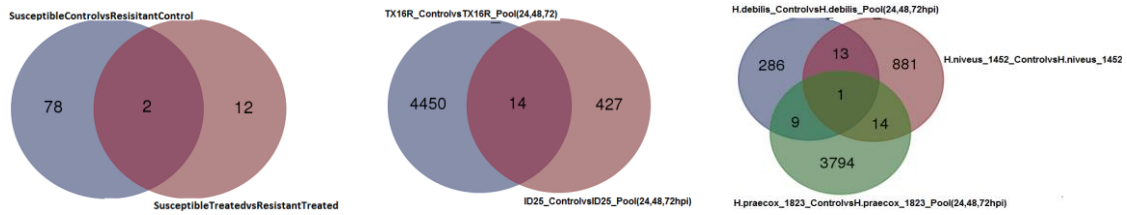


Fig. 2 Venn diagram showing commonly upregulated genes in different groups

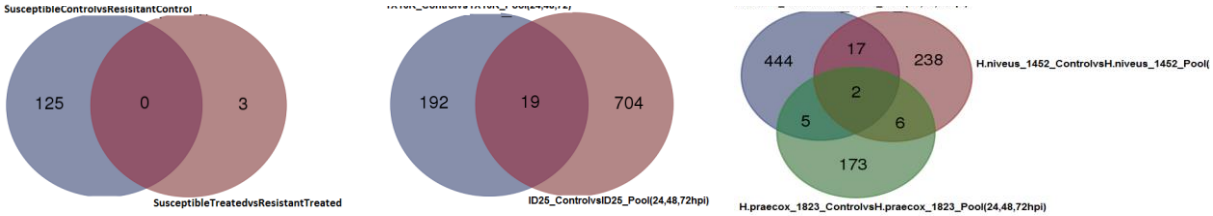


Fig. 3 Venn diagram showing commonly downregulated genes in different groups

Pathway enrichment was performed using Reactome database (Fig. 4). Pathway analysis indicated that the MAPK/MAPK6/MAPK4 signaling cascades are involved in *H. praecox;* Vesicle-mediated transport and membrane trafficking, regulation of HSF1-mediated heat shock response in *H. debilis*, mRNA splicing in TX16R, purine catabolism and detoxification of ROS in the susceptible genotype (PS 2023B).

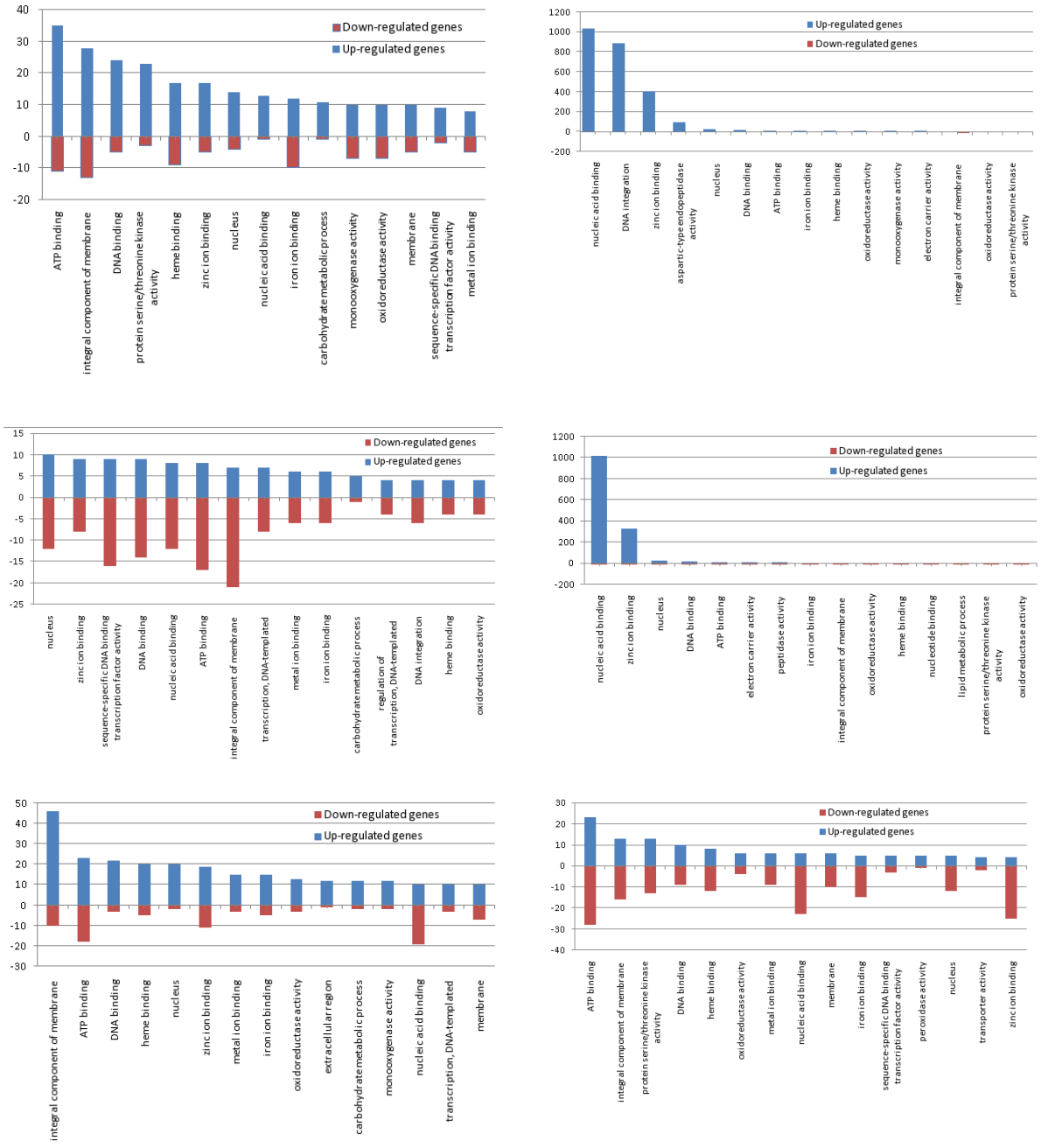


Fig. 4 Ontological analysis of differential expressed genes in PS 2023B, *H. praecox*, *H. debilis*, TX16R, *H. niveus* and ID-25 (in control vs infected)

The transcriptome data was explored for WRKY, Kinases and MAPK in the up and down regulated genes across all the pair-wise combinations. In ABSTRACT, there were 412 genes related to Kinases, 3 MAPK genes and 19 WRKY related genes from both up and down regulation. Work on validation of the key genes for their role in conferring resistance to powdery mildew in sunflower is underway.

TOWARDS MAPPING GENE(S) FOR RESISTANCE TO POWDERY MILDEW

Among the identified sources of resistance to powdery mildew, PI 642072 (TX16R) was selected as resistance source for mapping gene(s) that confer resistance to powdery mildew in sunflower. The F_1s were made by crossing PS 2023A (highly susceptible) and TX16R (resistant to powdery mildew) and further selfed to develop F_2 population. The F_1s showed resistance reaction to powdery mildew infection suggesting dominance nature. Variation for resistance to powdery mildew in F_2 population appeared to be quantitative (did not fit into Mendelian ratios) (Fig. 5) The F_2 population was advanced further by single seed descent method in order to develop recombinant inbred line (F_7-RIL) population for use in mapping of powdery mildew resistance in TX16R.

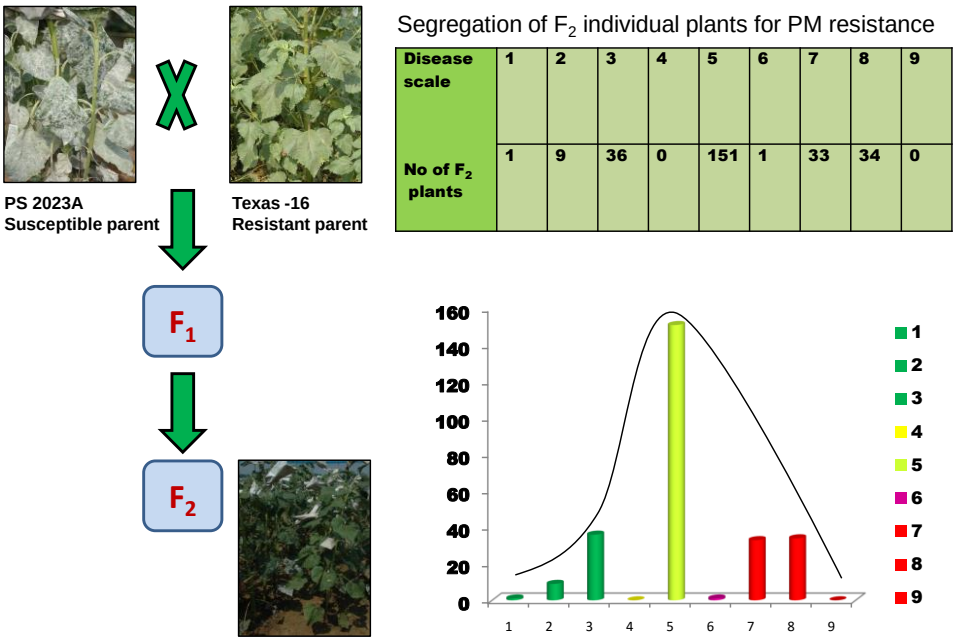


Fig. 5 Inheritance of resistance to powdery mildew in F_2 population produced from the cross: PS2033A x TX16R

Furthermore, interspecific crosses of cultivated sunflower with annual diploid species (*H. argophyllus*, *H. debilis* and *H. praecox*) were also made. The F_1s were confirmed for hybridity using SSR markers (ORS925, ORS505 and ORS898) and characterized for their reaction to powdery mildew. The F_1s involving *H. debilis* and *H. praecox* were highly resistant suggesting the dominance nature of resistance to powdery mildew in these sources. Development of backcross inbred line (BIL) populations is in progress towards mapping of powdery mildew resistance from wild sources. Till date, about 2100 sunflower specific SSR markers are available in public domain (Tang et al., 2002). The transcriptome data generated for the six genotypes has been mined for SSRs and SNPs and the additional markers would be used for trait mapping.

Thus, based on the importance and severity of the disease which is increasing over years and across seasons, the future line of research priorities would include determination of genetics

resistance to powdery mildew in different resistant donors including wild *Helianthus* species, introgression of resistance from the identified resistant donors into promising parental lines and molecular mapping of genes, which would enable marker-assisted selection (MAS) for resistance to powdery mildew in sunflower breeding.

ACKNOWLEDGEMENTS

The authors thank the Director, Indian Institute of Oilseeds Research, Hyderabad for extending all the facilities for carrying out this work. The financial support of the Department of Science and Technology, India for the project (SERB/SR/SO/PS/24/2012) is gratefully acknowledged. The authors are thankful to Dr. Loren Rieseberg, University of British Columbia, Canada for providing access to the sunflower genome data and Dr. Laura Marek, USDA-ARS, Ames, USA for providing the seeds of *Helianthus* species used in the study.

LITERATURE

Anonymous. (2007). *Annual Progress Report of AICRP on sunflower for the year 2006-07*. Directorate of Oilseeds Research, ICAR, Hyderabad, 230pp.

Anonymous. (2014). Annual Report 2013-14. Directorate of Oilseeds Research, Hyderabad.

Bardin, M., Carlier, J., Nicot, P.C. (1999). Genetic differentiation in the French population of *Erysiphe cichoracearum*, a causal agent of powdery mildew of cucurbits. Plant Pathology, *48:* 531-540.

Chen, R.S., Chu, C., Cheng, C.W., Chen, W.Y., Tsay, J.G. (2008). Differentiation of two powdery mildews of sunflower (*Helianthus annuus*) by PCR-mediated method based on ITS sequence. European Journal of Plant Pathology, *121*: 1-8.

Grote, D., Olmos, A., Kofoet, A., Tuset, J.J., Bertolini, E., Cambra, M. (2002). Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested-PCR. European Journal of Plant Pathology, *108:* 197-207.

Gulya, T.J., Woods, D.M., Bell, R., Mancl, M.K. (1991). Diseases of sunflower in California. Plant Disease, *75:* 572–574.

Karuna, K. (2010). Powdery mildew in sunflower. A training programme on screening methodology for major sunflower diseases. 25-26 August 2010, Directorate of Oilseeds Research, Rajendranagar, Hyderabad, India

Prathap Reddy, K., Chander Rao, S., Kirti, P.B., Sujatha, M. (2013). Development of a scoring scale for powdery mildew (*Golovinomyces cichoracearum* (DC.) V.P. Heluta) disease and identification of resistance sources in cultivated and wild sunflowers. Euphytica, *190:* 385-399.

Saliman, M., Yang, S. M., Wilson, L. (1982). Reaction of *Helianthus* species to *Erysiphe cichoracearum*. Plant Disease, *66:* 572–573.

Sujatha, M., Chander Rao, S., Karuna, K., Varaprasad, K.S. (2015). Sunflower Powdery Mildew. Technical Bulletin. 32p. Indian Institute of Oilseeds Research, Hyderabad, India

COMPARISON OF CYTOPLASMIC MALE STERILITY BASED ON PET1 AND PET2 CYTOPLASM IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

***Renate HORN*[1], *Antje REDDEMANN*[1], *Miglena DRUMEVA*[2]**

[1]*Institute of Biological Sciences, Department of Plant Genetics, University of Rostock, Albert-Einstein-Str. 3, 18059 Rostock, Germany*
[2]*Technical University of Varna, Department of Plant Production, Studentska Str. 1, 9010 Varna, Bulgaria*

ABSTRACT

Commercial sunflower hybrid breeding is exclusively based on the PET1 cytoplasm. However, diversity on the level of the cytoplasm is desired to reduce the susceptibility to potential pathogens. The PET2 cytoplasm represents a new CMS source with high potential for hybrid breeding. As the PET1 cytoplasm, CMS PET2 originates from an interspecific cross of *H. petiolaris* with *H. annuus*. However, rearrangements observed in the PET2 cytoplasm are totally different from the PET1 cytoplasm. The PET1 cytoplasm is characterized by the co-expression of *atpA* and *orfH522* and the presence of the CMS-specific 16-kDa-protein, whereas in the PET2 cytoplasm due to a duplication of the *atp9* gene, followed by an insertion of 271 bp of unknown sequences, two new open reading frames *orf288* and *orf231* are created. Both *orf*s, which share homology to the *atp9* gene, are co-transcribed and a clear reduction of this co-transcript can be observed in the anthers of fertility-restored hybrids. The *orf*s encode proteins of 11.1 kDa and 7.9 kDa, respectively. New markers linked to the *Rf1* gene and to the *Rf_PET2* restorer gene have been identified by AFLP analyses and have been developed from BAC-end sequences. Comparative mapping using SSR-markers demonstrated that both restorer genes are located on linkage group 13 close to each other, which facilitates cloning of both restorer genes.
Keywords: Cytoplasmic male sterility, fertility restoration, CMS PET1, CMS PET2, marker

INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited trait in higher plants in which these fail to produce or shed viable pollen (Horn 2006). In most cases mutations in the mitochondrial DNA lead to new open reading frames (ORFs) that encode CMS-specific proteins that interfere with the pollen development, a process that has a high demand on energy supply (Horn et al. 2014). For hybrid breeding, CMS is of special interest because it allows directed crosses when using CMS lines as mother lines. In addition, CMS can be restored by dominant nuclear genes, so called restorer of fertility (*Rf*) genes. This allows the restoration of fertility in F_1 hybrids. Improvements in yield and yield stability of sunflower hybrids require the development of new lines, which are resistant to diseases, e.g. fungal pathogens, or which have improved oil quality. Marker-assisted breeding can accelerate the back-cross programs and is especially useful if the trait like fertility restoration can only be assessed as late as the flowering stage, a rather advanced period in the plant development (Neuhaus & Horn 2004). Therefore the development of co-dominant markers closely linked to the fertility restorer gene is of great importance for sunflower breeding programs. In addition, the isolation of the fertility restorer gene is of general interest to the research community to understand the molecular mechanism behind fertility restoration (Horn 2006).

The development of co-dominant markers closely linked to the locus of the fertility restorer gene *Rf1* would present a great improvement for marker-assisted breeding in sunflower hybrid production. The selection for the presence of the restorer gene *Rf1* (homozygous or heterozygous) could be performed at a very early stage of plant development.
Worldwide, only one CMS source, the so called PET1 cytoplasm, has been used for commercial sunflower hybrid production, so far (Nichterlein & Horn 2005). This CMS source is the result of an interspecific cross between *Helianthus petiolaris* and *H. annuus* (Leclerq 1969). The PET1 cytoplasm is characterized by the *orfH522* that encodes for a 16-kDa-protein (Horn et al. 1991, Köhler et al. 1991, Laver et al. 1991). However, more than 70 CMS sources have been described for sunflower (Serieys 2005), but only about half of them have been analyzed for the molecular mechanisms leading to CMS (de la Canal 2001, Horn 2002, Horn & Friedt 1999, Horn et al. 2002). However, diversity on the cytoplasm side is desired to avoid the pathogen specialization as the one observed in the maize T-cytoplasm (Miller & Koeppe 1971), exclusively used up to then in maize hybrid production. In sunflower, the PET2 cytoplasm, which was also derived from an interspecific cross of *Helianthus petiolaris* and *H. annuus* (Whelan & Dedio 1980), might be an interesting alternative (Horn & Friedt 1997). However, molecular characterization of the PET2 cytoplasm as well as markers for the restorer gene *Rf_PET2* would be required.
Markers linked to the restorer gene *Rf1*, responsible for fertility restoration of hybrids based on the PET1 cytoplasm, have been identified (Horn et al. 2003, Kusterer et al. 2005). The restorer gene has been placed on linkage group 13 of the sunflower reference map using SSR-markers (Kusterer et al. 2005). In order to isolate the restorer gene *Rf1* by a map-based cloning approach (Kusterer et al. 2004 a, b) a bacterial artificial chromosome (BAC) library has been constructed for the restorer line RHA325 (Özdemir et al. 2002, 2004). Markers were hybridised against high density BAC filters of two BAC libraries (RHA325 and HA383) to identify positive BAC clones. Using BAC fingerprinting, cloning and sequencing of BAC ends, the BAC clones were organized into contigs around the restorer gene *Rf1* (Hamrit et al. 2008, Hamrit 2009).
All markers linked to the restorer gene *Rf1* described up to now are dominant markers, which do not allow distinguishing the homozygous from the heterozygous fertile plants. Here we present the development of a co-dominant CAPS-marker linked to the *Rf1* gene. In addition, the molecular mechanism behind the PET2 cytoplasm will be elucidated as well as the close location of the restorer genes *Rf1* and *Rf_PET2* on linkage 13 by comparative mapping.

MATERIALS AND METHODS

Plant material

HA89 (maintainer line of CMS PET1 and CMS PET2) was used to study the male fertile cytoplasm, CMS line PET2 (Whelan and Dedio, 1980) maintained by RHA265, which is a restorer line of CMS PET1, and CMS line PET1 (Leclercq, 1969), maintained with HA89 and the fertility-restored hybrid PET2 (RHA265) x IH-51 were used for comparing CMS PET1 and CMS PET2 source. Mapping of the *Rf_PET2* was performed in the F2 population RHA265(PET2) x IH-51.
The investigations on the restorer gene *Rf1* were performed using the fertility restorer line RHA325, homozygous for the dominant *Rf1* allele, the fertility maintainer line HA342, homozygous for the recessive allele *rf1*, and bulks of the F2 population of the cross HA342 x RHA325. Each of the F2 bulks consisted of 10 individuals from the F2 population, which were either homozygous for the recessive allele of the fertility restorer gene (S1 and S2) or for the dominant allele (R1 and R2). Total genomic DNA was extracted from leaves according to the protocol of Doyle & Doyle (1987).

Cloning and sequencing mitochondrial DNA

Mitochondrial DNA was isolated using the procedure of Köhler et al. (1991). The *HindIII* digested mtDNA was blotted on Hybond N+ membrane (GE Healthcare) after separation on a 0.8 % agarose gel. Hybridizations with *atp9* as probe were performed according to the manufacturer's instructions using ECL Direct™ Nucleic Acid Labeling and Detection System (GE Healthcare) for the detection of restriction polymorphisms. The *Hin*dIII digested mtDNA fragments were cloned into pUC18 vector and the resulting recombinant plasmids were used to prepare a mitochondrial DNA library. Positive clones were selected by *Hin*dIII hybridization pattern with *atp9* as probe and sequenced.

Analysis and use of BAC-end sequences

Eleven positive bacterial clones (BAC-end sequences) were included in the investigations, which had been identified by hybridizations with markers linked to the restorer gene *Rf1* (Hamrit 2009) on the basis of the two available BAC-libraries in sunflower (Özdemir et al. 2004; Clemson University Genomics Institute-http://www.genome.clemson.edu). The BAC ends had been sequenced using SP6 and T7 primers. The BAC-end sequences of 100L22, 94F15, 147A3, 67N4, 261F19, 126N19, 447N6, 59J13, 450J13, 450B06, 480G04 and 139A17 were included in this study. The software BioEdit 7 was used for processing and analysis of the BAC-end sequences with the aim to develop STS (Sequence-Tagged-Site)-markers for back-mapping of these BAC clones in the population. The design of STS-primers was carried out by the program Web primer (http://www.yeastgenome.org/cgi-bin/web-primer). The design of CAPS (Cleaved Amplified Polymorphic Sequence) markers was realized with the help of the program NEB cutter V2.0 (http://tools.neb.com/NEBcutter2/).

PCR amplification of STS- and CAPS-markers

PCR amplification with STS-primers was performed with 15 ng DNA in the PCR cycler 2700 under the following conditions: 3 min denaturation at 94°C, followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing using different temperatures depending on the primers (range between 55°C and 64°C), 30 sec polymerization at 72°C of polymerization; followed by a final 7 min period of elongation at 72°C. For CAPS-marker 480G04_BsrGI, the PCR products were directly digested with the restriction endonuclease BsrGI. The amplified products were separated by 2 % agarose gel electrophoresis at 100 V for 35 minutes. The PCR-products were visualized with ethidium bromide solution.

Cloning and sequencing of PCR products

PCR products were cloned using the pGEM®-T Easy vector (Promega). After minipreparation, plasmids were sequenced using T7 and SP6 primers.

AFLP and SSR analyses

Amplified fragment length polymorphism (AFLP) analyses were done as described by Vos et al. (1995). For the *Rf1* gene, three new preamplifications were used: (1) E02 and M01, (2) E02 and M02 and (3) E02 and M04. For the selective amplifications, 16 *Eco*RI primers were combined with 48 *MseI* primers. For the *Rf_PET2* gene, AFLP analyses were performed based on the preamplification of E01 and M02 as primers. For the selective amplification, 16 *Eco*RI primers (E31 to E46) and 16 *Mse*I primers (M47 to M62) were combined.

SSR analyses were performed as described in Sajer et al. (2013). Using the SSR primer combinations for ORS317, ORS630 and ORS1030 of linkage group 13 (Tang et al. 2005) and

the M13tailing procedure (Oetting et al. 1995) PCR products were labelled with IRD800 and separated on the DNA Analyzer 4300 (LI-COR, Biosciences).

RESULTS AND DISCUSSION

Molecular characterization of the PET2 cytoplasm

Comparing the Southern hybridization pattern of mitochondrial DNA (*HindIII* digested) from the PET2 cytoplasm and the male fertile cytoplasm a restriction polymorphism was detected using the *atp9* gene as probe. One fragment of 3.4 kb was identical in the male fertile cytoplasm and CMS PET2, but an additional fragment of 4.1 kb was only present in CMS PET2. Cloning and sequencing of the *HindIII* fragments showed that the 3.4-kb-fragment contained the regular copy of the *atp9* gene, whereas the PET2-specific 4.1-kb-fragment contained a split second copy of *atp9*, which resulted in two new open reading frames of 228 bp and 231 bp (Figure 1). Both *orf*s show partial homology to *atp9*. The insertion of 271 bp splitting the *atp9* represents sequences of unknown origin. The new *orf*s encode proteins of 11.1 kDa and 7.9 kDa, respectively. RT-PCR analyses showed that the two PET2-specific *orfs* are co-transcribed and that the co-transcript is specifically reduced in the anthers of fertility-restored hybrids. Mitochondrial genes of the F1F0 ATP synthase are the most frequent genes involved in creating cytoplasmic male sterility (Horn et al. 2014). In sunflower, one other CMS source, the PEF1 cytoplasm, which originates from an interspecific cross of *H. petiolaris* ssp. *fallax* and *H. annuus* (Serieys & Vincourt 1987), also showed changes in the *atp9* gene, here a 0.5-kb-insertion in the 3'-UTR, associated with the male sterility phenotype (de la Canal et al. 2001).

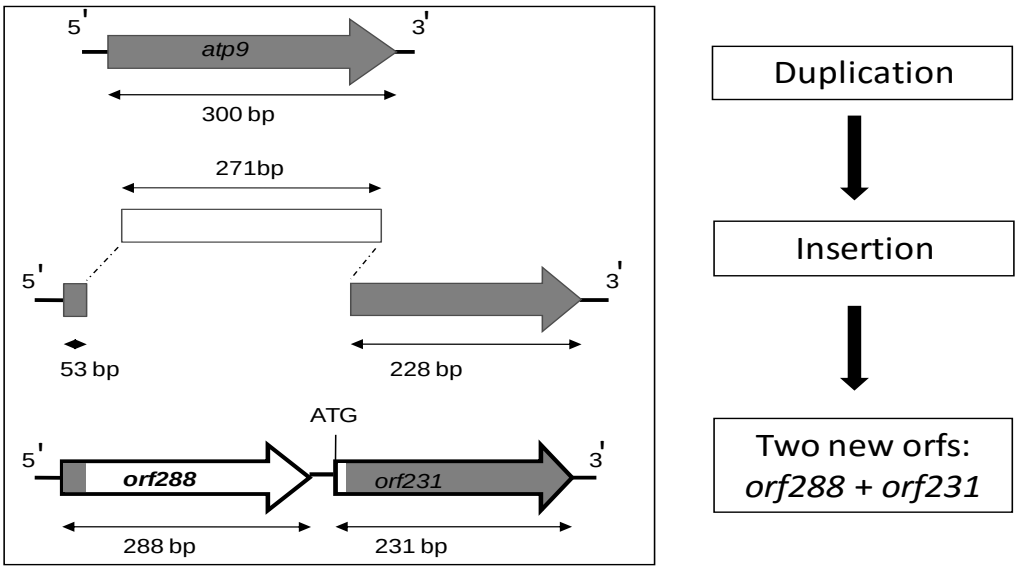


Figure 1: Model of the recombination events

Even though both CMS cytoplasm PET1 and PET2 were derived from an interspecific cross between *H. petiolaris* and *H. annuus*, the mechanism behind the male sterility of CMS PET2 is totally different than in the CMS PET1 cytoplasm, which makes it interesting to use the CMS PET2 cytoplasm for commercial sunflower hybrids. Now that the mechanism is known primers specifically differentiating the two CMS sources can be developed.

First round of developing of STS-markers for *Rf1* from BAC-end sequences

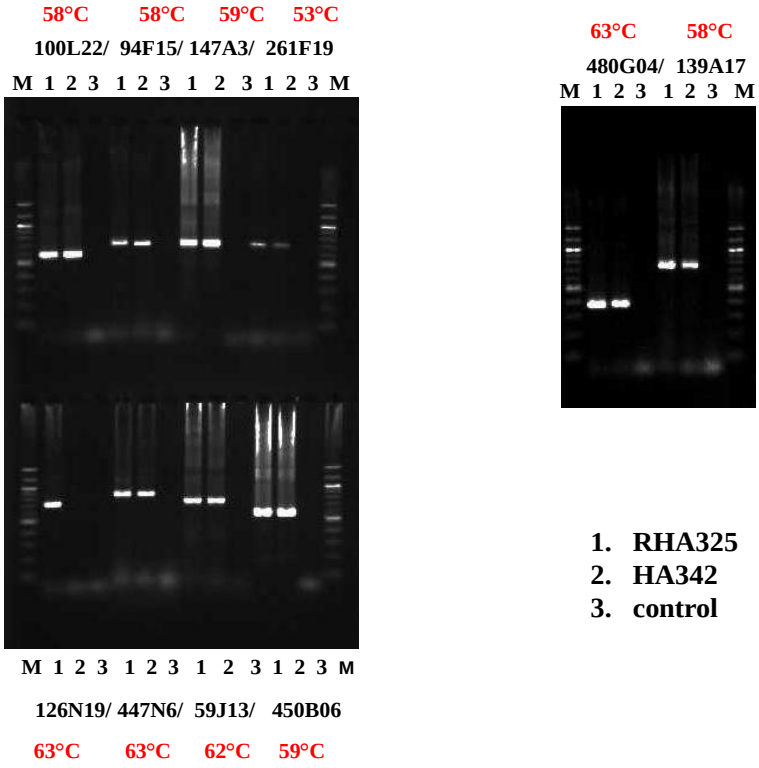
Based on the positive BAC-clones identified during the process of preliminary chromosome walking at the *Rf1* locus (Hamrit 2009), 11 STS-primer combinations were derived from the BAC-end sequences (Table 1). With the aim to improve the quality of the amplified products with the respective primers, a series of PCR-reactions with different annealing temperatures were tested. The optimal parameters for PCR-amplification for each of the investigated primers are given in Table 1.

Table 1: Design of STS-primers from the BAC-end sequences for back mapping the identified positive BACs

BAC-end	Primer name	Primer sequences 5' - 3'	T_A	Expected size of PCR product	Observed size of PCR product	Mono-/ Polymorph
100L22	100L22_for	GAACTTGCTAAATGTTAACGAG	58	573 bp	578 bp	monomorph
	100L22_rev	ATGCAAAAACCGCCTAAG				
94F15	94F15_for	TTAGTCGCCATGTGTACCGAT	58	712 bp	716 bp	monomorph
	94F15_rev	CCACTTTCGATGATGGAGTTG				
147A3	147A3_for	GTTATGCCCGATATCGTAAT	59	702 bp	709 bp	monomorph
	147A3_rev	ACCATTTTAAGTCCCGTAAG				
67N4	67N4_for	TTTCTTGTGTTTTACGATGCC	52-55	714 bp	-	-
	67N4_rev	TGTAACCGTCCGGAACAAAA				
261F19	261F19_for	ACCAAAAGGATCTAGAACTG	53	708 bp	717 bp	monomorph
	261F19_rev	CATTTTAAGGTCATATGGGC				
126N19	126N19_for	ACGCTGTGGCAATAAGACACA	63	701 bp	707 bp	monomorph
	126N19_rev	ACTTTGCAATTGTCACCAAAA				
447N6	447N6_for	TTCATGCTTTTAGCTGCCTGT	63	879 bp	884 bp	monomorph
	447N6_rev	TGCAGTTTAACTGCCCAAGA				
59J13	59J13_for	GCTTCTTGTGCTTCTTTTAAC	62	726 bp	726 bp	monomorph
	59J13_rev	TATCATGACGCTATCGGTTG				
450B06	450B06_for	AGCAGATTGTCAATCGGACAG	59	560 bp	564 bp	monomorph
	450B06_rev	GCTGAAAGATGAGCATCCAA				
480G04	480G04_for	GGTTCACATGGTGTGGATAA	63	361 bp	365 bp	monomorph
	480G04_rev	CTTCAATCAGACATCTATAGAGA				
139A17	139A17_for	GTAACGACTAGCAGGCAATAACA	58	717 bp	720 bp	monomorph
	139A17_rev	TGCGGACGTGAAATAGG				

The primers were tested with the parental lines RHA325 and HA342 to detect polymorphisms that would allow using them directly as markers. The PCR products showed the expected sizes (Table 1), but all showed monomorphic patterns between RHA325 and HA342 (Figure 2). The primers derived from 67N4 and 126N19 did not result in good PCR amplification products.

Figure 2: PCR products using the STS-primers derived from the BAC-end sequences for amplification from the lines RHA325 and HA342



Development of STS- and CAPS-markers for *Rf1* from cloned PCR-products

As the PCR amplification products with the derived STS-primers were monomorph for the parental lines (RHA325 and HA342) the PCR-products were cloned into the pGEM-T vector and sequenced to detect single nucleotide polymorphism (SNP) between the two lines that can be used to develop markers. Positive clones were obtained from the PCR products of the primers derived from the BAC-end sequences of 100L22, 147A3, 480G04, 450B06, 139A17, 59J13, 94F15. On the basis of the sequences analyses, variations in the nucleotide sequence of the investigated DNA fragments from RHA325 and HA342 were found (data not shown); these will be used to design specific polymorphic STS-markers and CAPS-markers. First results are shown for 480G04 (Table 2).

For the BAC clone 480G04, both types of markers could be designed. Based on variations in the 480G04 sequences of the two parental lines RHA325 and HA342, the specific STS-primers 480G04_RH325 and 480G04_HA342 were developed, which combined with the 480G04_rev primer specifically amplified a PCR product from RHA325 or HA342, respectively. Testing the primers in the bulks confirmed the association of the polymorphisms with fertility restoration (data not shown). However, these markers are again dominant.

More interesting was the development of a co-dominant marker based on a SNP in the restriction site of *BsrGI* between RHA325 and HA342. Whereas the PCR product of 361 bp of RHA325 (480G04_for/480G04_rev primer combination) is cut into a 138-bp-fragment and a 223-bp-fragment, the PCR product of HA342 remains uncut (Figure 3). The use of this CAPS-marker in the bulks showed its linkage to the fertility restorer gene. However, this marker still needs to be mapped to the restorer gene *Rf1* in the F2-population. This co-dominant marker will be very helpful in distinguishing plants, heterozygous or homozygous for the restorer gene *Rf1*.

Table 2: Development of STS- and CAPS-markers from cloned sequences

BAC-clone	STS-marker				
	Primer name	Forward primer sequences 5'-3' (reverse as before)	Expected size (Line)	Observed size (Line)	Monomorph/ Polymorph
480G04	480G04_ HA342	TTTGTGGGCTTCGTTTAATCGG	278 bp (HA342)	278 bp (HA342)	Polymorph (dominant)
480G04	480G04_ RHA325	TTTGTGGGCTTCGTTTAATAAC	277 bp (RHA325)	277 bp (RHA325)	Polymorph (dominant)
BAC-clone	CAPS-marker				
	Restriction enzyme	Cutting sequence	Expected size (Line)	Observed size (Line)	Monomorph/ Polymorph
480G04	BsrGI	5′…T˘GTACA…3′ 3′…ACATG˘T…5′	138 bp/223 bp (RHA 325) 361 bp (HA342)	138 bp/223 bp (RHA 325) 361 bp (HA342)	Polymorph (co-dominant)

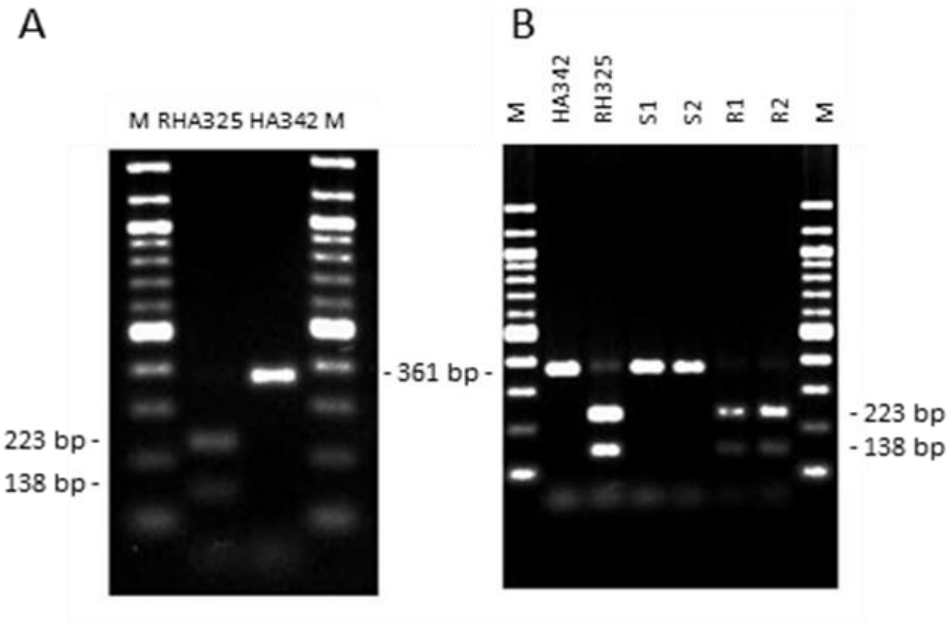


Figure 3: CAPS-marker 480G04_BsrGI. DNA was amplified using the primer combination 480G04_for/480G04_rev and digested with the restriction endonuclease *BsrGI*. A. parental lines (RHA325 and HA342) and B. RHA352 and HA342 as well as bulks (S1, S2, R1, R2). M: 100 bp marker

Comparative mapping of the *Rf1* and *Rf_PET2* gene

New AFLP-markers linked to the restorer gene *Rf1* have been identified as well as first AFLP-markers closely mapping to the *Rf_PET2* gene. Some of these have been cloned and sequenced.

The restorer gene *Rf1* had been mapped to the linkage group 13 (Kusterer et al. 2005) using SSR-markers of the general genetic map of sunflower (Tang et al. 2003). In order to see if the *Rf_PET2* restorer gene might be also located on the same linkage group, three SSR markers (ORS317, ORS630 and ORS1030) bordering the *Rf1* gene were mapped in the F2 population RHA265(PET2) x IH-51 segregating for the *Rf_PET2* gene. Comparative mapping showed that *Rf_PET2* maps less than 10 cM from the *Rf1* gene. This will facilitate cloning of the two restorer genes. Comparing the mechanism of fertility restoration from *Rf1* and *Rf_PET2* will give a better understanding about the processes behind fertility restoration in sunflower.

Conclusions

The molecular basis of the new CMS source PET2 was revealed, which now allows the development of markers differentiating between CMS PET1, CMS PET2 and the fertile cytoplasm. In combination with the identification of AFLP-markers linked to the restorer gene *Rf_PET2* this new CMS source would be now ready to be used for the development of commercial sunflower hybrids.

In addition, a step forward has been made in chromosome walking at the fertility restorer locus *Rf1*. The BAC-end sequences can be used to obtain new overgo probes for subsequent hybridizations against BAC-filters for identification of new positive BAC-clones. However, BAC clones have still to be back-mapped by markers. Their arrangement in contigs will ultimately result in a complete contig around the *Rf1* gene. The newly identified AFLP-markers for the *Rf1* gene will be helpful for this work as well. All markers described up to now have been dominant markers, which do not allow differentiation between homozygous and heterozygous fertile plants. Therefore, the development of the co-dominant CAPS-marker 480G04_BsrG I for the fertility restorer gene *Rf1* is especially interesting. Both, the two new STS-markers as well as the CAPS-marker will be used to back-map the BAC-clone 480G04 in the F2-population and to confirm its position in the region of gene *Rf1*. Interestingly, the restorer gene *Rf_PET2* seems to be located less than 10 cM from the *Rf1* gene.

ACKNOWLEDGEMENTS

We like to thank Prof. Dr. Wolfgang Friedt, University of Giessen, for providing the seeds and plant material used in this study as well as for the field studies in Groß-Gerau near Frankfurt am Main, Germany.

LITERATURE

de la Canal L., Crouzillat D., Quetier F., Ledoigt G. (2001) A transcriptional alteration on the *atp9* gene is associated with a sunflower male-sterile cytoplasm. Theoretical and Applied Genetics 102: 1185-1189

Doyle J.J., Doyle J.L. (1990) Isolation of plant DNA from fresh tissue. Focus 12:13-15

Hamrit S. (2009) Chromosome walking at the fertility restorer locus *Rf1* in sunflower (*Helianthus annuus* L.) PhD thesis, University of Rostock, Institute of Biological Sciences, Department of Plant Genetics, pp. 154

Hamrit S., Kusterer B., Friedt W., Horn R. (2008) Verification of positive BAC clones near the *Rf1* gene restoring pollen fertility in the presence of the PET1 cytoplasm in sunflower (*Helianthus annuus* L.) and direct isolation of BAC ends. In: Proceedings of the 17th International Sunflower Conference, Cordoba, Spain, 8.-12.06.2008

Horn R. (2002) Molecular diversity of male sterility inducing and male-fertile cytoplasms in the genus *Helianthus*. Theoretical and Applied Genetics 104: 562-570

Horn R. (2006) Cytoplasmic male sterility and fertility restoration in higher plants. Progress in Botany 67: 31-52

Horn R., Friedt W. (1997) Fertility restoration of new CMS sources in sunflower. Plant Breeding 116: 317-322

Horn R., Friedt, W. (1999) CMS sources in sunflower: different origin but same mechanism? Theoretical and Applied Genetics 98: 195-201

Horn R., Gupta K.J., Colombo N. (2014) *Mitochondrion role in molecular basis of cytoplasmic male sterility. Mitochondrion 19: 198-205*

Horn R., Kohler R.H., Zetsche K. (1991) A mitochondrial 16 kDa protein is associated with cytoplasmic male sterility in sunflower. Plant Molecular Biology 17: 29-36

Horn R., Kusterer B., Lazarescu E., Prüfe M., Özdemir N., Friedt W. (2002) Molecular diversity of CMS sources and fertility restoration in the genus *Helianthus*. Helia 25 (36):29-40

Horn R., Kusterer B., Lazarescu E., Prüfe M., Friedt W. (2003) Molecular mapping of the *Rf1* gene restoring pollen fertility in PET1-based F_1 hybrids in sunflower (*Helianthus annuus* L.). Theoretical and Applied Genetics 106: 599-606

Köhler R.H., Horn R., Lössl A., Zetsche, K. (1991) Cytoplasmic male sterility in sunflower is correlated with the co-transcription of a new open reading frame with the *atpA* gene. Molecular and General Genetics 227: 369-376

Laver H.K., Reynolds S.J., Moneger F., Leaver C.J. (1991) Mitochondrial genome organization and expression associated with cytoplasmic male sterility in sunflower (*Helianthus annuus*). Plant Journal 1: 185-193

Leclercq P. (1969) Une sterilite male chez le tournesol. Annales de l'Amelioration des Plantes 19: 99-106

Kusterer B., Horn R., Friedt W. (2005) Molecular mapping of the fertility restoration locus *Rf1* in sunflower and development of diagnostic markers for the restorer gene. Euphytica 143: 35-43

Kusterer B., Rozynek B., Brahm L., Prüfe M., Tzigos S., Horn R., Friedt W. (2004a) Construction of a genetic map and localization of major traits in sunflower (*Helianthus annuus* L.). Helia 27: 15-23

Kusterer B., Friedt W., Lazarescu L., Prüfe M., Özdemir N., Tzigos S., Horn R. (2004b) Map-based cloning strategy for isolating the restorer gene *Rf1* of the PET1 cytoplasm in sunflower (*Helianthus annuus* L.). Helia 27: 1-14

Miller R.J., Koeppe D.E. (1971) Southern corn leaf blight: susceptible and resistant mitochondria. Science 173(3991): 67-9

Neuhaus G., Horn R. (2004) Implications of single nucleotide polymorphisms for plant breeding. Progress in Botany 65: 55-71

Nichterlein K., Horn R. (2005) Haploids in the improvement of Linaceae and Asteraceae. In: Biotechnology in Agriculture and Forestry: Haploids in Crop Improvement II, Palmer D., Keller W., Kashal K. (eds), Springer Verlag, Vol 56: 277-294

Oetting W.S., Lee H.K., Flanders D.J., Wiesner G.L., Sellers T.A., King R.A. (1995) Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. Genomics 30: 450-458

Özdemir N., Horn R., Friedt W. (2002) Isolation of HMW DNA from sunflower (*Helianthus annuus* L.) for BAC cloning. Plant Molecular Biology Reporter 20: 239-249

Özdemir N., Horn R., Friedt W. (2004) Construction and characterization of a BAC library for sunflower (*Helianthus annuus* L.). Euphytica 138: 177-183

Sajer O., Scorza R., Dardick C., Zhebentyayeva T., Abbott A.G., Horn R. (2012) Development of sequence-tagged site markers linked to the pillar growth type in peach [*Prunus persica* L. (Batsch)]. Plant Breeding 131: 186-192

Serieys H., Vincourt P. (1987) Characterization of some new cytoplasmic male sterility sources from Helianthus genus. Helia 10: 9-13

Serieys H. (2005) Identification, study and utilisation in breeding programs of new CMS sources. FAO Subnetwork; Proc. 2005 Sunflower Subnetwork Progress Report, 17-20 July 2005; Rome Italy: FAO, p. 47-53

Tang S.X., Kishore V.K., Knapp S.J. (2003) PCR-multiplexes for a genome-wide framework of simple sequence repeat marker loci in cultivated sunflower. Theoretical and Applied Genetics 107: 6-19

Vos P., Hogers R., Bleeker M., Reijans M., Lee T.V., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. (1995) AFLP: A new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407–4414

Whelan E.D.P., Dedio W. (1980) Registration of sunflower germplasm composite crosses CMG-1, CMG-2, and CMG-3. Crop Science 20: 832

IDENTIFICATION OF *HADELLA, HAGID1* AS WELL AS *HASLEEPY* AND *HASNEEZY* GENES INVOLVED IN GIBBERELLIN SIGNALING IN SUNFLOWER

Ralph EWALD, Nina GEHM, Lisa POPIOLKOWSKI, Amira ANTELMANN, Renate HORN[1]

[1]*Institute of Biological Sciences, Department of Plant Genetics, University of Rostock, Albert-Einstein-Str. 3, D-18059 Rostock, Germany,*

renate.horn@uni-rostock.de

ABSTRACT

Mutations in the gibberellin (GA) biosynthesis or signaling pathway lead to significant changes in shoot growth (dwarfism/gigantism) and were responsible for the so-called 'green revolution' in cereals. Knowledge about the GA metabolism offers starting-points for targeted breeding in sunflower. However, so far we know little about the components of the GA signaling pathway in sunflower. Using the sunflower genome sequence database, kindly provided by the Loren Rieseberg group (www.heliagene.org), we screened for similar sequences to known DELLA, GID1 and SLEEPY proteins from other plant species. Next, identified sequences of the HA-412-HO scaffold database were used for primer design and the existence of the genes was verified in the line HA383 using DNA as well as cDNA. Here we describe the identification and characterization of four DELLA (HaDELLA1-2 and HaDELLA-like1-2), five HaGID1A-E and nine F-box genes (HaSLEEPY1A-F and HaSNEEZY1-3). For functional analysis, we studied sunflower SLEEPY and DELLA homologs in Arabidopsis thaliana. Our complementation studies of the Arabidopsis SLEEPY mutant sly1-10 demonstrated that HaSLY1A encodes a functional F-box protein, which is fully able to compensate the sly1-10 mutant phenotype. Overexpression of HaDELLA1 in wild-type Arabidopsis induced dwarfism and late-flowering.

Keywords: Gibberellins, *Helianthus annuus*, plant hormones, signal transduction

INTRODUCTION

Three major regulators are responsible for perception of GAs by the GA signaling pathway: the GA receptor GID1 (GA-insensitive dwarf1), the DELLA protein and SLY1/GID2 (SLEPPY1/GA-insensitive dwarf2) as part of the SCF E3 ubiquitin ligase. GID1 was characterized as soluble receptor for GA by studies of GA-insensitive dwarf rice mutants (Ueguchi-Tanaka et al., 2005). In Arabidopsis, three GID1 encoding genes (AtGID1A, AtGID1B and AtGID1C) were identified (Nakajima et al., 2006). Analyses of the gid1 double and triple mutants as well as interacting studies with DELLA proteins revealed that GID1 orthologs have redundant but also distinct roles in plant development (Iuchi et al., 2007). In contrast, there is very little knowledge available about GID1 encoding genes in sunflower. Recently, Blackman et al. (2011) has published one partial GID1-like sequence (about 37%, HaGID1B), which shows high similarities to GID1B (At3g63010) in Arabidopsis. However, transcriptome analysis with the focus on GA synthesis and signaling genes of Gerbera hybrida indicate that species of the compositae family contain various homologous GID1 genes (Kuang et al., 2013). In Gerbera, nine different GID1 transcripts were detected and in H. annuus three corresponding hits were found by local BLASTN indicating the existence of more than one GID1 gene in sunflower.

DELLA proteins are nuclear transcriptional regulators that repress the GA signaling pathway and belong to the GRAS gene family (Tian et al., 2004). Several plant species like rice (SLR1) and tomato (Pro) contain only a single gene encoding for DELLA (Ikeda et al., 2001; Jasinski et al., 2008). In contrast, other species like A. thaliana have multiple DELLA genes (Tyler et al., 2004). Ramos et al. (2013) have recently reported that the semidominant mutant sunflower allele, Rht1, which maps to linkage group 12 of the sunflower public consensus map, completely cosegregated with a haplotype of HaDELLA1. Phenotypic effects of this allele include shorter height and internode length, insensibility to exogenous gibberellin application, normal skotomorphogenetic response, and reduced seed set under self-pollinating conditions (Ramos et al., 2013). In addition to the known HaDELLA1 sequence similar EST sequences provide initial indications of a second HaDELLA gene (Blackman et al., 2011), but it remains to be determined whether that C-terminal sequence is part of a true DELLA protein or belongs to another member of the large GRAS gene family.

The F-box protein GID2/SLY1 interacts with DELLA proteins, especially in the presence of GA activated GID1, via the C-terminal GGF and LSL domains (Sun, 2008). The N-terminal F-box domain is necessary for the interaction with the SCF complex (Smalle & Vierstra, 2004). However, the molecular mechanisms behind GA signaling have been extensively studied in plants such as Arabidopsis and rice, which have only a single gene encoding SLY1/GID2. In sunflower, just one putative SLY1 sequence (HaSLY1) has been described so far (Blackman et al., 2011), but it seems to be very likely that sunflower, like Gerbera hybrida with six putative GID2/SLY1 transcripts (Kuang et al., 2013), will have more than one SLY1 homolog.

MATERIAL AND METHODS

Screening of the sunflower genome for GID1, DELLA and SLEEPY homologs

The genome database of the sunflower line HA-412-HO provided by Loren Rieseberg (www.heliagene.org) was screened for similar sequences to known GID1, DELLA1 and SLY1 sequences of Arabidopsis thaliana. Therefore the 3.1 GB scaffold fasta file (Celera_14libs_sspace2_ext.final.scaffolds) was converted into a local nucleotide database by using the stand alone blast program Blaststation-Local (www.blaststation.com). We also used published sequences of GA signaling genes of Lactuca sativa (Sawada et al., 2008) due to its close relationship to sunflower as well as previously published EST sequences of putative sunflower genes involved in GA signaling (Blackman et al., 2011).

In silico analyses of sunflower GA signaling homologs

To compare the hits of the scaffold database showing highest E-values with GID1, DELLA or SLY1 proteins we performed phylogenetic analyses. Therefore, we choose GID1, DELLA and SLY1 homologs of sequenced higher land plant genomes, which were described in the literature or detected by BLASTP and TBLASTN tools of the databases NCBI (National Center for Biotechnology Information) and Phytozome (http://www.phytozome.org). We also included amino acid sequences of Selaginella moellendorffii (lycophyte) as well as homologous protein sequences of Physcomitrella patens (moss). Several studies dealt with the subject of evolutionary origin of GA signaling. These studies indicate that the GA signaling evolved after the divergence of bryophytes from land plants (Wang et al., 2015). Furthermore, it was demonstrated that homologs of GID1, DELLA, and SLY1 work similar in S. moellendorffii and in flowering plants, whereas no evidences were found for functional conservation of genes in P. patens (Hirano et al., 2007). For this reason, sequences of P. patens were used as outgroup, whereas S.

moellendorffii sequences served as exclusion limits to eliminate similar but unrelated sequence, like other GRAS or F-box proteins. The "one click" mode of Phylogeny.fr (Dereeper et al., 2008) offered a quick and easy method for handling large volumes of sequences and was used to get a first impression how much of the hits really belonged to the GA signaling members. Next, Neighbor-joining trees were computed using the JTT matrix-based method and bootstrap test of 1000 replicates to confirm the evolutionary distance.

Verification of GA signaling scaffold sequences in HA383

Based on scaffold sequences forward and reverse primers were designed encompassing the respective coding sequence of GID1, DELLA and SLY1 homologs. Genomic DNA from leaves of HA383 was subjected to standard PCR (Taq DNA polymerase NEB). The obtained fragments were cloned in pGEM®-T Easy vector (Promega) and sequenced using SP6 and T7 primers designed for sequencing inserts cloned into this vector. Nucleotide polymorphisms were verified by using a proof reading Phusion DNA polymerase (Thermo Scientific). To get accurate information on the exon-intron structure of the genes, RNA from HA383 was isolated according to the protocol for RNA extraction from different tissues of grapevine and other woody plants (Gambino et al., 2008). All RNA samples have undergone a DNAse treatment (DNAse I; Fermentas) to remove DNA contaminations. Complementary DNA (cDNA) was obtained from total RNA by using RevertAid H minus cDNA synthesis kit (MBI Fermentas) and used for amplification of coding sequences.

Functional analyses of the sunflower SLEEPY and DELLA homologs HaSLY1A and HaDELLA1

PCR amplicons of HaSLY1A and HaDELLA1 coding sequences flanked by restriction sites were ligated into corresponding cloning sites of the 35S cassette. The entire 35S cassettes, now including cds of HaSLY1A or HaDELLA1, were excised and inserted into the vector pGREEN0029 via the EcoRV restriction site (http://www.pgreen.ac.uk). These vectors were used to transform Arabidopsis mutant sly1-10 or Landsberg erecta wild-type plants by using Agrobacterium tumefaciens strain GV3101psoup. After selection with BASTA (phosphinotricine, 0.1%), integration of the 35S:HaSLY1A or 35S:HaDELLA1 transgene was confirmed by PCR using a primer specific for the 35S promoter and reverse primers for the respective coding sequences (cds). In the screen for HaSLY1A complemented plants that were homozygous for sly1-10, we used the specific primer pairs sly1-10f/2-63r and sly1-10f/sly1-10r2 for PCR with genomic DNA to verify the absence of the wild-type Arabidopsis AtSLY1 gene and the presence of the sly1-10 allele, respectively (McGinnis et al., 2003). Primer for the gene At2g09990 encoding the 40S ribosomal protein S16 were used as positive control for DNA content. Arabidopsis plants were grown under a 10-h-light/14-h-dark (22°C/18°C) cycle at 100 to 150 µmol photons m^{-2} s^{-1} in controlled environment chambers, or a 12-h-light/12-h-dark cycle in the case of HaDELLA1-overexpression (OE) lines and the corresponding Arabidopsis wild-type.

RESULTS AND DISCUSSION

Identification of GID1 homologs in the sunflower genome

GID1, the soluble receptor for GA, was first characterized in rice (Ueguchi-Tanaka et al., 2005). A reason for this surely was that no other GID1 isoform was able to compensate phenotypic alterations based on mutations in this single gene. In contrast, in Arabidopsis three GID1 proteins

(AtGID1a, AtGID1b and AtGID1c) with redundant but also distinct roles in plant development were identified (Nakajima et al., 2006). In 2011, one sunflower EST sequence (BU028290) was published, which encodes the C-terminal part of an Arabidopsis AtGID1b similar protein (Blackman et al., 2011). However, our genome databases screenings revealed that the genomes of most eudicots contain at least two different genes encoding GID1 homologous proteins. In addition, Kuang et al. (2013) found three corresponding hits to GID1 homologs in Gerbera hybrida. In H. annuus, local BLASTN indicated several HaGID1 isoforms. By using the sunflower EST BU028290, which is similar to AtGID1b, for BLAST searches in the HA-412-HO scaffold database we detected an ORF of 1005 bp, which was identical to BU028290 in the 3'-end. The resulting amino acid sequence showed more than 90% identities when compared to Lactuca sativa LsGID1A and LsGID1B sequences (Sawada et al., 2012). Nevertheless the start ATG, as well as the next 13 amino acids could not be detected in that scaffold. By using the Lactuca GID1 sequences (LsGID1A and LsGID1B) we found a second scaffold, which was identical to the first scaffold in the 3'-end. In addition, this scaffold contained a start ATG followed by a 39-bp-sequence, which showed high similarities in the amino acid sequence to LsGID1A and LsGID1B, 890 bp upstream from the 1005-bp-ORF. To confirm the exon-inron structure we used RT-PCR with primers encompassing the full-length cds (binding in the 5'- and 3'-UTR region) and amplified the mature HaGID1B cds. We were thus able to guarantee that both scaffolds contained parts of HaGID1B, which consists of two exons and one intron of 890 bp. We utilized the HaGID1B sequence and found four other highly similar sequences (HaGID1A, C, D and E) within the HA-412-HO genome (Figure 1). Like HaGID1B, HaGID1A and HaGID1E consist of two exons separated by an intron of 577 bp and 885 bp, respectively. We anticipate that HaGID1C and HaGID1D have similar structures but the first exon and the following intron are missing due to truncated scaffold sequences. Phylogenetic analyses using the second exons, the main parts of HaGID1C and HaGID1D cds, have clearly shown that the resulting protein sequences belong to GID1 receptors (tree not shown). Consequently, our findings reveal that sunflower contains at least five HaGID1 isoforms and it seems to be very likely that HaGID1s, like Arabidopsis GID1 proteins, mediate a complex network with overlapping but also distinct functions.

Identification of DELLA homologs in the sunflower genome

DELLA proteins belong to a subfamily of the plant specific GRAS gene family. DELLAs contain a conserved C-terminal GRAS domain and a unique N-terminal DELLA domain, which is essential for GA-induced degradation (Sun, 2011). In rice and barley, one single gene codes for the only DELLA protein, called SLENDER1 due to the elongated stem phenotype (Ikeda et al., 2001; Chandler et al., 2002). In contrast, Arabidopsis contains five DELLA proteins with partly overlapping but also distinct functions (Daviere & Achard, 2013). There is some evidence that the genome of sunflower contains at least two different genes (HaDELLA1 and HaDELLA2) coding for DELLA proteins (Blackman et al., 2011). However, functional evidence for the HaDELLA proteins is missing, although Ramos et al. (2013) could show that the reduced height in some dwarf sunflower lines is based on a SNP in the DELLA motif of HaDELLA1, which leads to a single amino acid change from DELLA to DELPA (Ramos et al., 2013). At the beginning of our search for DELLA homologs in sunflower, we first aligned the EST sequences of HaDELLA1 published by Blackman et al. (2011). The ESTs coded for an N-terminal HaDELLA part as well as for a C-terminal GRAS domain, but did not overlap in the middle. For that reason, we tried to amplify the whole cds by using primers binding in the 5'- and 3'-UTRs and were able to fill the observed gap of 41 amino acids between the N- and C-terminal part. The HaDELLA1 amplicons differed in size by about 300 bp, when we used DNA or cDNA of the

line HA383. This is due to the fact that the HaDELLA1 cds is divided into two parts by a 325-bp-intron. In the next step, we wanted to find out whether the putative HaDELLA2 EST (CD850340), lacking the DELLA domain, is part of a true DELLA protein and searched for identical scaffold parts in the HA-412-HO genome. Indeed, CD850340 is part of an 1701-bp-ORF, not interrupted by introns, with a typical DELLA domain. In addition to HaDELLA1 and HaDELLA2, we detected two other coding sequences having putative DELLA domains. These sequences were named HaDELLA-Like1 and HaDELLA-Like2 due to the modified DELLE and DELLF motifs, respectively. Besides these DELLA sequences, we found many other hits of unrelated putative GRAS proteins, which were excluded by phylogenetic analyses. The HaDELLA-Like1 coding sequence consists of 1710 nucleotides. The HaDELLA-Like2 cds still remains fragmentary (1521 bp), lacking about 50-60 amino acid at the end. Despite many attempts to amplify transcripts, we could not detect expression of HaDELLA-like2 in different organs or developing stages of the line HA383, a prerequisite for RACE analyses. Like for HaDELLA-Like2, HaDELLA-Like1 transcripts could not be detected. However, it seems rather unlikely that HaDELLA-Like1 is just a pseudogene because HaDELLA-Like1 (also referred to as RGL2 by Mandel) was identified as a gene of likely agronomic importance in evolutionary analyses of crop-related traits in sunflower (Mandel et al., 2014). The authors speculated that HaDELLA-Like1, which co-localized with a QTL for seed dormancy, might perhaps have something to do with lesser or no dormancy of primitive and improved varieties compared to the strong seed dormancy observed in wild sunflowers. Investigations of Arabidopsis RGL2 revealed that RGL2 transcript levels rise rapidly following seed imbibition and then decline rapidly as germination proceeds (Lee et al., 2002). Further studies are needed to find out whether HaDELLA-Like1 is a functional equivalent of AtRGL2. Hower, it is conceivable that the expression level of HaDELLA-Like1 may be lower in improved varieties without dormancy.

Identification of SLEEPY and SNEEZY homologs in the sunflower genome

The two orthologous F-box genes GID2 and SLY1 of rice and Arabidopsis are needed for GA-stimulated DELLA degradation by the 26S proteasome (Wang & Deng, 2011). A second F-box protein SNE (SNEEZY) in Arabidopsis is only partially able to compensate the sly1 phenotype by overexpression. Extensive studies of AtSLY1 and AtSNE overexpression in the Arabidopsis sly1-10 mutant suggest that one reason, why SNE is unable to fully compensate the mutant phenotype, is that SLY1 regulates a broader spectrum of DELLA proteins than SNE (Ariizumi et al., 2011). However, rice and Arabidopsis have only a single gene for GID2/SLY1 and SNE, respectively, whereas other plant species like Gerbera hybrida have more SLY1 homologs (Kuang et al., 2013). In sunflower, one full-length cds of a putative SLEEPY protein (HaSLY1, now named HaSLY1A) was described so far (Blackman et al., 2011) but initial work of our group revealed the existence of a second HaSLY1 isoform (HaSLY1B).

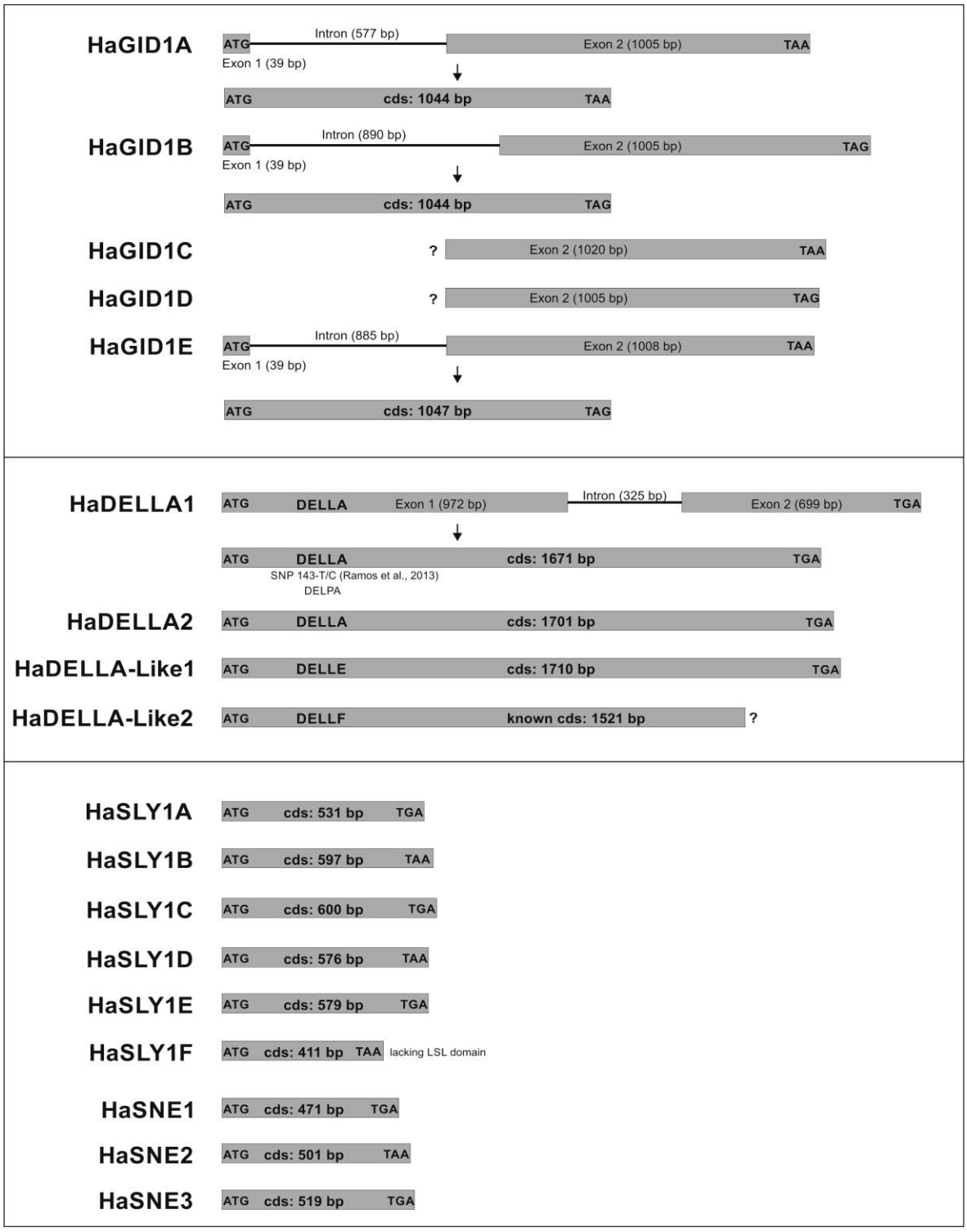


Fig. 16: Schematic overview of genes involved in GA signaling in the sunflower line HA383

Through the access to the genome database we were able to identify all in all nine coding sequences showing similarities to Arabidopsis SLY1, some of them being incomplete. Like HaSLY1A and HaSLY1B, four other coding sequences also showed high identity values to AtSLY1 and were named HaSLY1C, D, E and F. The last three related sequences, on the other hand, had a significantly lower correlation to AtSLY1 and HaSLY1s. We assumed that these

three proteins (now HaSNE1, 2 and 3) could be hitherto undetected sunflower SNEEZY homologs due to amino acid identities of about 50% to AtSNE. The coding sequence of HaSNE3 was incomplete in the scaffold database assembled by the program Celera but we detected the full-length cds in the Ha412Newbler20120907_gene database (www.heliagene.org). The complete cds of HaSLY1E, lacking the 5'-end in the scaffold databases, was obtained by RACE-PCR. Assuming that the HaSLY1F sequence was unusually short due to sequencing or assembly errors, we were surprised to find the same TAA stop codon in the sunflower line HA383 at position 409, which leads to a shortened F-box protein. Attempts to obtain RACE-PCR products as well as RT-PCR amplicons have failed. In contrast to all other HaSLY1s, HaSLY1F seemed to be not expressed. Complementation studies of truncated AtSLY1 forms provided evidence for malfunction of SLEEPY proteins without the LSL domain (Ariizumi et al., 2011). Together with the fact that no HaSLY1F expression was detectable the unusually short form suggests that HaSLY1F may represent a pseudogene.

In summary, our findings of nine F-box proteins in sunflower, as well as the detection of six putative SLY1 transcripts in the transcriptome of Gerbera hybrida ray florets (Kuang et al., 2013) suggest that in the Asteraceae family genomes may contain a large number of duplicated F-box proteins.

Functional analyses of sunflower SLEEPY and DELLA homologs

The very low regeneration rates in sunflower make it almost impossible to generate stable knockout or knockdown mutant lines. Therefore, we chose the model plant Arabidopsis for functional analyses of the sunflower GA signaling homologs. In this work, we describe our general approach for functional analyses using HaSLY1A and HaDELLA1 as examples. The sly1-10 mutant provides a good basis for complementation studies with SLY1 homologs. In Arabidopsis the overexpression of AtSLY1 under the control of the 35S-promoter fully complemented the dwarfism of the loss-of-function mutant sly1-10 (McGinnis et al., 2003). The full-length ORF of HASLY1A gene was cloned into plant transformation vector pGREEN0229 including the 35S-promoter and terminator (Fig. 17 A). The sly1-10 mutant was transformed with these constructs.

The genotyping analyses clearly showed that all lines contained the respective T-DNA and were homozygous for the sly1-10 mutation (Fig. 17 C). The growth of overexpression lines was compared with untransformed sly1-10 plants as well as wild-type (Ler) plants. HaSLY1A-overexpression lines grew significant faster than untransformed sly1-10 plants and resulted in a similar growth type as the wild-type plants (Fig. 17 E). These results show that the HaSLY1A protein was able to rescue the genetic defects of sly1-10.

Accumulation of DELLA proteins results in dwarfism and often in delayed flowering onset. Typical examples of this are GID1 and SLY1/GID2 knockout lines (Sasaki et al., 2003; Strader et al., 2004), as well as mutations in DELLA genes responsible for the so called 'green evolution' in cereals (Boss & Thomas, 2002). Therefore we tested the phenotypic changes of Arabidopsis wild-type plants (ecotype Ler) by overexpressing HaDELLA1 under the control of the 35S-promoter. The HaDELLA1 overexpression lines showed a delayed flowering onset, slightly decreased rosette diameter and shorter stems (Fig. 17 D). We have not yet made statistical evaluations (waiting for the T3-generation), but the phenotypic changes of HaDELLA1-OE line are in line with observation of plants showing DELLA accumulation.

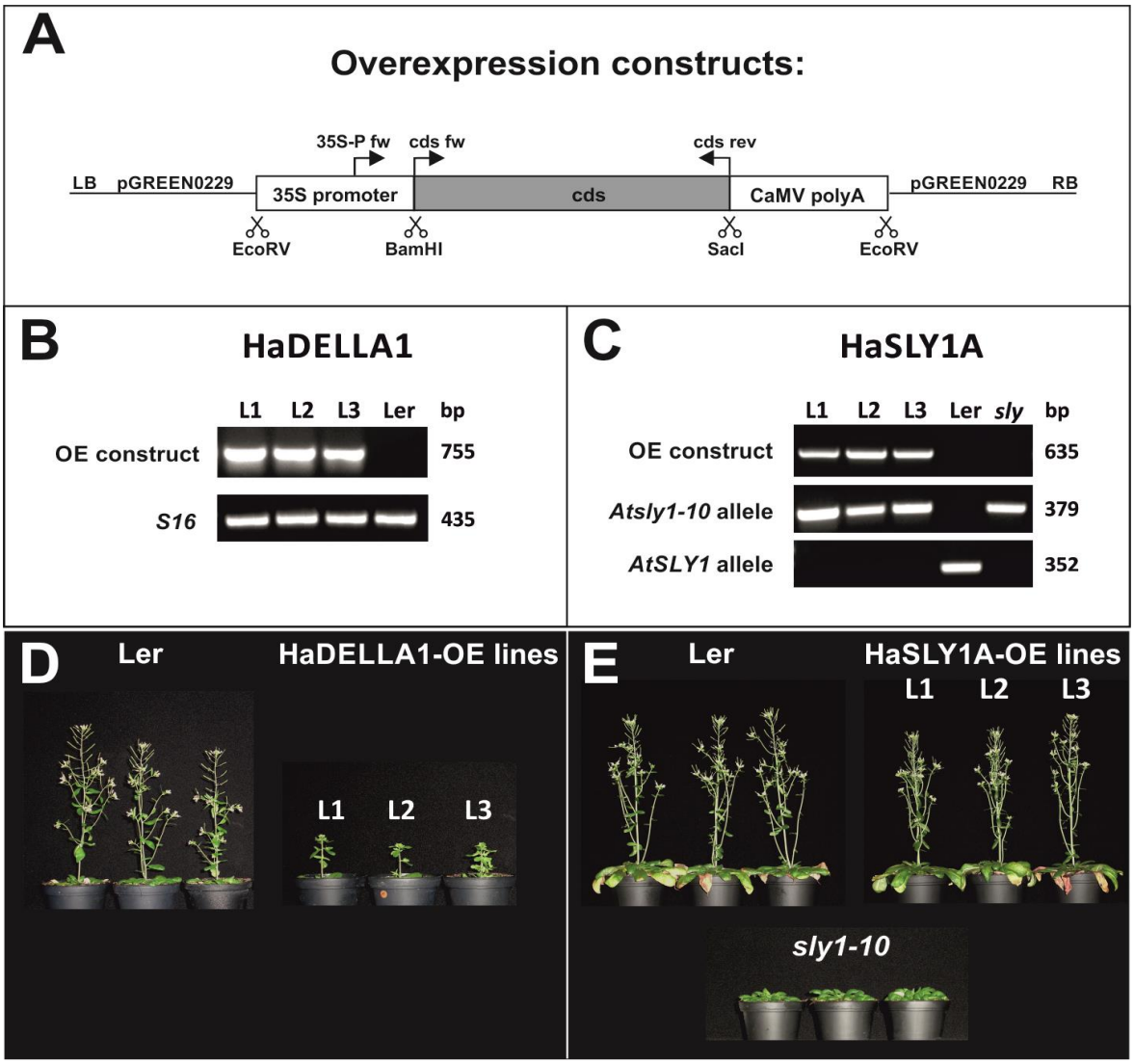


Fig. 17: Overexpression of HaDELLA1 in wild-type Arabidopsis induced dwarfism and late-flowering, whereas overexpression of HaSLY1A rescued sly1-10 dwarf phenotype. A: Schematic overview about cloning strategy of overexpression constructs (see methods for detailed information). B, C: Genotyping of HaDELLA1 (Ler background) and HaSLY1A (sly1-10 background) overexpression lines (L1-L3). D, E: Phenotyping of overexpression lines compared to corresponding Arabidopsis wild-type and sly1-10 mutant plants.

Currently, we analyze these overexpression lines, as well as overexpression lines of the other GA signaling genes detected in sunflower to get more detailed knowledge about their functions.

LITERATURE

Ariizumi T, Lawrence PK, Steber CM. 2011. The role of two F-box proteins, SLEEPY1 and SNEEZY, in Arabidopsis gibberellin signaling. Plant Physiol 155(2): 765-775.

Blackman BK, Rasmussen DA, Strasburg JL, Raduski AR, Burke JM, Knapp SJ, Michaels SD, Rieseberg LH. 2011. Contributions of flowering time genes to sunflower domestication and improvement. Genetics 187(1): 271-287.

Boss PK, Thomas MR. 2002. Association of dwarfism and floral induction with a grape 'green revolution' mutation. Nature 416(6883): 847-850.

Chandler PM, Marion-Poll A, Ellis M, Gubler F. 2002. Mutants at the Slender1 locus of barley cv Himalaya. Molecular and physiological characterization. Plant Physiol 129(1): 181-190.

Daviere JM, Achard P. 2013. Gibberellin signaling in plants. Development 140(6): 1147-1151.

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, et al. 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 36(Web Server issue): W465-469.

Gambino G, Perrone I, Gribaudo I. 2008. A Rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. Phytochem Anal 19(6): 520-525.

Hirano K, Nakajima M, Asano K, Nishiyama T, Sakakibara H, Kojima M, Katoh E, Xiang H, Tanahashi T, Hasebe M, et al. 2007. The GID1-mediated gibberellin perception mechanism is conserved in the Lycophyte Selaginella moellendorffii but not in the Bryophyte Physcomitrella patens. Plant Cell 19(10): 3058-3079.

Ikeda A, Ueguchi-Tanaka M, Sonoda Y, Kitano H, Koshioka M, Futsuhara Y, Matsuoka M, Yamaguchi J. 2001. slender rice, a constitutive gibberellin response mutant, is caused by a null mutation of the SLR1 gene, an ortholog of the height-regulating gene GAI/RGA/RHT/D8. Plant Cell 13(5): 999-1010.

Iuchi S, Suzuki H, Kim YC, Iuchi A, Kuromori T, Ueguchi-Tanaka M, Asami T, Yamaguchi I, Matsuoka M, Kobayashi M, et al. 2007. Multiple loss-of-function of Arabidopsis gibberellin receptor AtGID1s completely shuts down a gibberellin signal. Plant J 50(6): 958-966.

Jasinski S, Tattersall A, Piazza P, Hay A, Martinez-Garcia JF, Schmitz G, Theres K, McCormick S, Tsiantis M. 2008. PROCERA encodes a DELLA protein that mediates control of dissected leaf form in tomato. Plant J 56(4): 603-612.

Kuang Q, Li L, Peng J, Sun S, Wang X. 2013. Transcriptome analysis of Gerbera hybrida ray florets: putative genes associated with gibberellin metabolism and signal transduction. PLoS One 8(3): e57715.

Lee S, Cheng H, King KE, Wang W, He Y, Hussain A, Lo J, Harberd NP, Peng J. 2002. Gibberellin regulates Arabidopsis seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition. Genes Dev 16(5): 646-658.

Mandel JR, McAssey EV, Nambeesan S, Garcia-Navarro E, Burke JM. 2014. Molecular evolution of candidate genes for crop-related traits in sunflower (Helianthus annuus L.). PLoS One 9(6): e99620.

McGinnis KM, Thomas SG, Soule JD, Strader LC, Zale JM, Sun TP, Steber CM. 2003. The Arabidopsis SLEEPY1 gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. Plant Cell 15(5): 1120-1130.

Nakajima M, Shimada A, Takashi Y, Kim YC, Park SH, Ueguchi-Tanaka M, Suzuki H, Katoh E, Iuchi S, Kobayashi M, et al. 2006. Identification and characterization of Arabidopsis gibberellin receptors. Plant J 46(5): 880-889.

Ramos ML, Altieri E, Bulos M, Sala CA. 2013. Phenotypic characterization, genetic mapping and candidate gene analysis of a source conferring reduced plant height in sunflower. Theor Appl Genet 126(1): 251-263.

Sasaki A, Itoh H, Gomi K, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Jeong DH, An G, Kitano H, Ashikari M, et al. 2003. Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. Science 299(5614): 1896-1898.

Sawada Y, Katsumata T, Kitamura J, Kawaide H, Nakajima M, Asami T, Nakaminami K, Kurahashi T, Mitsuhashi W, Inoue Y, et al. 2008. Germination of photoblastic lettuce seeds is regulated via the control of endogenous physiologically active gibberellin content, rather than of gibberellin responsiveness. J Exp Bot 59(12): 3383-3393.

Sawada Y, Umetsu A, Komatsu Y, Kitamura J, Suzuki H, Asami T, Fukuda M, Honda I, Mitsuhashi W, Nakajima M, et al. 2012. An unusual spliced variant of DELLA protein, a negative regulator of gibberellin signaling, in lettuce. Biosci Biotechnol Biochem 76(3): 544-550.

Smalle J, Vierstra RD. 2004. The ubiquitin 26S proteasome proteolytic pathway. Annu Rev Plant Biol 55: 555-590.

Strader LC, Ritchie S, Soule JD, McGinnis KM, Steber CM. 2004. Recessive-interfering mutations in the gibberellin signaling gene SLEEPY1 are rescued by overexpression of its homologue, SNEEZY. Proc Natl Acad Sci U S A 101(34): 12771-12776.

Sun TP. 2008. Gibberellin metabolism, perception and signaling pathways in Arabidopsis. Arabidopsis Book 6: e0103.

Sun TP. 2011. The molecular mechanism and evolution of the GA-GID1-DELLA signaling module in plants. Curr Biol 21(9): R338-345.

Tian CG, Wan P, Sun SH, Li JY, Chen MS. 2004. Genome-wide analysis of the GRAS gene family in rice and Arabidopsis. Plant Mol Biol 54(4): 519-532.

Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, Ecker JR, Sun TP. 2004. DELLA proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. Plant Physiol 135(2): 1008-1019.

Ueguchi-Tanaka M, Ashikari M, Nakajima M, Itoh H, Katoh E, Kobayashi M, Chow TY, Hsing YI, Kitano H, Yamaguchi I, et al. 2005. GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. Nature 437(7059): 693-698.

Wang CY, Liu Y, Li SS, Han GZ. 2015. Insights into the Origin and Evolution of the Plant Hormone Signaling Machinery. Plant Physiol 167(3): 872-886.

Wang F, Deng XW. 2011. Plant ubiquitin-proteasome pathway and its role in gibberellin signaling. Cell Res 21(9): 1286-1294.

QUANTITATIVE DETERMINATION OF SUNFLOWER IN MIXED CONCENTRATE FEEDS BY REAL TIME PCR

Muhammet KAYA [1], ***Zekeriya KIYMA*** [2]

[1] *Eskişehir Osmangazi University, TURKEY*
[2] *Eskişehir Osmangazi University Faculty of Agriculture Department of Animal Science, TURKEY*

muhammetkaya@ogu.edu.tr

ABSTRACT

Feeding farm animals such as, high-yielding dairy, poultry, swine and beef cattle at certain periods with a balanced and adequate mixed concentrate feed is a must. Concentrate feed is produced from grain feed materials that goes through various processes and mixed with oilseed meals, food industry by-products and feed additives. The aim of this study was to determine proportional amounts of sunflower in mixed concentrate feeds by DNA-based Real Time Polymerase Chain Reaction (RT-PCR) in order to monitor compliances of mixed feed labels, investigate the appropriateness of the required quality criteria, detect and avoid unintended feed materials that are mixed in imported plant raw materials. For this purpose, concentrate feed samples that were prepared at the laboratory by weight to weight and also the samples with known ingredients from a local feed plant were used to test quantity of sunflower. Genomic DNA (gDNA) extracted using commercial kits and evaluated for quality and quantity prior to use in a PCR assay. For the specific detection of sunflower and taxon specific (for the relative quantification) a fragment of the *helianthinin* and *actin* genes were selected respectively using gene specific primers and probes. The calibration curve was established on the basis of five samples. The average value of the slope of the standard curve was within the range of -3.1 to -3.6, and the R2 coefficient was ≥ 0.98. This study, first time showed that sunflower in a mixed concentrate feed can be quantified by DNA-based RT-PCR with a high precision.

Key Words : Mixed Concentrate Feeds,Sunflower, RT-PCR, Quantitative Determination

EVALUATION OF WRKY AND MYB TRANSCRIPTION FACTORS IN SOME DOWNY MILDEW INFECTED SUNFLOWER LINES; MICROARRAY DATA ANALYSIS

Ertugrul FILIZ [1]***, İbrahim İlker ÖZYİĞİT*** [2]***, Recep VATANSEVER*** [2]

[1] *Düzce University, TURKEY*
[2] *Marmara University, TURKEY*

ABSTRACT

Downy mildew, which is caused by *Plasmopara halstedii*, is a major plant disease significantly affecting the sunflower (*Helianthus annuus*) production. Yield losses can reach up to 100% in heavily contaminated fields, even could lead the abandoning of cultivation. When considering the worldwide cultivation of sunflower as oil crop, plant losses have serious impact on the country economy as well as on the global food security. So far, thirty-six*P. halstedii* pathotypes/races were identified all over the world. In this study, expression profiles of *WRKY* and *MYB* transcription factor (TF) genes were analyzed in four downy mildew (race 334 and 710) infected sunflower genotypes such as PSC8 (susceptible), XRQ (resistant), and RIL43 and 88 (cross between XRQ and PSC8). Microarray data, including 60 normal vs disease samples were retrieved from NCBI GEO Data Sets (access. GSE25717). For array analyses, sunflower lines were grown in compost under 70% humidity, 20°C temperature and 16h day/8h night light periods. Germinated seeds (2-3 days) were inoculated with downy mildew or water for control for 3hrs, and then following 6 or 10 days of inoculation, plants were harvested for RNA extraction and array analyses. Data were normalized with GCRMA algorithm in Bioconductor package. Analyses demonstrated the 18 putative *WRKY* and 49 putative *MYB* TFs in downy mildew infected sunflower genotypes. In *WRKY* TFs, *WRKY-b* (Heli023654), *WRKY4* (Heli013712 and Heli000574), *WRKY1* (Heli029273) and *WRKY30*(Heli009222) TFs demonstrated significant downregulation in most downy mildew-sunflower interactions while the rest mainly upregulated in infected lines. Hierarchical clustering with Euclidean distance showed that*WRKY-b* (Heli023654) and *WRKY4* (Heli013712 and Heli000574) TFs in genes, and 10 days PSC8, RIL43 and RIL88 lines infected with race 710 in conditions have similar expression profiles. In addition, without line consideration, 6 or 10 days plants infected with race 334 had similar expression pattern and clustered closely, however, plants infected with 710 race demonstrated divergence in clustering based on plant age (6 or 10 days). In*MYB*, hierarchical clustering of TFs demonstrated three main clusters, including considerably up- and downregulated genes, and the genes with mosaic pattern. In addition, plant age seemed to have determinative effect in gene expression patterns. All these implicate that infection period (plant age) and pathotype (race 334 or 710) play an important role in modulation of expression profiles of *WRKY* and *MYB* TFs. However, further molecular and physiological studies are required to elucidate the relationship between these two TFs as well as with their interactors. Comparative analyses of *WRKY* and *MYB* TFs in susceptible, resistant and cross sunflower lines could significantly provide valuable insights to understand the gene regulatory elements in downy mildew-sunflower interactions as well as could pave the way for their biotechnological manipulation to improve the commercially important sunflower lines.

Key Words : Pathotype, race, array, mildew, inoculation, TF

DE NOVO SEQUENCING OF THE HELIANTHUS ANNUUS AND OROBANCHE CUMANA GENOMES

Stephane Munos [1], ***Sunrise Consortium*** [2], ***Sunflower Genomic Ressources Consortium*** [3], ***Helior Consortium*** [3]

[1] *INRA, FRANCE*
[2] *Sunrise Consortium, FRANCE*

stephane.munos@toulouse.inra.fr

ABSTRACT

The sunflower (*Helianthus annuus*) genome is diploid, large (N=17 chromosomes spanning 3.6Gb) and complex. It has been shown to be composed of over 81% of transposable elements (Staton et al., 2012). The strategy using short Roche and Illumina reads from a range of library sizes coud not completely solve the great complexity of this genome. In this context, within the SUNRISE project, we started an ambitious approach to generate a high quality genome sequence of the INRA line XRQ, by exploiting the PacBio RSII sequencing progresses. In the frame of the HeliOr project, PacBio sequencing was also used to sequence the genome of another sunflower INRA line (PSC8) and the genome of the parasitic plant *Orobanche cumana* (the broomrape of sunflower).*Orobanche cumana* is a non-chlorophyll plant that specifically infests sunflower by fixing to its roots. It then uses a part of the mineral nutrients absorbed by sunflower and a part of the assimilates produced by the sunflower. Broomrape can cause yield loses up to 90%. Very few genomic resources are available for this species but its genome is diploid and around 40% of the sunflower genome size (N=19 chromosomes, 1.5Gb). In this paper, we will present our approach, the overall assembly process and a first analysis of the genomes annotation.

Key Words : genome sequencing, PacBio, sunflower, PacBio

IN VITRO POLLEN VIABILITY IN SOME WILD TYPE SUNFLOWER GENOTYPES (HELIANTHUS SPP)

Seda ÖZER [1], Nazan DAGUSTU [1]

[1] Uludag University, Field Crops Department, Bursa, TURKEY

ndagustu@uludag.edu.tr

ABSTRACT

The evaluation of pollen viability is one of the essential criteria for pollinator's characterization. This study was carried out to understand the relationship between *in vitro* pollen viability and pollination and/or seed set in eight genotypes of wild type sunflower [(*Helianthus petiolaris* subsp. *petiolaris,* (1), (*H. petiolaris,* (1), *H. annuus* subsp. *lenticularis*, (1), *H. petiolaris* subsp. *petiolaris*, (1), *H. argophyllus*, (3), *H. maximiliani*, (1)] Two pollen viability tests, [TTC (2,3,5-triphenyl tetrazolium chloride), Asetocarmine] were used to estimate pollen viability. The data taken from three pollen characters (viable, semi viable, death) were analysed statistically by Jump statistical programme. Significant differences among genotypes, dies and interactions between genotypes and dies at 1% probability levels were found at all examined characters. The percentage of pollen viability varied from 48.6 to 99.1% by acetocarmine test and from 25.3 to 68.5% by TTC test. The highest pollen viability was obtained from diffent origin of *H. argophyllus* number 34 (% 79.53*)* and 35 genotypes (% 75.33) while *H. petiolaris* subsp. *petiolaris* had the lowest (%42.2). The acetocarmine had the highest pollen viability (83.3%) followed by TTC (%37.7). When the interactions examined acetocarmine died the wild sunflower pollens at 99.1%, TTC died the lowest rate of *H. petiolaris* pollens (%25.3). Among the wild type sunflowers studied *H. argophyllus* appeared to be suitable pollinators with respect to the criteria investigated.

Key Words : Wild type sunflower genotypes, pollen viability

CHARACTERIZATION OF SUNFLOWER INBRED LINES WITH HIGH OLEIC ACID CONTENT BY DNA MARKERS

***Behiye Banu BILGEN*[1]**

[1] *Namık Kemal University, Faculty of Agriculture, Agricultural Biotechnology Department, 59000, Tekirdağ TURKEY*

**bbilgen@nku.edu.tr*

ABSTRACT

Sunflower is one of the most important oilseed crops due to high oxidative stability of its oil with high oleic acid content. Screening high oleic sunflower genotypes by standard methods such as Gas Chromatography (GC) is time consuming and expensive. Using molecular markers associated with high oleic acid trait is a useful tool in order to facilitate sunflower breeding program. For the purpose of genotyping the sunflower lines for high oleic content, two markers were chosen; SSR marker and HO PCR specific fragment. The results showed that high oleic containing hybrids expressed a specific SSR band at 246 bp length, and also HO PCR specific fragment at 870 bp length. The results were confirmed by determining the fatty acid composition. The results of this work allowed to validation of two DNA markers in sunflower inbred lines for high oleic acid traits.

Key words: *Helianthus annuus* L., Marker-assisted selection, SSR, Oleic acid composition

INTRODUCTION

Sunflower is one of the most important oil crops in the world. It is produced in the world generally for human and non-food purposes (cosmetics, paints, etc.) due to the oil and fatty acid composition of the seed being adapted to these uses. Sunflower oil contains high level of unsaturated fatty acids (88%); linoleic acid (48-74%), oleic acid (14-40%) and also saturated fatty acids; palmitic acid (4-9%) and stearic acid (1-7%) (Singchai et al., 2013; Nagarathna et al., 2011). It is desirable for human consumption because of its favorable fatty acid composition (Baydar and Erbas 2005). Oleic sunflower production and consumption started rapidly both for healthy frying oil, and also non-food purposes in recent years. Non-food applications in particular require oleic acid content that is stable and higher than 90% (Vannozzi 2006). Diets containing vegetable oil with high oleic acid content have been reported to be most effective for preventing cardiovascular diseases (Delplanque et al., 1997; Broun et al., 1999). Increase of oleic acid content has become one of the major goals to improve vegetable oil quality (Lacombe et al., 2004). In order to reach this aim, Sunflower lines and hybrids which have high oleic acid content in their seeds have been obtained by selection programs from HO (High oleic) Pervenet mutant by chemical mutagenesis (Soldatov 1976). The mean content of oleic acid of the seeds from Pervenet population is higher than 65% whereas this content in normal LO varieties is about 20% (Berville et al., 2009). Because of the interest in oleic acid and also the agronomic performance of HO varieties carrying the Pervenets mutation compared with the LO varieties, these varieties are widely used in the world (about 1.2 million ha, CETIOM 2002). The phenotypic determination (fatty acid analysis) does not allow rapid and early determination of HO genotypes and also cannot provide differentiation of homozygotes from heterozygotes for

the mutation. The use of molecular markers has become popular tool for the genetic and breeding studies and it is rapid, cheaper and simple when suitable markers were developed (Varshney et al., 2005). Therefore, marker assisted selection (MAS) analysis is necessary at genomic level allowing rapid and earlier determination of homozygous HO genotypes for sunflower breeding studies.

The aims of this study are characterization of sunflower inbred lines with high oleic acid content by DNA markers and evaluate the effectivity of two marker types developed by Berville et al. (2009).

MATERIALS AND METHODS

Plant materials

For the purpose of screening on high oleic acid genotypes, around 300 sunflower F_3 (K2-R-SN-9/13) individuals obtained from a cross between high oleic acid and low oleic acid lines were used. Leaves were collected from the field, labeled with individual number and stored kept at -80ºC until further use.

DNA isolation

Before DNA isolation leave samples were homogenized with Retsch® Model MM300 Mixer Mill. Different manual DNA isolation methods (Dellaporta et al., 1983; Doyle and Doyle 1987; Li et al., 2007; Azmat et al., 2012; Souza et al., 2012; Healey et al., 2014) and also DNA isolation kits (NANObiz Plant Genomic DNA Isolation Kit, Vivantis GF-1 Plant DNA Extraction Kit and i-genomic Plant DNA Extraction Mini Kit) were tested in order to obtain high quality and quantity DNA for PCR analysis. Finally i-genomic Plant DNA Extraction Mini Kit was selected and used for DNA isolation from all samples. Concentration of each DNA was measured with Qubit® 2.0 Fluorometer and the quality of DNA was checked by 1% agarose gel electrophoresis, stained with RedSafe Nucleic Acid Staining Solution and visualized by Gel Imaging System Vilber Lourmat Quantum ST5. Each of the extracted DNA was diluted as 50 ng per µl and was stored at -20 ºC for later uses.

PCR analysis

Genotyping of high oleic (HO) and low oleic (LO) sunflower individuals was performed with two primer pairs; SSR (N1-1F/N1-1R) and HO PCR specific fragment (N1-3F/N2-1R) that were chosen from the patent obtained by Berville et al. (2009) (Table 1). PCR amplification was carried out using 20 µl volume containing 100 ng of template DNA, 2 mM $MgCl_2$, 1X reaction buffer, four dNTPs (each 0.2 mM), 10 pmol of each primer (forward primer WellRed D4 fluorescent dye labeled) and 1.5 U of Taq-polymerase. The PCR profile for SSR (N1-1F/N1-1R) consisted of 5 min denaturing at 94 ºC, followed by 35 cycles of 1 min denaturing at 94 ºC, 1 min annealing at 50 ºC and 1 min extension at 72 ºC, with a final extension of 10 min at 72 ºC. The PCR profile for HO PCR specific fragment (N1-3F/N2-1R) consisted of 5 min denaturing at 94 ºC, followed by 35 cycles of 1 min denaturing at 94 ºC, 1 min annealing at 58 ºC and 1 min extension at 72 ºC, with a final extension of 10 min at 72 ºC. Amplified PCR products were controlled by 2% agarose gel electrophoresis, stained with RedSafe Nucleic Acid Staining Solution and visualized by Gel Imaging System Vilber Lourmat Quantum ST5 (Figure 1 and

Figure 2). SSR (N1-1F/N1-1R) fragments were scored in a Beckman Coulter GenomeLab™ GeXP Genetic Analysis System and fragment sizes were calculated by its Software.

Table 1. Characteristics of markers used to analyze HO and LO sunflower genotypes

No	Primer type	Primer name	Primer sequences (5′-3′)
1	SSR	N1-1F	TTGGAGTTCGGTTTATTTAT
		N1-1R	TTAGTAAACGAGCCTGAAC
2	HO PCR specific fragment	N1-3F	GAGAAGAGGGAGGTGTGAAG
		N2-1R	AGCGGTTATGGTGAGGTCAG

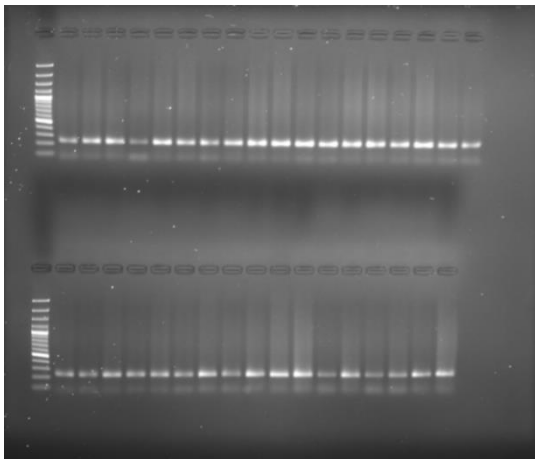


Figure 1. Amplified fragments with SSR (N1-1F/N1-1R) primer for sunflower individuals (First lane is 100 bp DNA Ladder)

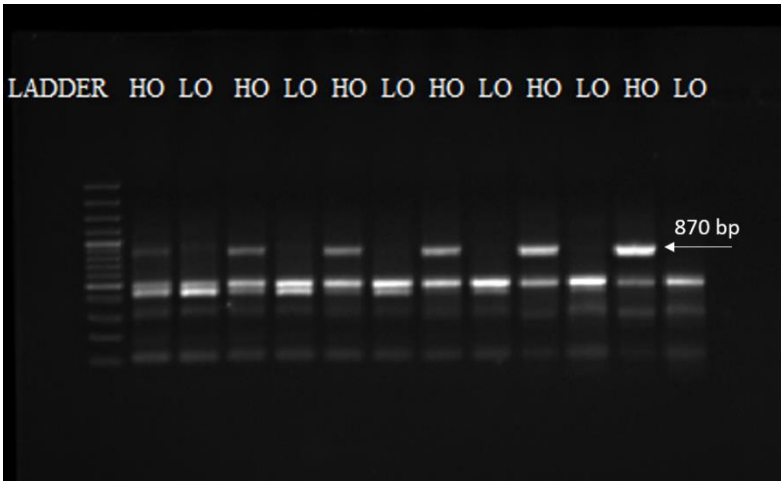


Figure 2. PCR amplification of HO and LO genotypes with HO PCR specific fragment (N1-3F/N2-1R) (First lane is 100 bp DNA Ladder)

PCR amplification of SSR (N1-1F/N1-1R) primer leads to 243/246/249 bp fragments corresponding to 15/16/17 TTA repeats, respectively. The HO genotypes has 16 TTA repeats whereas LO genotypes has 17 TTA repeats for the studied sunflower individuals. DNA sequence analysis with reverse primer was carried out to confirm repeat motifs corresponding to HO genotypes (Figure 3 and 4).

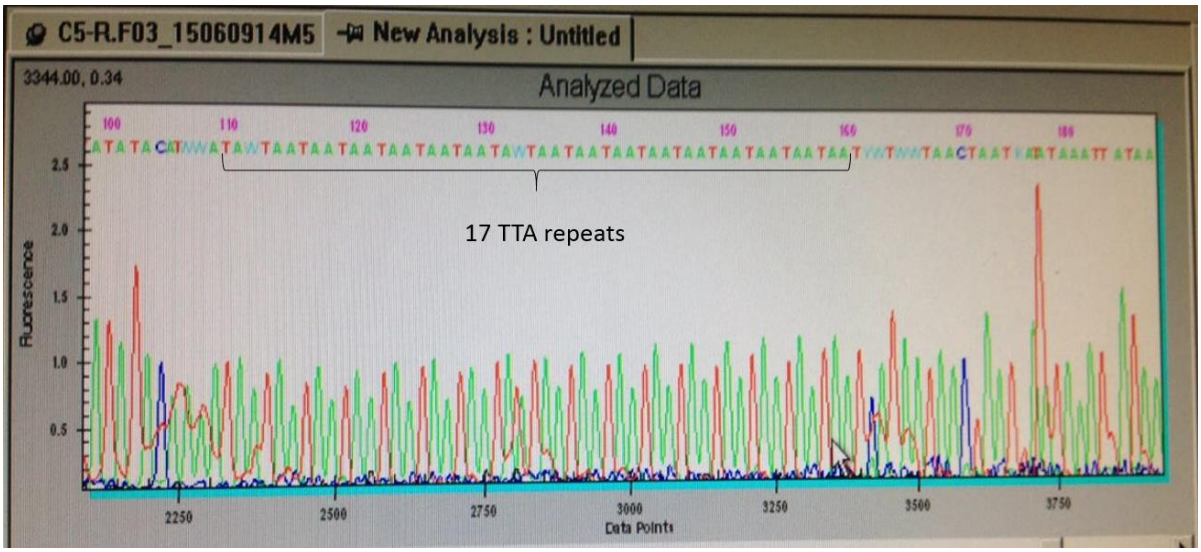


Figure 3. DNA sequence analysis of LO genotypes that have 17 TTA repeats (249 bp)

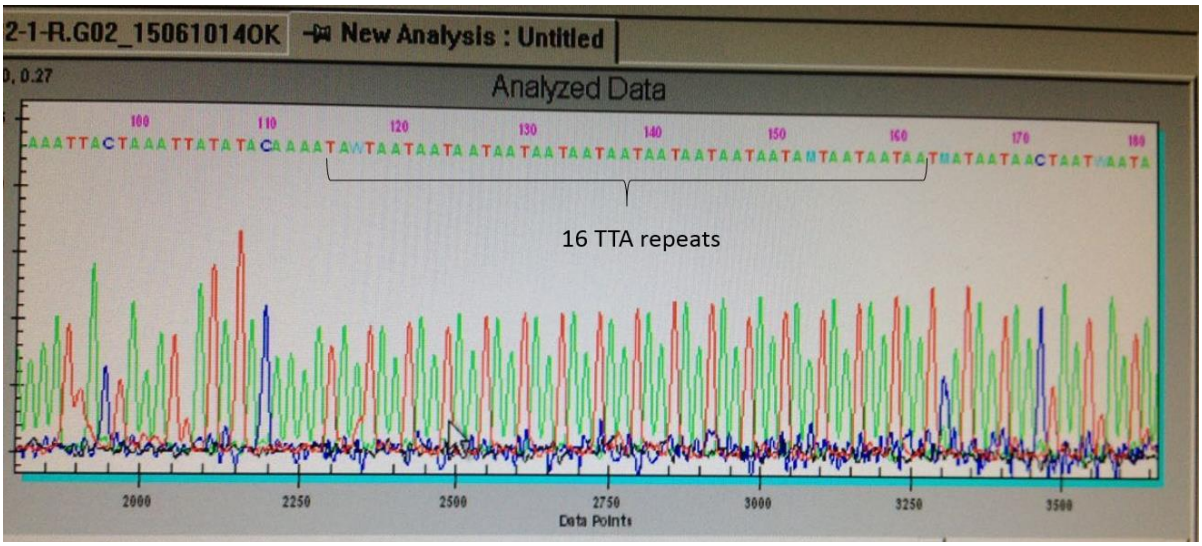


Figure 4. DNA sequence analysis of HO genotypes that have 16 TTA repeats (246 bp)

RESULTS AND DISCUSSION

The Pervenet mutation was labelled by the polymorphism of the SSR locus located on the Δ12-desaturase gene intron (Berville et al., 2009). Alleles and genotypes of studied sunflower individuals were determined for analyzed SSR (N1-1F/N1-1R) locus. According to DNA fragment analysis for SSR locus 246/246 Homozygous, 249/249 Homozygous and 246/249

Heterozygous genotypes were identified (Figure 5). In order to confirm HO sunflower genotypes, all studied individuals were screened with HO PCR specific fragment (N1-3F/N2-1R). The Pervenet mutation was labelled by the 870 bp PCR fragment across the 5' insertion point by HO PCR specific fragment (N1-3F/N2-1R) (Berville et al., 2009). The results showed that high oleic containing sunflower individuals (HO genotypes) showed a specific band at about 870 bp length which was absent in low oleic (LO) genotypes (Figure 2).

After evaluation of all studied sunflower individuals (Totally 300 F_3), 183 of them were HO genotypes, the others were LO genotypes. The results were confirmed by determination of fatty acid composition using gas chromatography in all the studied individuals. According to fatty acid analysis, the oleic acid content was obtained from 60-92% for HO genotypes and below 60% (minimum 22.8%) for LO genotypes.

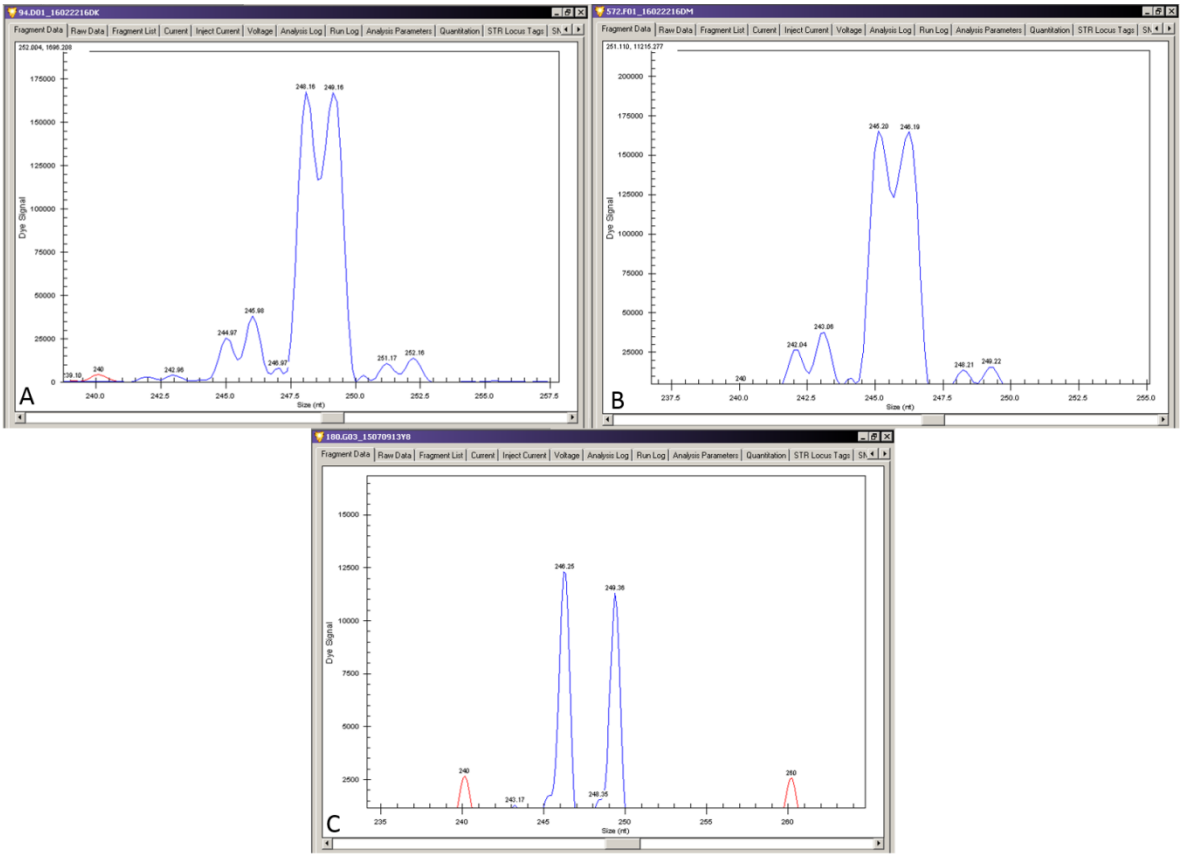


Figure 5. DNA fragment analyses results for (N1-1F/N1-1R) primer A) 249 bp (Homozygous LO genotype), B) 246 bp (Homozygous HO genotype), C) 246 bp/249 bp (Heterozygous HO genotype)

Various sunflower lines and hybrids have been studied to distinguish HO genotypes from LO genotypes by different researchers and molecular marker types such as RAPD or SSR (Dehmer and Friedt 1998; Nagarathna et al., 2011; Grandon et al., 2012; Singchai et al., 2013). Nagarathna et al., (2011) studied around 350 sunflower genotypes including RHA-lines, cms lines, inbreds and germplasm lines to screening on high oleic acid. In Nagarathna et al., (2011) For the purpose of genotyping the sunflower lines for high oleic content, HO PCR specific fragment (N1-3F/N2-1R) were chosen and also the seeds were used for the determination of fatty acids (linoleic acid, oleic acid, palmitic acid and stearic acid) using gas chromatography. They

reported that the genotypes having a specific band (at 800 to 900 bp) showed high oleic content. Singchai et al., (2013) studied the developed lines that used as the representative of low and high oleic acid sunflowers for genotyping. They screened thirty seven SSR primers including 34 primers of ORS set, 2 primers of ha set and N1-3F/N2-1R primer to identify DNA samples from two lines (high and low oleic acid contents). Out of the 37 SSR primers screened for polymorphism, 10 SSR primers including N1-3F/N2-1R generated differentiating bands between the high and low oleic content lines. With the 10 SSR markers they studied, Singchai et al., (2013) reported that it is possible to identify the genetic markers linked to high oleic acid trait which may be useful for further sunflower breeding program.

As a conclusion PCR analysis with selected primers enabling to amplify either the Δ12HOS allele and thus the Pervenet mutations (N1-3F/N2-1R) or the SSR locus (N1-1F/N1-1R), lead to discriminate HO and LO genotypes. Consequently, these primers may be used in selection programs to identify genotypes carrying the Pervenet mutation. However, these markers especially SSR (N1-1F/N1-1R) need to be further validation in different sunflower populations in order to confirm their capability to identify high and low oleic acid contents.

ACKNOWLEDGEMENTS

This study was supported by The Scientific and Technical Research Council of Turkey (TUBITAK) (Project No: 114O974).

LITERATURE

Azmat, M.A., Khan, I.A., Cheema, H.M.N., Rajwana, I.A., Khan, A.S., Khan, A.A. 2012. Extraction of DNA suitable for PCR amplifications from mature leaves of *Mangifera indica* L.. Journal of Zhejiang University-Science B. 13(4): 239-243.

Baydar, H., Erbas, S. 2005. Influence of seed development and seed position on oil, fatty acids and total tacopherol contents in sunflower (*Helianthus annuus* L.). Turk. J. Agric. For. 29: 179-186.

Berville, A., Lacombe S., Veillet S., Granier C., Leger S., Jouve P. 2005. Method of selecting sunflower genotypes with high oleic acid content in seed oil, The Patent Cooperation Treaty (PCT), WO 2005/106022 A2.

Broun, P., Gettner, S. and Somerville, C., 1999. Genetic engineering of plant lipids. Annual Review of Nutrition, 19:197-216

CETIOM, 2002. Technical Center for Oilseed Crops and Industrial Hemp

Dehmer, K.J., Friedt, W. 1998. Development of molecular markers for high oleic acid content in sunflower (*Helianthus annuus* L.). Industrial Crops and Products. 7: 311-315.

Dellaporta, S.L., Wood J., Hicks J.B. 1983. A Plant DNA Minipreparation: Version II. Plant Mol. Biol. Rep., 1: 19-21.

Delplanque, B., Le Roy, B., Senault, C. and Lemort, N., 1997. Reduced capacity of cholesterol efflux, delayed postprandial lipid response, and abnormal Apo-CIII distribution in normolipemic sujects with premature coronary heart disease. Atherosclerosis, 134(1,2): 338-4, pp. 200.

Doyle, J.J., J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phyto. Bull. 19: 11-15.

Grandon, N.G., Moreno, M.V., Scorcione, M.C., Gieco, J.O., Alvarez, D., Paniego, N., Heinz, R. 2012. Characterization of sunflower inbred lines (*Helianthus annuus* L.) for high oleic acid content using SSR markers. INTA.

Healey, A., Furtado, A., Cooper, T., Henry, R.J. 2014. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. Plant Methods. 10: 21.

Lacombe, S., Kaan, F., Griveau, Y., Berville, A. 2004. The Pervenets high oleic mutation: Methodological studies. Helia, 40: 41-54.

Li, J.T., Yang, J., Chen, D.C., Zhang, X.L., Tang, Z.S. 2007. An optimized mini-preparation method to obtain high-quality genomic DNA from mature leaves of sunflower. Genet. Mol. Res. 6(4): 1064-1071.

Nagarathna, T.K., Shadakshari, Y.G., Ramanappa, T.M. 2011. Molecular analysis of sunflower (*Helianthus annuus* L.) genotypes for high oleic acid using microsatellite markers. Helia 34: 63-68.

Singchai, A., Muangsan, N., Machikowa, T. 2013. Evaluation of SSR markers associated with high oleic acid in sunflower. International Journal of Biological, Food, Veterinary and Agricultural Engineering, 7: 631-634.

Soldatov, K. I. 1976. Chemical mutagenesis in sunflower breeding. Proc. 7th International Sunflower Conference, 27 June - 3 July, Krasnodar, Russia, Pp. 352-357.

Souza, H.A.V., Muller, L.A.C., Brandão, R.L., Lovato, M.B. 2012. Isolation of high quality and polysaccharide-free DNA from leaves of *Dimorphandra mollis* (Leguminosae), a tree from the Brazilian Cerrado. Genet. Mol. Res. 11 (1): 756-764.

Vannozzi, G.P., 2006. The perspectives of use of high oleic sunflower for oleochemistry and energy raws. Helia 29: 1-24.

Varshney, R.K., Graner, A., Sorrels M.E., 2005. Genic microsatellite markers in plants: features and applications. Trends Biotechnol 23(1): 48-55.

GENETIC ENGINEERING STUDIES ON SUNFLOWER

Mehmet Emin ÇALIŞKAN[1], Sarbesh Das DANGOL[1]

[1]Department of Agricultural Genetic Engineering, Faculty of Agricultural Science and Technologies, Nigde University, 51240 Merkez, Nigde, Turkey

**caliskanme@nigde.edu.tr*

ABSTRACT

Domestication of sunflowers (*Helianthus annuus*) by humans for particular structures that are desirable to humans in a relatively benign environmental conditions and stresses have forced these plants to undergo evolutionary increases in the yield, but at the cost of reduced defense mechanisms against biotic and abiotic stresses and diseases. A multitude of factors such as insects and diseases reduce the sunflower yield, and research to develop pest resistance, herbicide resistance, increasing oil per acre yield of sunflower holds indispensable. Wild species of sunflower contain rich source of useful genes, which needs to be transferred to cultivated ones. Though improved transformed techniques have been reported, more efficient transformation protocol needs to be explored. There are some studies involving transgenic sunflower plants to increase yield, oil content, insect/fungal resistance, stress tolerance and production of biopharmaceutical proteins. Studies involving ecological impact of *Bt* sunflowers with regards to "gene flow" remains controversial. Stable transformation is relatively time-consuming with low regeneration rate and has left sunflower transformation recalcitrant. For this reason, transient expression in sunflowers have gained attention in studies of function of promoters, regulation of gene, subcellular localization of proteins, protein stability, protein-protein interactions and small RNA function. In this presentation, we will attempt to give an overview of the genetic engineering studies in sunflower with main challenges, achievements and future prospects.

Key words: Transgenic sunflower, Transient expression, Stress resistance, Insect resistance, Oil yield, Biopharming.

INTRODUCTION

Cultivated sunflower (*Helianthus annuus* L.) has its origin from North America and is one of the few major food crops in the world (Harter *et al*., 2004; Blackman *et al*., 2011). Domestication of sunflowers by humans for particular structures that are desirable to humans in a relatively benign environmental conditions and stresses have forced these plants to undergo evolutionary increases in the yield, but at the cost of reduced defense mechanisms against biotic and abiotic stresses and diseases (Mayrose *et al*., 2011). Mayrose *et al*. (2011) studied growth traits of sunflower under benign environmental condition which they found to be greater for the domesticated genotype population than that for the wild population, but with a drop in defense response in domesticated sunflowers when exposed to biotic and abiotic stresses. Additionally, it was found that lepidopteran pests preferred domesticated sunflowers more than the wild sunflowers in experimental agriculture fields (Chen and Welter, 2002). It was also revealed that

Botrytis cinerea and drought had more negative consequences on domesticated sunflowers than the native plants (Mayrose *et al*., 2010). A multitude of factors such as insects and diseases reduce the sunflower yield, and molecular biology research with a focus on transgenic sunflowers to develop pest resistance, herbicide resistance, increasing oil per acre yield of sunflower holds indispensable as well as its study on ecological impact is pressing.

VARIOUS TECHNIQUES IN GENETIC ENGINEERING OF SUNFLOWERS

Various approaches in production of transgenic plants have been used, but with low efficiencies in transformation: Polyethylene glycol (PEG)-induced vector uptake of pCAMVNEO into protoplasts isolated from sunflower seedling hypocotyls (Moyne *et al*., 1988), microprojectile bombardment (Knittel *et al*., 1994; Laparra *et al*., 1995; Hunold *et al*., 1995) and electroporation (Kirches *et al*., 1991). PEG-induced vector uptake turned out to be mainly labor intensive and some other protocols with *Agrobacterium*-mediated transformation of sunflower plants have been used (Bidney *et al*., 1992; Laparra *et al*., 1995; Rao *et al*., 1999; Weber *et al*., 2003; Ikeda *et al*., 2005; Mohamed *et al*., 2006).

One possibility as to the reason for less studies with stable transformation experiments with sunflowers could be because of no efficient and reproducible protocol for sunflower transformation (Radonic *et al*., 2008). Selection of transformants, tissue regeneration, long life cycle of sunflower plants, time-consuming homozygous lines generation mainly as compared to the time required to obtain homozygous transgenic *Arabidopsis* plants have possibly made stable transformation of sunflower to be used to elucidate metabolic or signal pathways of sunflowers recalcitrant. This has led to some studies to choose *Arabidopsis* or tobacco heterologous system or transient expression in sunflower leaves to further unravel gene functions in sunflowers (Manavella and Chan, 2009; Cabello *et al*., 2012; Cabello *et al.*, 2016; Tata *et al*., 2016).

THE DEVELOPMENT OF INSECT TOLERANT TRANSGENIC SUNFLOWERS

A polyphagous insect *Helicoverpa armigera* (Noctuidae; Lepidoptera) is reported to cause 20-25% yield losses in sunflowerss and sometimes upto 40-70% in severe conditions (Ranasingh and Mahalik, 2008). Westdal (1975) found that the sunflower beetle *Zygogramma exclamationis* (25 larvae per plant) reduced seed production in sunflower as much as 30%.

Cry1F-transgenic sunflowers were obtained with a *Cry1F* gene ("Bt" gene) isolated from *Bacillus thuringiensis* which conferred resistance against *Spilosoma virginicia* and *Rachiplusia nu*. Compared to the control, increased tolerance of transgenic plants against larvae at the seedling and preflowering stages were found during the feeding assay with transgenic leaf discs. *Cry1AC* gene was used to develop a transgenic line of *Bt* sunflowers by Pioneer Hi-Bred and Dow AgroSciences which produce CrylAc protein that is lethal to *Lepidopteran* (moth) larvae (Snow *et al*., 2003). Snow *et al.* (2003) reported that the transgenic plants yielded considerably more inflorescences with more mature seeds in more inflorescences and higher number of viable seeds per plant as compared to non-transgenic controls. They observed that the transgenic plants in a greenhouse experiment even without the insect pests produced no difference in the seeds or inflorescences. The study concluded that the transgene itself didn't actually cause the benefit in these transgenics, but the protection from lepidopeterian resulted in the gain of fecundity in transgenics. They suggested that the wild sunflowers and weedy populations near to the cultivated transgenic sunflowers would render recurring events of "gene flow" from the transgenics and it could have detrimental effects on the native lepidopteran herbivores and other populations of coleopteran and dipteran herbivores.

THE DEVELOPMENT OF FUNGAL RESISTANT TRANSGENIC SUNFLOWERS

Charcoal rot disease caused by *Macrophomina phaseolina* in sunflower causes losses on more than 500 cultivated and wild plant species (Khan, 2007). *Alternaria* blight caused by *Alternaria helianthi* is reported to reduce seed and oil yield by 27-80% and 17-33% respectively (Reviewed by Mukhtar, 2009). *Sclerotinia* has been reported to cause damage upto 50% in sunflower in UK (Tu, 1989). Fungal pathogen *Plasmopara halstedii* causes Downy mildew and can lead to more than 50% yield loss (Hvarleva *et al*., 2009).

Oxo-transgenic sunflower plants were obtained by introducing wheat germin gf2.8 *OXO* gene to confer resistance against fungal disease *Sclerotinia* head rot (Lane *et al*., 1991; Lu *et al*., 2000; Hu *et al*., 2003). However, it has been of concern if OXO enzyme could be a human allergen (Jensen-Jarolim *et al*., 2002). The probability of transgenic wild plants being a worse weed is scarce as *OXO* transgene will diffuse neutrally on its escape because the transgenic wild plants do not produce ample number of seeds than the wild population (Burke and Rieseberg, 2003). Chapman and Burke (2006) also ruled out the possibility of "gene flow" concluding that the natural selection is the key in spread of favorable transgene alleles. Human lysozyme gene under CaMV 35S promoter and *Nos* terminator in a binary vector containing *NPTII* and *GUS* marker genes was incorporated in sunflowers using hypocotyl explants with *Agrobacterium*-mediated transformation conferred resistance against mold disease causing *Sclerotinia sclerotiorum* (Sawahel and Hagran, 2006). Lectin or proteinase inhibitor genes have been used to engineer sunflower with insect resistance (Schuler *et al.*, 1998).

THE DEVELOPMENT OF ABIOTIC STRESS TOLERANT TRANSGENIC SUNFLOWERS

Yeast metallothionein gene (*CUP1*) from yeast was incorporated into sunflower to evaluate tolerance of transgenic plants to heavy metals at the callus stage and selected heavy metal-tolerant lines of the transgenic sunflower calli. The results showed use of transgenics to obtain abiotic stress tolerance in sunflowers (Watanabe *et al*., 2005). LBA4404 strain harboring T-DNA containing dsRNA-suppressor of proline dehydrogenase gene, produced based on the *ProDH1* gene of Arabidopsis, was integrated into the genome of sunflower plants transformed *in vitro* and *in planta* to increase sunflower tolerance level to water deficiency and salinity (Tishchenko *et al*., 2014**)**.

MOLECULAR PHARMING IN SUNFLOWERS

Plants have the ability to bring about protein stability and bioactivity by glycosylation and posttranslational modification, and plant and animal cell protein synthesis pathway are alike; it is estimated that to synthesize pharmaceutical proteins in plants is highly economical than using fermentation techniques and mammalian cell cultures (Rybicki *et al*., 2010; Ma *et al*., 2003). Guan and Wang (2014) successfully expressed CTB-LK (Cholera toxin B subunit-Lumbrokinase), peeled seeds of which if administered orally to rats and mice had significant antithrombotic effect, in sunflower seeds using *Agrobacterium* mediated transformation. This study also concluded that the CTB-LK expression in sunflower seeds eradicated the requirement for protein downstream processing. Similarly, the use of *Agrobacterium rhizogenes*-mediated transformation of topinambour in sunflower plants, callus and "hairy" root cultures proved sunflower plants to be a good source of recombinant interferon alpha 2b protein. The plasmid vectors with interferon gene fused with *Nicotiana plumbaginifolia* L. calreticulin apoplast

targeting signal driven by *35S* CaMV promoter or root-specific *Mll* promoter to obtain transgenic *H. tuberosus* cultures with high antiviral activity (Maistrenko *et al*., 2015).

THE DEVELOPMENT OF SUNFLOWERS WITH INCREASED FATTY ACID OIL CONTENT BY MUTAGENESIS

Modifying oil quality is crucial as it is one of the edible oils worldwide known for its salubrious quality and lipid peroxidation (Moschen *et al*., 2014). Many sunflower lines have been developed with elevated saturated fatty acid content with greater than 25% of fatty acids compared to 12% in normal sunflower using physical or chemical mutagenesis. Osorio *et al*. (1995) developed CAS-3 and CAS-5 mutants with high amount of stearic acid and palmitic acid contents respectively. Fernández-Martínez *et al*. (1997) reported CAS-12 mutants with high palmitic acid and oleic acid contents. Fernández-Moya *et al*. (2002) developed CAS-14 mutants with upto 37% stearic acid content. Velasco *et al*. (2008) used ethylmethane sulfonate as a chemical mutagen and obtained M2 seeds from a single M1 plant with 5-39% palmitic acid content. 10-30% of palmitic acid was obtained from the progenies of all selected M2 seeds.

THE DEVELOPMENT OF TRANSGENIC SUNFLOWERS WITH INCREASED FATTY ACID OIL CONTENT

The Δ9-stearoyl-(acyl carrier protein) desaturase coding sequence from *Ricinus communis* was transferred in sunflower under the control of seed-specific promoter and terminator sequences of *Hads10*. Seed oil composition analysis showed significant decrease in stearic acid content in the seeds obtained from transgenic plants. Some progenies exhibited saturated fatty acid content below 10% whereas other plants had elevated palmitic acid content with reduced stearic acid content (Rousselin et al., 2002). Hydroxymethylglutaryl-CoA (*Hmgr*-CoA) and *Erwinia uredovora* phytoene desaturase (*Crtl*) genes were introduced into sunflower to obtain potential increase in oil quality (Dagustu *et al*., 2008).

OTHER MOLECULAR STUDIES IN TRANSGENIC SUNFLOWERS

Post-transcriptional gene silencing (PTGS) in transgenic sunflower expressing *glucuronidase* (GUS) activity has been performed using grafting procedure. In two weeks silencing was observed and the study showed that the RNA infiltration in sunflower induces transient silencing and is not transmitted to offspring (Hewezi *et al*., 2005). Shulga *et al*. (2015) reported first transgenic sunflower with alteration in *HAM59* expression to study the function of *HAM59* MADS-box gene in sunflower which is involved in formation of reproductive organs of flower.

The elucidation of role of PLFOR48 sequence in resistance to mildew in sunflower was studied assessing loss of function, by expressing antisense cDNA PLFOR48 construct in RHA 266 sunflower line. Transgenic sunflower lines displayed severe developmental abnormalities. The same antisense expression in transgenic tobacco lines resulted in higher susceptibility to *Phytophthora parasitica*. It was reported that TIR-NBSLRR R genes in sunflower and tobacco have a dual role in plant development and fungal resistance (Hewezi *et al*., 2006).

CURRENT STIUATION AND CHALLENGES

A progress is still being made in efficiently transforming sunflower crops but stable transformation of sunflower plants is just yet time-consuming in generating homozygous lines and in regeneration of tissue. Also, sunflower has a long life cycle and transient expression of genes can be an alternative method in elucidating molecular mechanisms such as function of promoters, regulation of gene, subcellular localization of proteins, protein stability, protein-protein interactions and small RNA function (Manavella and Chan, 2009). Despite of this constraint, several studies by developing transgenic sunflowers are still being conducted.

Wild sunflower species provide greater contribution as a rich source of genes in crop improvement to bring about economic viability in cultivates species as major oilseed global crop (Seiler and Fredrick, 2011). As having a narrow background in domesticated sunflowers with deficient genes, discovery of unique genes from wild sunflower plants is indispensable and is still underway. This could help in developing transgenic sunflowers with desired traits from wild population.

Clearfield and Express Sun technologies saw restriction on growing of genetically modified crops for "not being biotech product" (Reviewed by Kaya, 2015). Genetically modified crops have always been a matter of debate and public acceptance regarding this remains divided with some people being reluctant on the use of biotechnology in crop amelioration.

FUTURE DIRECTIONS IN GENETIC ENGINEERING OF SUNFLOWER

Transgenic technology holds imperative role in sunflower breeding and exerts strong promises to increase yield, oil content, insect/fungal resistance, stress tolerance and production of biopharmaceutical proteins. Albeit having improved transformed techniques in sunflower, more efficient transformation protocol needs to be explored for generating increased success rates in obtaining transgenic sunflowers as well as search for candidate genes with elite traits in developing transgenic crops does remain apparent.

Traits that are being studied in sunflower for environment release is sparse. Sunflower is known to have a high exposure to gene flow ultimately generating continuous variability. Strict environmental monitoring is inevitable to preclude undesired outcomes (Cantamutto and Poverene, 2007).

LITERATURE

Bidney D., Scelonge C., Martich J., Burrus M., Sims L., Huffman G. (1992). Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. Plant Molecular Biology, *18*: 301-313.

Blackman B.K., Scascitellic M., Kanec N.C., Lutona H.H., Rasmussena D.A., Byed R.A., Lentze D.L., Rieseberg L.H. (2011). Sunflower domestication alleles support single domestication center in eastern North America. PNAS, *108*: 14360-14365.

Burke J.M., Rieseberg L.H. (2003). Fitness effects of transgenic disease resistance in sunflower. Science, *300*: 1250.

Cabello J.V., Arce A.L., Chan, R.L. (2012). The homologous HD-Zip I transcription factors *HaHB1* and *AtHB13* confer cold tolerance via the induction of pathogenesis related and glucanase proteins. Plant Journal, *69*: 141– 153.

Cabello J.V., Giacomelli J.I., Piattoni C.V., Iglesias A.A., Chan R.L. (2016). The sunflower transcription factor HaHB11 improves yield, biomass and tolerance to flooding in transgenic Arabidopsis plants. Journal of Biotechnology, *222*: 73-83.

Cantamutto M., Poverene M. (2007). Genetically modified sunflower release: Opportunities and risks. Field Crops Research, *101*: 133–144.

Chapman M.A., Burke J.M. (2006). Letting the gene out of the bottle: the population genetics of genetically modified crops. New Phytologist, *170*: 429-443.

Chen Y.H., Welter S.C. (2002). Abundance of a native moth *Homoeosoma electellum* (Lepidoptera: Pyralidae) and activity of indigenous parasitoids in native and agricultural sunflower habitats. Environmental Entomology, *31*:626-636.

Dağüstü N., Fraser P., Enfissi E., Bramley P. (2008). Screening for high callus induction and *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.). Biotechnology and Biotechnological Equipment, *22*: 933-937.

Fernández-Martínez J.M., Mancha M., Osorio J., Garcés R. (1997). Sunflower mutant containing high levels of palmitic acid in high oleic acid background. Euphytica, *97*: 113-116.

Fernández-Moya V., Martínez-Force E. and Garcés R. (2002). Temperature effect on a high stearic acid sunflower mutant. Phytochem, *59*: 33-37.

Guan C., Ji J., Wang G. (2014). Expression of cholera toxin B subunit–Lumbrokinase in edible sunflower seeds–The use of transmucosal carrier to enhance its fusion protein's effect on protection of rats and mice against thrombosis. American Institute of Chemical Engineers, *30*: 1029-1039.

Harter A.V., Gardner K.A., Falush D., Lentz D.L., Bye R.A., Rieseberg L.H. (2004). Origin of extant domesticated sunflowers in eastern North America. Nature, *430*: 201–205.

Harter A.V., Gardner K.A., Falush D., Lentz D.L., Bye R.A., Rieseberg L.H. (2005). Introduction of yeast metallothionein gene *(CUP1)* into plant and evaluation of heavy metal tolerance of transgenic plant at the callus stage. Soil Science and Plant Nutrition, *51*: 129-133.

Hewezi T., Alibert G., Kallerhoff J. (2005). Local infiltration of high- and low-molecular weight RNA from silenced sunflower (*Helianthus annuus* L.) plants triggers post-transcriptional gene silencing in non-silenced plants. *3*: 81-89.

Hewezi T., Mouzeyar S., Thion L., Rickauer M., Alibert G., Nicolas P., Kallerhoff J. (2006). Antisense expression of a NBS-LRR sequence in sunflower (*Helianthus annuus* L.) and tobacco (*Nicotiana tabacum* L.): evidence for a dual role in plant development and fungal resistance. Transgenic Research, *15*: 165-180.

Hu X., Bidney D.L., Yalpani N., Duvick J.P., Crasta O., Folkerts O., Lu G. (2003). Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. Plant Physiology, *133*: 170-181.

Hunold R., Burrus M., Bronner R., Duret J., Hahne G. (1995). Transient gene expression in sunflower (*Helianthus annuus* L.) following microprojectile bombardment. Plant Science, *105*: 95-109.

Hvarleva T., Tarpomanova I., Hristova-Cherbadji M., Hristov M., Bakalova A., Atanassov A., Atanasov I. (2009). Toward marker assisted selection for fungal disease resistance in

sunflower. Utilization of *H. Bolanderi* as a source of resistance to Downy mildew. Biotechnology and Biotechnological Equipment, *23*: 1427-1430.

Ikeda M., Matsumura M., Kamada H. (2005). Suitability of small and branching sunflower varieties for molecular genetic experiments and their transformation by *Agrobacterium* infection. Plant Biotechnology, *22*: 97-104.

Jensen-Jarolim E., Schmid B., Bernier F., Berna A., Kinaciyan T., Focke M., Ebner C., Scheiner O., Boltz-Nitulescu G. (2002). Allergologic exploration of germins and germin-like proteins, a new class of plant allergens. Allergy, *57*: 805-810.

Kaya Y. (2015). Herbicide resistance breeding in sunflower, current situation and future directions. Journal of ASM Life Sciences, *2*: 101-106.

Khan, S.K. 2007. Macrophomina phaseolina as causal agent for charcoal rot of sunflower. Mycopath, *5*: 111-118.

Kirches E., Fre N., Schnabl H. (1991). Transient gene expression in sunflower mesophyll protoplasts. Botanica acta, *104*: 212-216.

Knittel N., Gruber V., Hahne G., Lénée P. (1994). Transformation of sunflower (*Helianthus annuus* L.): a reliable protocol. Plant Cell Reports, *14*: 81-86.

Lane B.G., Bernier F., Dratewka K., Shafai R., Kennedy T.D., Pyne C., Munro J.R., Vaughan T., Walters D., Altomare F. (1991). Homologies between members of the germin gene family in hexaploid wheat and similarities between these wheat germins and certain *Physarum spherulins*. Journal of Biological Chemistry, *266*: 10461–10469.

Laparra H., Burrus M., Hunold R., Damm B., Bravo-Angel A.M., Bronner R., Hahne G. (1995). Expression of foreign genes in sunflower (*Helianthus annuus* L.): evaluation of three transfer methods. Euphytica, *85*: 63-74.

Ma J.K., Drake P.M., Christou P. (2003). The production of recombinant pharmaceutical proteins in plants. Nature Reviews Genetics, *4*: 794–805.

Maistrenkoa O.M., Luchakivskaa Y.S. , Zholobakb N.M., Spivakb M.Y., Kuchuka M.V. (2015). Obtaining of the transgenic *Heliantus tuberosus* L. plants, callus and "hairy" root cultures able to express the recombinant human interferon alpha-2b gene. Cytology and Genetics, *49*: 308–313.

Manavella P.A., Chan R.L. (2009). Transient transformation of sunflower leaf discs via an *Agrobacterium*-mediated method: applications for gene expression and silencing studies. *4*: 1699-1707.

Mayrose M., Kane N.C., Mayrose I., Dlugosch K.M., Rieseberg L.H. (2011). Increased growth in sunflower correlates with reduced defences and altered gene expression in response to biotic and abiotic stress. Molecular Ecology, *20*: 4683-4694.

Mohamed S., Boehm R., Schnabl H. (2006). Stable genetic transformation of high oleic *Helianthus annuus* L. genotypes with high efficiency. Plant Science, *171*: 546-554.

Moyne A.L., Thor V., Pelissier B., Bergounioux C., Freyssinet G., Gadal P. (1988). Callus and embryoid formation from protoplasts of *Helianthus annuus*. Plant Cell Reports, 7: 427 - 440.

Osorio J., Fernández-Martínez J., Mancha M., Garcés R. (1995). Mutant sunflowers with high concentration of saturated fatty acids in the oil. Crop Science, *35*: 739-742.

Ranasingh, N. and J. K. Mahalik. 2008. Insect pests management in sunflower. Orissa Review November 2008. p. 51-52.

Rao K., Rohini V. (1999). *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): A simple protocol. Annals of Botany, *83*: 347-354.

Rousselin P., Molinier J., Himber C., Schontz D., Prieto-Dapena P., Jordano J., Martini N., Weber S., Horn R., Ganssmann M., Grison R., Pagniez M., Toppan A., Friedt W., Hahne, G. (2002). Modification of sunflower oil quality by seed-specific expression of a heterologous 9-stearoyl-(acyl carrier protein) desaturase gene. Plant Breed., *121*: 108-116.

Rybicki E.P. (2010). Plant-made vaccines for humans and animals. Plant Biotech. J., *8*: 620–637.

Sawahel W., Hagran A. (2006). Generation of white mold disease-resistant sunflower plants expressing human lysozyme gene. Biologia Plantarum, *50*: 683-687.

Schuler T.H., Poppy G.M., Kerry B.R., Denholm, I. (1998). Insect-resistant transgenic plants. Trends in Biotechnology, *16*: 168-175.

Seiler G., Fredrick M.L. (2011). Germplasm resources for increasing the genetic diversity of global cultivated sunflower. Helia, *34*: 1-20.

Shulga O.A., Neskorodov Y.B., Shchennikova A.V., Gaponenko A.K., Skryabin K.G. (2015). Antisense expression of a NBS-LRR sequence in sunflower (*Helianthus annuus* L.) and tobacco (*Nicotiana tabacum* L.): Evidence for a dual role in plant development and fungal resistance. *461*: 480-484.

Snow A.A., Pilson D., Rieseberg L.H., Paulsen M.J., Pleskac N., Reagon M.R., Wolf D.E. (2003). A Bt transgene reduces herbivory and enhances fecundity in wild sunflowers. Ecological Application, *13*: 279-286.

Tishchenko O.M., Komisarenko A.G., Mykhalska S.I., Sergeeva L.E., Adamenko N.I., Morgun B.V., Kochetov A.V. (2014). *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.) *in vitro* and *in planta* using *LBA4404* strain harboring binary vector pBi2E with dsRNA-suppressor of proline dehydrogenase gene. Cytology and Genetics, *48*: 218-226.

Tata S.K., Jung J., Kim Y., Choi J.Y., Jung J., Lee I., Shin J.S., Ryu S.B. (2016). Heterologous expression of chloroplast-localized geranylgeranyl pyrophosphate synthase confers fast plant growth, early flowering and increased seed yield. Plant Biotech. Journal, *14*: 29–39.

Tu, J.C. (1989). Management of white mold of white beans in Ontario. Plant Dis, *73*: 281–285.

Velasco L., Pérez-Vich B., Fernández-Martínez J.M. (2008). A new sunflower mutant with increased levels of palmitic acid in seed oil. Helia, *31*: 55-60.

Watanabe M., Shinmachi F., Noguchi A., Hasegawa I. (2005). Introduction of yeast Metallothionein gene *(CUP1)* into plant and evaluation of heavy metal tolerance of transgenic plant at the callus stage. Soil Science, *51*: 129-133.

Weber S., Friedt W., Landes N., Molinier J., Himber C., Rousselin P., Hahne G., Horn R. (2003). Improved *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. Plant Cell Reports, *21*: 475-482.

Westdal, P.H. (1975). Insect pests of sunflowers. In: Harapiak, J.T. [ed.], Oilseed and Pulse Crops in Western Canada - a Symposium. Modern, Saskatoon, Sask. 479-495.

MAPPING OF A BROOMRAPE RESISTANCE GENE IN SUNFLOWER LINE LIV-17

Ivana IMEROVSKI [1], ***Aleksandra DIMITRIJEVIĆ*** [1], ***Dragana MILADINOVIĆ***[1], ***Sandra CVEJIĆ*** [1], ***Siniša JOCIĆ*** [1], ***Bosko DEDIĆ*** [1]

[1] *Institute f Field And Vegetable Crops, Novi Sad, SERBIA*

ivana.imerovski@gmail.com

ABSTRACT

Broomrape (*Orobanche cumana* Walr.) is a parasitic plant that causes severe yield loses in Southern and Eastern Europe and in some areas of Asia. While the application of herbicides is limited for environmental and economic reasons, breeding for resistance is regarded as the most effective solution. Closely linked molecular markers can facilitate and accelerate the process of introducing resistance genes into breeding material. Here we report mapping of a new resistance gene in LIV-17, sunflower inbred line which was resistant to broomrape populations from Spain, Romania and Turkey. Bulk segregant analyses was done on F2 population from a cross LIV-17/HA-26-PR using 210 SSR markers. Preliminary results showed that the resistance gene was placed in LG3 of the sunflower genetic map. Identification of closely linked molecular markers which will enable marker-assisted selection is underway.

Key Words : sunflower, broomrape, SSR

SCREENING OF THE PRESENCE OF OL GENE IN NS SUNFLOWER COLLECTION

Aleksandra DIMITRIJEVIĆ[1]*, Ivana IMEROVSKI[1], Dragana MILADINOVIĆ[1], Milan JOCKOVIĆ[1], Sandra CVEJIĆ[1], Siniša JOCIĆ[1], Tijana ZEREMSKI[1], Zvonimir SAKAČ[1]

[1]*Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia*

**aleksandra.dimitrijevic@nsseme.com*

ABSTRACT

Providing high quality oil is of great interest for oil companies. When it comes to sunflower oil, there are two types of oil on the market: high linoleic and high oleic. High oleic oil is considered a healthier version of oil, since it rich in omega-9 fatty acids that are oxidative more stable than linoleic fatty acid (omega-6 fatty acid), dominant in common sunflower oil. Development of high oleic sunflower genotypes was enabled by the discovery of Pervenets mutant sunflower population. In the IFVCNS, there is a great collection of sunflower inbred lines with wide range of oleic acid content (OAC). From the collection, we have chosen 62 genotypes for determination of OAC. In addition we used molecular marker reported by Schuppert et al. (2006) to screen for presence of the mutation that led to increase in OAC. The OAC in lines in which the presence of the mutation was detected ranged between 36.48 and 88.61% (mutant lines derived from high oleic line L31 – 36.48 – 56.58 and standard inbred lines 58.25 – 88.61%); while in lines where OAC varied between 14.24 and 34.46% this mutation was not detected. These results will help in choosing the best parental lines in future breeding programs, while the marker used will enable quick detection of the mutation. In addition it showed that the mutation in mutant lines most likely did not affect the analyzed part of the FAD2-1D sequence.
Key words: Oleic acid, Helinathus annuus L., marker assisted selection

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the third most important oilcrop in the world. Sunflower oil is naturally rich in polyunsaturated omega-6 fatty acid, linonelic acid, ranging between 50 and 70%, while the content of monounsaturated omega-9 fatty acid, oleic acid, ranges between 20 and 25% (Kabbaj et al., 1996). The creation of mutant sunflower cultivar Pervenets (Soldatov, 1976), obtained by dimethyl-sulfate (DMS) treatment lead to broadening of sunflower breeding programs and allowed creation of high oleic sunflower lines and hybrids. Today, the end user is dictating what type of oil is in high demand. Therefore, breeders create and conduct their breeding programs in compliance with the market demand.

In today's market, high oleic oil is considered to be healthier than high linoleic, since it rich in omega-9 fatty acids that are oxidative more stable than linoleic fatty acid. This trait is important in food industry since a lot of processing activities include higher temperature treatment or converting some unsaturated fats into saturated fats in order to achieve higher melting point. The conversion is important in margarine production because oil stays solid at room temperature. Bearing in mind benefits that high oleic oil has on human health and in industry due to their temperature stability, introduction of *Ol* gene in breeding programs originating from Pervenets became the basis for sunflower breeding for high oleic lines and hybrids, since Pervenets mutant has oleic acid content (OAC) greater than 65% (Soldatov, 1976; Lacombe and Bervillé, 2001, Lacombe et al., 2004).

There are different reports about the inheritance of OAC. Initially, Urie (1984) reported dominant mode of inheritance, while Fick (1984) reported partially dominant mode of inheritance. Later on, there were reports of existence of a modifier gene (Urie, 1985; Miller et al., 1987; Fernández et al., 1999) or one or more genes that influence OAC (Fernández-Martínez et al., 1989; Pérez-Vich et al., 2002; Velasco et al., 2000). In general, OAC varies depending on the genetic background of the recipient genotype.

The molecular change underlying the increase in OAC in Pervenets is the duplication of the *FAD2-1* allele. FAD2 (oleoyl-phosphatidyl choline desaturase) is an enzyme that catalyses synthesis of linoleic acid from oleic acid (Okuley et al., 1994). Three FAD genes are present in sunflower genome: *FAD2-1*, *FAD2-2*, *FAD2-3* (Hongtrakul et al., 1998; Martínez-Rivas et al., 2001). Of those three, only *FAD2-1* is strongly expressed in developing seeds (Hongtrakul et al., 1998). Partial duplication of this gene led to silencing of the *FAD2-1* gene, thus decreasing the activity of FAD enzyme leading to accumulation of oleic acid (Lacombe et al., 2002). *FAD2-1* was reported to cosegregate with *Ol* gene at LG14 (Lacombe and Bervillé, 2001; Pérez-Vich et al., 2002; Schuppert et al., 2006). Hongtrakul et al. (1998) and Schuppert et al. (2006) reported that duplicated sequence of *FAD2-1* does not differ from the corresponding wild type sequence.

So far, different molecular tools were used for analysis of *FAD2-1* gene (Hongtrakul et al., 1998; Dehmer, and Friedt, 1998; Lacombe and Bervillé, 2001; Lacombe et al., 2000; 2002; 2004; 2009, Schuppert et al., 2006). Some of these reports include identification or development of molecular markers for detection of *FAD2-1*. At the Institute of Field and Vegetable Crops there are 2 registered high oleic sunflower hybrids, however we are expanding our breeding program for creation of greater variety of high oleic hybrids.

In present work sunflower inbred lines were selected from a considerable sunflower collection developed at the Institute of Field and Vegetable Crops, Novi Sad (IFVCNS) and was screened for presence of *Ol* mutation by use of INDEL molecular marker reported by (Schuppert et al., 2006). This marker is developed to detect presence of *Ol* mutation since forward primer corresponds to the intergenic region present in *Ol* mutation and reverse primer is complementary to coding region of *FAD2-1*. To verify molecular results, OAC of chosen lines was analyzed by use of gas chromatography (GC). The main goal of present work was to evaluate the efficiency of the INDEL marker for marker assisted selection in IFVCNS lines and to identify the best high oleic parental lines for future crossings.

MATERIAL AND METHODS

Plant material

Chosen plant material for analysis includes lines that are used in current breeding program at the IFVCNS and vary in OAC (Table 1). Additionally, four mutant lines (M-1, M-2, M-3, M-4) derived from high oleic proprietary line developed at the IFVCNS were analyzed in order to try do detect changes on a molecular level that underlined decrease in OAC.

Plants were grown in growth chamber in Klasmann Deilmann Substrat 1 until reaching two leaf-pair stage when leaves were sampled for DNA extraction. Out of each examined sunflower line a bulk sample of 10 plants was formed and plant leaves were kept at -70^{o}C until DNA extraction.

Oleic acid content

Oil samples were obtained by pressing of 2 grams of seeds in a hydraulic press (Sirio, Mikodental 10 tons stregth, cc 400 bars) to yield approximately 0.5 ml of oil available for GC analysis. In the reaction vial 270 µl of TMSH (transesterification agens) was added to exactly 30 µl of oil, well shaked in the vortex, and kept at room temperature for an hour.

Table 1. Tested sunflower genotypes, their oleic acid content and obtained molecular profiles (presence or absence of a part of the *FAD2-1D* sequence)

Genotype - sunflower line	Oleic acid content in %	Presence of *FAD2-1D* mutation*	Genotype - sunflower line	Oleic acid content in %	Presence of *FAD2-1D* mutation *
L1	88.61	+	L34	79.17	+
L2	87.74	+	L35	78.98	+
L3	87.39	+	L36	78.56	+
L5	87.04	+	L37	77.35	+
L6	86.63	+	L38	77.06	+
L7	86.62	+	L39	76.42	+
L8	86.56	+	L40	75.33	+
L9	86.29	+	L41	74.15	+
L10	85.94	+	L42	70.22	+
L11	85.55	+	L43	69.40	+
L12	85.41	+	L44	69.33	+
L13	85.10	+	L45	68.29	+
L14	84.92	+	L46	68.29	+
L15	84.72	+	L47	63.45	+
L16	84.69	+	L48	62.04	+
L17	84.17	+	L49	58.25	+
L18	84.04	+	M-4	56.58	+
L19	83.63	+	M-3	50.13	+
L20	83.49	+	M-1	49.93	+
L22	83.45	+	M-2	36.48	+
L23	82.91	+	L50	34.46	-
L24	82.88	+	L51	28.30	-
L25	82.31	+	L52	24.52	-
L26	81.64	+	L53	22.03	-
L27	81.39	+	L54	21.53	-
L28	81.29	+	L55	21.47	-
L31	80.57	+	L56	17.31	-
L30	80.37	+	L57	17.03	-
L32	79.51	+	L58	14.24	-
L33	79.47	+			

* presence of amplified band (+), absence of amplified band (-)

The oleic acid was identified using a reference mixture of fatty acids methyl esters (FAME). A multi-standard from Supelco (FAME RM-1, Cat. no. O7006) containing the methyl esters of palmitic, stearic, oleic, linoleic, linolenic and arachidic fatty acids was used to confirm the retention times as well as to confirm that the peak areas reflected actual composition of these mixtures.

Oleic acid content analysis was performed on Agilent 5890 gas chromatograph equipped with flame ionization detector (FID) and split/splitless injector (split ratio of 1:50). The separation was performed on a fused silica capillary column (HP-INNOWAX, 30m×0.25mm i.d., and 0.25µm film thickness). Helium was used as carrier gas at a constant pressure of 53kPa at 50°C min). The temperature program was as follows: initial temperature of 50°C was held for 1 min, increased to 200°C at a rate of 25°C/min, then increased to 230°C at a rate of 3°C/min, and and hold for 18 min. The injector and detector temperatures were set at 250 and 280°C respectively. The sample volume injected was 1 µl. The results were processed using ChemStation software and expressed as the percentage of individual fatty acids in the oil sample.

Molecular analysis

DNA was extracted from leaves by use modified CTAB protocol (Permingeat et al., 1998). For detection of *FAD2-1D* sequence primer pair F4-R1 was used (Schuppert et al., 2006). PCR was performed as described by Schuppert et al. (2006) in mix described by Dimitrijević et al. (2010). Products of PCR amplification were run on 2% agarose gels and visualized with the BIO-Print system (Vilber Lourmat, Marne-La-Vallée, France).

RESULTS AND DISCUSSION

Oleic acid content varied between tested lines, ranging from 14.24 to 88.61% (Table 1, Figure 1). Thirty one sunflower line (L1-L31) had OAC higher that 80% , 22 lines (L32-L49 and M-1, M-2, M-3, M-4), had OAC ranging between 36 and 80% and 9 lines (L50-L58) had less than 36% OAC. Even though mutant lines, (M-1, M-2, M-3, M-4) originate from high oleic line, GC analysis showed significant decrease in OAC (Table 1).

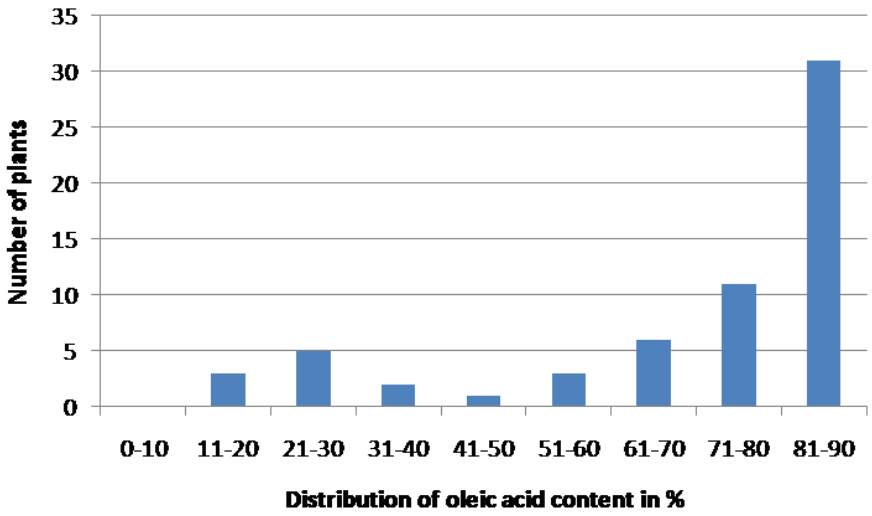


Figure 1. Distribution of oleic acid content (%) in examined sunflower genotypes

In order to examine the presence of *Ol* mutation in tested lines F4-R1 primer was used (Schuppert et al., 2006). Out of 62 chosen genotypes, seeds of three lines did not germinate; consequently they were excluded from the molecular analysis. Molecular marker used amplified a band of expected length (approximately 650 bp) in all sunflower lines, except in lines L50-L58 that had low OAC ranging from 14.24 to 34.46% (Figure 2). Presence of an amplified band in all tested mutant lines showed that there is an *Ol* mutation present in examined lines, consequently

some other changes on a molecular level must have happened and caused significant decrease in OAC. Since EMS was used for treatment of wild-type line, small nuclear changes could have occurred in *FAD2-1D* sequence, as EMS most frequently induces SNPs (G to A and C to T point mutations) (McCallum et al., 2000), as was the case with high oleic mutant lines developed by León et al. (2013). Consequently, there is a possibility that some small changes occurred in amplified sequence which could not be detected by electrophoresis. Alternatively, some changes might have occurred in other parts of *FAD2-1D* sequence or somewhere else in sunflower genome. However, this is unlikely since most of the reports on molecular changes in fatty acid composition occurred in the sequence of encoding enzymes (León et al., 2013).

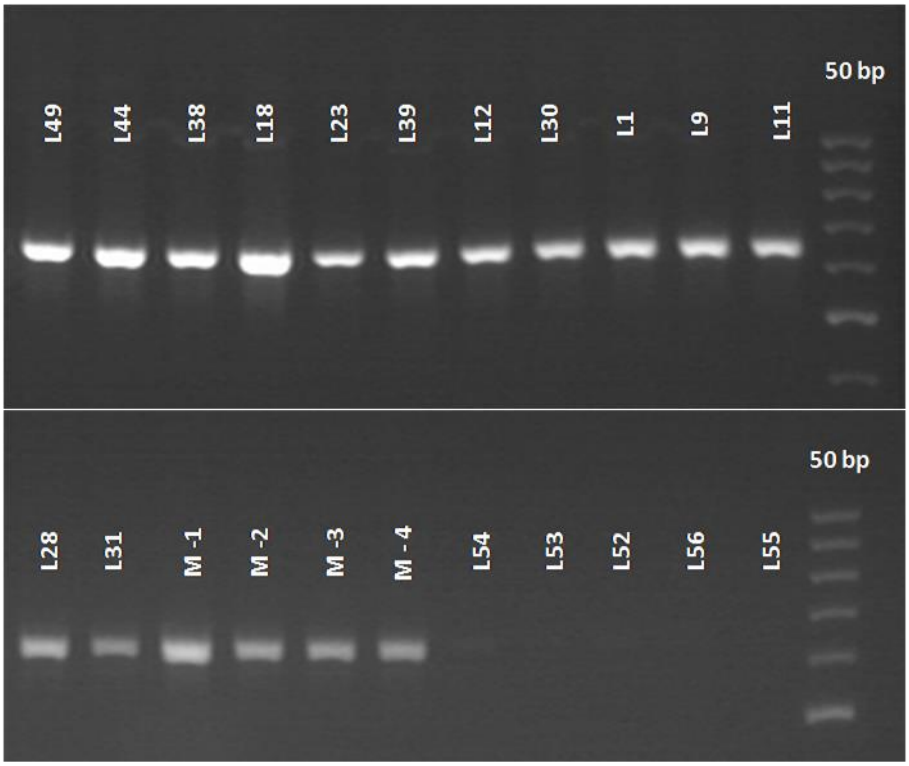


Figure 2. Molecular profiles of high oleic, low oleic and mutant sunflower lines obtained by amplification with F4-R1 (Schuppert et al., 2006) (DNA ladder 50 bp, Thermo Scientific)

In this study, we examined OAC in a set of sunflower lines and established that there is a great variation in OAC in comparison to studies performed by Lacombe et al. (2004), since in this research high oleic lines with OAC ranging from 83 to 91%. and low oleic lines with OAC ranging from 23 to 39% were used for molecular studies. The great variation in OAC could be explained by the fact that OAC is influenced not only by genetic background (Lancombe et al., 2001; Schuppert et al., 2006), but also by the environmental conditions, primarily temperature, but also by sowing date etc. (Triboï-Blondel et al., 2000; Flagella et al., 2002; Izquierdo et al., 2002; Del Gatto et al., 2015).

Molecular marker used in this study successfully identified high oleic genotypes and could therefore be used in marker assisted selection in IFVCNS. However, *Ol* mutation was detected in mutant lines that had lower OAC, as well. This means that molecular breeders should always be aware of the genetic background used in breeding and verify results with GC.

Acknowledgment: This work was supported by Ministry of Education, Science and Technological Development, Republic of Serbia, project TR 31025.

LITERATURE

Dehmer, K.J., Friedt, W. (1998). Development of molecular markers for high oleic acid content in sunflower (*Helianthus annuus* L.). Industrial Crops and Products 7(2): 311-315.

Del Gatto, A., Mengarelli, C., Pedretti, E.F., Duca, D., Pieri, S., Mangoni, L., Signor, M., Raccuia, S.A., Melilli, M.G., 2015. Adaptability of sunflower (Helianthus annuus L.) high oleic hybrids to different Italian areas for biodiesel production. Industrial Crops and Products, 75: 108-117.

Dimitrijevic, A., Imerovski, I., Miladinovic, D., Tancic, S., Dusanic, N., Jocic, S., Miklic, V. (2010). Use of SSR markers in identification of sunflower isogenic lines in late generations of backcrossing. Helia 33(53): 191-198.

Fernández, H., Baldini, M., Olivieri, A.M. (1999). Inheritance of high oleic acid content in sunflower oil. Journal of Plant Breeding and Genetics 53: 99–103.

Fernández-Martínez, J.M., Jiménez, A., Domínguez, J., García, J.M., Garcés, R., Mancha, M. (1989). Genetic control of the high oleic acid content in cultivated sunflower. Euphytica 41: 39-51.

Fick, G.N. (1984). Inheritance of high oleic acid in the seed oil of sunflower. Proc. 6th Sunflower Res. Workshop. Natl. Sunflower Assoc., Bismarck, USA. 9.

Flagella, Z., Rotunno, T., Tarantino, E., Di Caterina, R., De Caro, A. (2002). Changes in seed yield and oil fatty acid composition of high oleic sunflower (*Helianthus annuus* L.) hybrids in relation to the sowing date and the water regime. European journal of agronomy 17(3): 221-230.

Hongtrakul V., Slabaugh M.B., Knapp S.J. (1998). A seed specifc D12 oleate desaturase gene is duplicated, rearranged, and weakly expressed in high oleic acid sunflower lines. Crop Science 38: 1245-1249.

Izquierdo, N., Aguirrezábal, L., Andrade, F., Pereyra, V. (2002). Night temperature affects fatty acid composition in sunflower oil depending on the hybrid and the phenological stage. Field Crops Research 77(2): 115-126.

Kabbaj, A., Vervoort, V., Abbott, A.G., Tersac, M., Bervillé, A. (1996). Polymorphism in *Hetianthus* and expression of stearate, oleate and linoleate desaturase genes in sunflower with normal and high oleic contents. Helia 19(25): 1-18.

Lacombe, S. Bervillé, A. (2001). A dominant mutation for high oleic acid content in sunflower (*Helianthus annuus* L.) seed oil is genetically linked to a single oleate-desaturase RFLP locus. Molecular Breeding 8(2): 129-137.

Lacombe, S., Guillot, H., Kaan, F., Millet, C. Bervillé, A. (2000). Genetic and molecular characterization of the high oleic content of sunflower oil in Pervenets. Proc. 15th Int. Sunflower Conf, Toulouse, France. 12-15.

Lacombe, S., Kaan, F., Griveau, Y. Bervillé, A. (2004). The pervenets high oleic mutation: methodological studies. Helia 27(40): 41-54.

Lacombe, S., Leger, S., Kaan, F., Berville, A. Sas, M. (2002). Genetic, molecular and expression features of the Pervenets mutant leading to high oleic acid content of seed oil in sunflower. Oléagineux, Corps gras, Lipides 9(1): 17-23.

Lacombe, S., Souyris, I. and Bervillé, A.J. (2009). An insertion of oleate desaturase homologous sequence silences via siRNA the functional gene leading to high oleic acid content in sunflower seed oil. Molecular Genetics and Genomics 281(1): 43-54.

León, A.J., Zambelli, A.D., Reid, R.J, Morata, M.M., Kaspar, M., Martínez-Force, E., Garcés, R., Salas, J.J., Venegas-Caleron, M. (2013). Isolated Mutated Nucleotide Sequences That Encode a Modified Oleate Destaurase Sunflower Protein, Modified Protein, Methods and Uses. WIPO Patent WO/2013/004280, Jan 10, 2013.

Martínez-Rivas, J.M., Sperling, P., Luehs, W., Heinz, E. (2001). Spatial and temporal regulation of three different microsomal oleate desaturase genes (FAD2) from normal-type and higholeic varieties of sunflower (*Helianthus annuus* L.). Molecular Breeding 8 (2): 159-168.

McCallum, C.M., Comai, L., Greene, E.A., Henikoff, S. (2000). Targeted screening for induced mutations. Nature biotechnology 18(4): 455-457.

Miller, J.F., D.C. Zimmerman, Vick, B.A. (1987). Genetic control of high oleic acid content in sunflower oil. Crop Science 27(5): 923-926.

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E., Browse, J. (1994). Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell 6(1): 147–158.

Pérez-Vich, B., R. Garcés, Fernández-Martínez, J.M. (2002). Inheritance of high palmitic acid and its relationship with high oleic acid content in the sunflower mutant CAS 12. Plant Breeding 121: 49-56.

Permingeat, H.R., Romagnoli, M.V., Vallejos, R.H. (1998). A simple method for isolating high yield and quality DNA from cotton (*Gossypium hirsutum* L.) leaves. Plant Molecular Biology Reported 16: 1-6.

Schuppert, G.F., Tang, S., Slabaugh, M.B., Knapp, S.J. (2006). The sunflower high-oleic mutant Ol carries variable tandem repeats of FAD2-1, a seed-specific oleoyl-phosphatidyl choline desaturase. Molecular Breeding 17(3): 241-256.

Soldatov, K.I. (1976). Chemical mutagenesis in sunflower breeding. Proc 7th Intl Sunflower Conf Krasnodar, USSR, Vlaardingen, the Netherlands: Intl. Sunflower Assoc., pp. 352–357.

Triboï-Blondel, A., Bonnemoy, B., Falcimagne, R., Martignac, M., Messaoud, J., Philippon, J. Vear, F. (2000). The effect of temperature from flowering to maturity on seed composition of high oleic sunflower inbreeds and mid oleic hybrids. In Proc of the 15th Intern. Sunf. Conf. Toulouse, France. A67-A72.

Urie, A.L. (1985). Inheritance of high oleic acid in sunflower. Crop Science 25(6): 986-989.

Urie, A.L. (1984). Inheritance of very high oleic acid content in sunflower. Proc 6th Sunflower Res. Workshop. Natl. Sunflower Assoc., Bismarck, USA. 9-10.

Velasco, L., Pérez-Vich, B., Fernández-Martínez, J.M. (2000). Inheritance of oleic acid content under controlled environment. Proc. 15th Int. Sunflower Conf, Toulouse, France. A31-A36.

SEASONAL TIME-COURSE OF EXPANSIN EXPRESSION IN FLOWERS AND GROWING GRAINS OF SUNFLOWER (*HELIANTHUS ANNUUS* L.)

***Francisca CASTILLO*[1,2] *- Santiago VÁSQUEZ*[1,2] *– Alex GONZALEZ*[2] *- Carolina LIZANA*[2] *- Alejandro CLAUDE*[3] *- Daniel CALDERINI*[2]**

[1]*Graduate School, Faculty of Agricultural Sciences, Universidad Austral de Chile, Valdivia, Chile*
[2]*Plant Production and Plant Protection Institute, Faculty of Agricultural Science, Universidad Austral de Chile, Valdivia, Chile*
[3]*Biochemistry and Microbiology Institute, Faculty of Sciences, Universidad Austral de Chile, Valdivia, Chile*

**castillo.francisca88@gmail.com*

ABSTRACT

Grain weight is a key component of yield and quality of sunflower. Taking into account that the ovaries of flowers became the pericarp of grains in grasses and dicots such as sunflower, it has been proposed that the maternal tissues impose a physical restriction to growing grains in these crops. The physiological processes supporting the hypothesis that the pericarp controls grain weight (GW) are only starting to be understood. Expansins (Expns.) are proteins that play a key role in plant cell growth by inducing the loosening of cell walls in plants, which determines the cellular growth expansion. The objective of the present study was to assess the seasonal time-course expression of Expn. genes in ovary, pericarp and embryo of flowers and growing grains of sunflower. Two sunflower genotypes contrasting in grain size and weight were sown in a split-plot design with three replicates at the Agricultural Research Station of the Universidad Austral de Chile in Valdivia, Chile. Ovaries and grains (divided in pericarp and embryo) were sampled at pre-anthesis and post-anthesis, respectively. Relative quantification of mRNA levels of Expns. was evaluated by qPCR. Final GW was different between genotypes (80 and 150 mg). Expression analysis by qPCR showed that specifics Expns. (Expn.3, 4 and 5) are associate better with grain growing dynamics in sunflower, and a variation in the expression of Expns. between genotypes contrasting in GW and in flower and grain tissues across the developmental stages.

Keywords: grain weight, expansin expression, yield, sunflower

INTRODUCTION

Expansins (Expns.) are proteins inducing cell wall extension (McQueen-Mason et al. 1992). They have been known as "factors that loosen the cell wall", allowing relaxation of the cell wall during cell expansion, playing a major role in growth. Expns. are involved in different physiological processes as the cell wall disassembly during fruit ripening (Brummel et al. 1999), organogenesis of leaves (Fleming et al., 1997), differentiation of vascular cells (Cho and Kende, 1998) and root system architecture (Ma et al., 2013; Marowa et al., 2016); however, there is little information about the role of Expansins in specific tissues like grains.

A recent study shows the evolutionary divergence between classes of Expns. especially α and β groups in grass and dicots plants, due to the difference in the composition of the cell wall,

highlighting that the β Expns. family has expanded considerably in grasses (Sampedro et al., 2015). This was important to consider when characterize Expns. in reproductive structures of sunflower.

In wheat, it has been estimated that there are at least 30 α and 65 β Expns. Generally, a multigene family suggests that different members may play unique developmental or tissue-specific roles. This may be the situation of the α Expn. group, which has shown organ or development stage specificity in wheat (Liu et al., 2007). The present study hypothesizes that some Expns. will be specifically involved in the pericarp extension, and that their expression could be related to grain growth dynamics in sunflower. Previous experiments found expression of 6 different Exp. cDNA sequences in pericarp tissues of wheat at 14 days after anthesis. Out of the 6, 4 matched known wheat Expn., whereas the other 2 were novel wheat Expns., similar to sequences described for *Festuca pratensis* and *Oryza sativa* (Calderini et al., 2006; Lizana et al., 2010).

A strong relationship between grain size dynamics, water content and Expn. expression was found in wheat grains during grain filling. Water content of grains was consistent with high abundance of Expns. gene transcripts (Lizana et al., 2010). Plant cell expansion is turgor-driven and regulated by cell wall mechanical properties and is related to Expns. proteins (Cosgrove, 2015), a key component in grain enlargement according to Lizana et al. (2010).

From the background outlined above, the expression of TaExpA6 transcript is consistent with grain elongation in wheat (Lizana et al., 2010). Therefore, the following questions arise:

i) Are there Expns. associated with grain growth in sunflower?
ii) Is the elongation of the pericarp and embryo driven by different Expns.?
iii) Is the timing of Expn. expression similar between the pericarp and embryo?

The objectives of this work was to identify the expression of Expns. And their time course in ovary, pericarp and embryo of sunflower during the growth of the reproductive organs.

The study of Expns. at both pre- and post-pollination and their relationship with the dynamics of grain growth will be key to a better understanding of processes controlling GW. It will also make it possible to establish the relationships among dry matter, water content, and Expns. expression in flowers and growing grains of sunflower.

MATERIALS AND METHODS

Plant material and field experiment

Two sunflower genotypes contrasting in GW and adapted to the south of Chile, Alybro (oilseed) and RHA280 (confectionery) were sown under field conditions at the Experimental Station of the Universidad Austral de Chile in Valdivia (39◦47′S, 73◦14′W). The genotypes were laid out in a randomized complete block design with three replicates. Sowing date were 20 of October as in previous evaluations in Valdivia. Plots consisted of seven rows, 0.70 m apart and 5 m long with a plant population density of 6 plants m^{-2}. Plots were fertilized at sowing with N, P and K based on soil analysis, ensuring that the crop was not affected by any nutrient limitation. Weeds, insects and diseases were prevented or controlled, and regular watering was supplied to complement rainfall throughout the experiment to avoid water stress.

Phenology, flower and grain sampling

Phenology of the crop was followed during the growing season according to the scale by Schneiter and Miller (1981). Individual plants were tagged at R3 (Schneiter and Miller, 1981) to evaluate the seasonal time-course of development, dry matter, water content and dimensions of

flowers and grains as in previous studies (Rondanini et al., 2009). Grains were split into pericarp and embryo at each sampling. Two capitula per replicate were harvested at 2 or 3 days intervals, florets and grains were sampled from two places in the capitulum at each sampling date (grains from peripheral position). Harvested flowers and grains were immediately processed (to measure fresh weight and dimensions) or preserved into cryotubes and quickly immersed in liquid nitrogen for molecular analysis. Samples were stored at -80°C until processing.

Grain weight and dimension measurements

Four flowers and grains from the peripheral position were weighed after harvest at each sampling date to determine fresh weight. In the case of grains, they were immediately separated into pericarp and embryo and each component weighed. In early phases of grain development the embryo included the aqueous endosperm (present at the early stages of embryo development and consumed during the embryo growth). Plant material were dried for 72 h at 60°C to determine dry weight.

Absolute water content (mg) of flowers and grains (pericarp and embryo) were calculated as the difference between fresh and dry weight, while water concentration (%WC, on a fresh weight basis) was estimated as the ratio between absolute water content and fresh weight, expressed as a percentage. Flower and grain dimensions (length, width, and height) were recorded quickly after sampling using an electronic caliper; this was measured in four grains.

Molecular analysis: In silico analysis and primer design

The first objective was to identify *in silico* the Expns. sequence genes expressed in grain of sunflower. Public databases of sunflower genome (https://www.heliagene.org/) enabled a search for putative Expns. using the BLAST tool with *Helianthus annuus* trascriptome and Zinnia elegans Exp. 3 mRNA sequence (GenBank: AF230333.1) because this is a related species of sunflower. The sequences obtained from BLAST were evaluated using the bioinformatic tool option "expression patterns" making it possible to consider Expns. putative expression patterns in plant organs. Expns. sequences expressed mainly in grain were chosen.

Selected sequences were aligned using the program Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to reveal the number of unique sequences. Sequences were searched against the non-redundant GenBank DNA and protein database using BLASTn and BLASTX (Altschul et al., 1990, 1997) and against the Uni Prot database resources using BLASTX. The best matches were used as the foundation for sequence identity-based annotations. Sequences were used in BLASTx searches to confirm that they correspond to Expn. Transcripts. In addition, nucleotide sequences were translated into protein with the ExPASy bioinformatic tool (http://web.expasy.org/translate/) to mark off the coding region for the design of specific primers. These primers were designed using the "PRIMIQUE" tool to detect different sequences of the gene family (Fredslund and Lange, 2007). Two primer pairs were chosen for the same sequence.

A bibliographic search were conducted for housekeeping genes of sunflower for use as an endogenous control to normalize the data for differences in input RNA and the efficiency of reverse transcription between the various samples. Primers reported by previous studies for elongation factor 1 (EF1), S19 protein, β-tubulin, actin, ubiquitin and 18S of sunflower grains were evaluated (Brunner et al., 2004; Layat et al., 2014; Meimoun et al., 2014; Oracz et al., 2008; Pramod et al., 2012).

RNA extraction and RT-PCR

Total RNAs were isolated with the RNeasy Plant Mini kit (Qiagen) according with the manufacturer's instructions. The kit provides a choice of lysis buffers depending on the amount and type of secondary metabolites in the tissue; therefore, the RNA extraction protocol should be standardized. The quality and concentration of RNA were measured by spectroscopy with Nanodrop (nd-1000, Thermo Fisher Scientific, USA).

The isolated RNA was pretreated with DNasaI. First-strand cDNA was synthesized from 250 ng RNA using the ImProm-II™ Reverse Transcription System. The oligo(dt)16-18 primer/template mix was thermally denatured at 70°C for 5 minutes and chilled on ice. A reverse transcription reaction mix was assembled on ice to contain nuclease-free water, reaction buffer, reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor. 1u/µl of Recombinant RNasin® Ribonuclease Inhibitor was added. The template-primer combination was added to the reaction mix on ice. Following an initial annealing at 25°C for 5 minutes, the reaction was incubated at 42°C for up to one hour. The synthesized cDNA (20µl) was stored at -20°C. As a negative control, an RNA sample was replaced by water in this procedure.

Quantification of mRNA levels by using real time PCR (qPCR)

The PCR reaction was performed in a final volume of 25µL and containing 12,5µL Brilliant II SYBR Green PCR Master Mix (Stratagene, Agilent technologies), 1 µL 10 µM forward and reverse primers and 8,5 µL of sterile deionized water. After an initial DNA polymerase activation step at 95◦C for 10 min, the samples were subjected to 35 amplification cycles (95◦C for 15 s,60◦C for 15 s, and 72◦C for 15 s). No-template and no-transcriptase controls were included to detect genomic DNA contamination.

A melting curve was generated by incubating the reaction at 95°C for 15 s, 25°C for 1 s, and 70◦C for 15 s and then slowly increasing the temperature to 95 ◦C. The target gene expression was quantified with the method proposed by Livak (2001) by using the Agilent AriaMx software to calculate the transcript abundance relative to the calibrator, which becomes sample 1, and all other quantities are expressed as an n-fold difference relative to the calibrator.

After confirming the amplified specific products, a standard curve of each pair of primers was created with the product from the previous amplification. A dilution of 1: 1000 was prepared and then 7 serial dilutions were prepared by a factor of 10 starting from the 1:1000 dilution of the previously amplified product. This was achieve the efficiency of the primers.

Samples were subjected to service sequencing. Resulting sequencing chromatograms be viewed, evaluated and aligned. Sequence alignments and database searches were carried out using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and https://www.heliagene.org/.

Statistical analysis

Final grain weight was estimated using a bilinear model as in Calderini et al. (1999). The model was fitted using the iterative optimization technique of Table curve V 3.0 (Jandel, 1991). Data of final grain weight and variables from grain growth dynamics were assessed by ANOVA using the software STATISTICA v. 7.0 (Stat Soft, Inc., 2004). The LSD test (5%) was employed for differences among genotypes.

RESULTS AND DISCUSSION

Grain weight and dimensions were significantly different between genotypes ($P \leq 0.001$) as we expected. Grains of the confectionery genotype (RHA280) were havier in RHA280 (149 mg) than in Alybro (79 mg) as well as grain dimensions (Fig. 1a and b). These differences were evident as early as 3 days after antehsis (DAA) (Fig. 1). Moreover, a linear association ($r^2= 0.77$)

between final grain weight and ovary weight was found in this study (data not shown). These results agree with previous studies where positive relationships between grain weight and the weight/size of carpels at anthesis have been reported in sunflower (Cantagallo et al., 2004), sorghum (Yang et al., 2009) and wheat (Hasan et al., 2011). Therefore, the pre-anthesis period has proven critical to determining GW (Calderini et al., 1999; Ugarte et al., 2007). RHA280 had a mean of 465 filled grains and 184 empty grains per capitulum, and Alybro, 1497 filled grains and 80 empty grains per capitulum (data not shown).

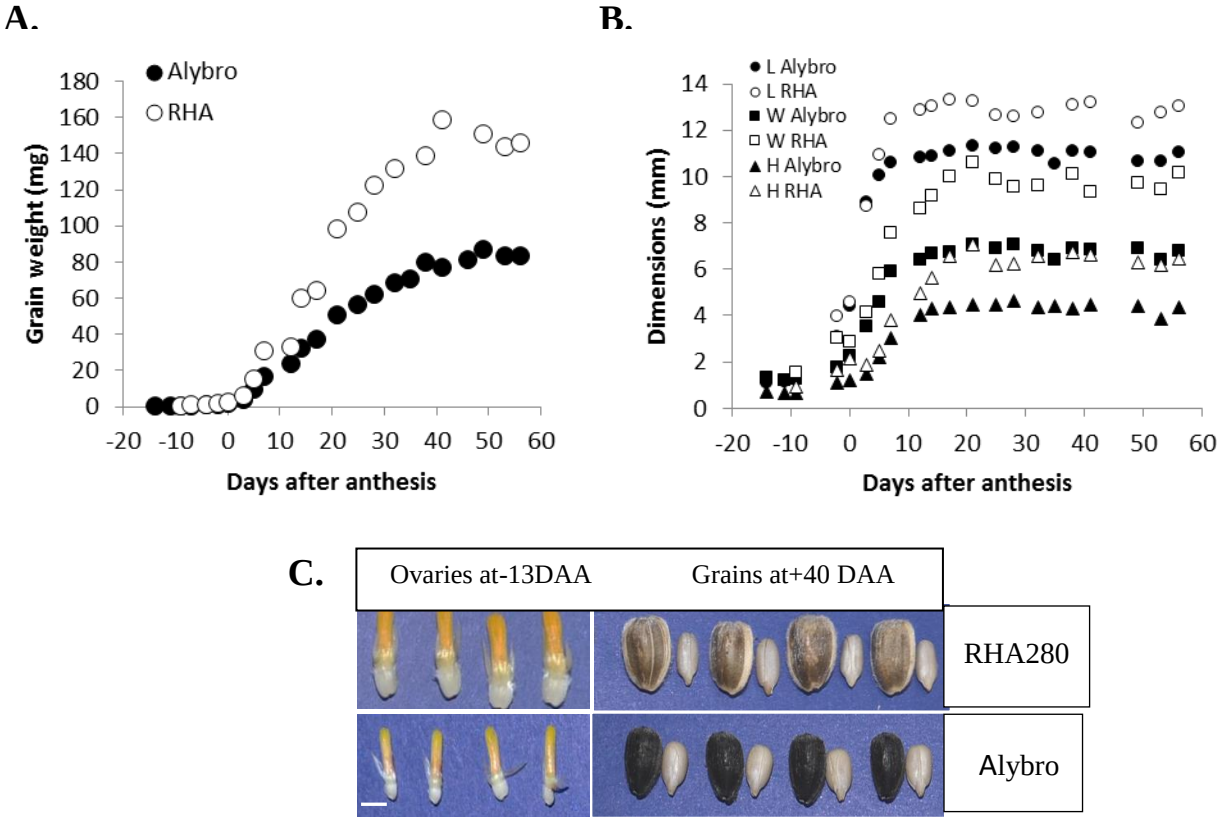


Figure 1. **Time-course of grain weight and grain dimensions of peripheral grains of two sunflowers genotypes (RHA280 and Alybro).** Dynamics of grain dry weight (A) and dimensions (B) of RHA280 (open symbols) and Alybro (closed symbols) are shown. The photos of ovaries (-13 DAA) and grains (+40 DAA) of both genotypes are also shown, Scale bar: 4 mm. (C). Grains were separated into pericarp and embryo. L: Length, W: width, H: high.

The expression of three putative Expns. Genes, named by us Expn. 3, Expn. 4 and Expn. 5 (accession in Heliagene database: Ha412T4l900C0S1, HaT13l007346, HaT13l009552, respectively) according to the name of the similar Expn. sequence with a maximum value of identity in the results of a BLAST, were chosen and evaluated by qPCR along the development of the reproductive organs in both genotypes.

Taking into account the dynamics showed in Figs. 1 and 2, it was found that some Expns. could be specifically involved in the grain tissues extension, suggesting their expression would control grain size of sunflower. For example, Expn. 4 follows a similar time course than the grain growth dynamic of genotypes (Fig. 2). Lizana et al., 2010 showed that the expression of few Expns. is associated with grain elongation when the grain is growing in wheat. The present is the first study showing parallelism between growing grains and Expns. expression in grain tissues of sunflower.

In addition to the time course of expression, specifics Expns. Seem to be controlling the extension of ovary, pericarp and embryo. Expn. 3 and Expn. 4 was found specific to maternal tissues (ovary and pericarp) and Expn. 3 was more abundant in the pericarp. On the other hand, Expn. 5 was found more abundant in the embryo (Fig. 2). The timing of Expn. 3 and Expn.4 in the pericarp showed a higher abundance at +7 DAA when in sunflower, the growth of the pericarp levels off soon after flowering, i.e., 8 days after anthesis at R5.1 (Rondanini et al., 2009; Lindström and Hernández, 2015). Interestingly, the confectionery genotype showed higher abundance of Expns. genes later than the oil genotype Alybro (Fig. 2), suggesting that Expns. isoforms control the growth in flowers and grains of sunflower. Furthermore, it has been hypostatized that the pericarp imposes a physical restriction to growing grains in grasses (Calderini et al., 1999; Ugarte et al., 2007; Yang et al., 2009), which might explain the importance of the pre-flowering phase for GW determination.

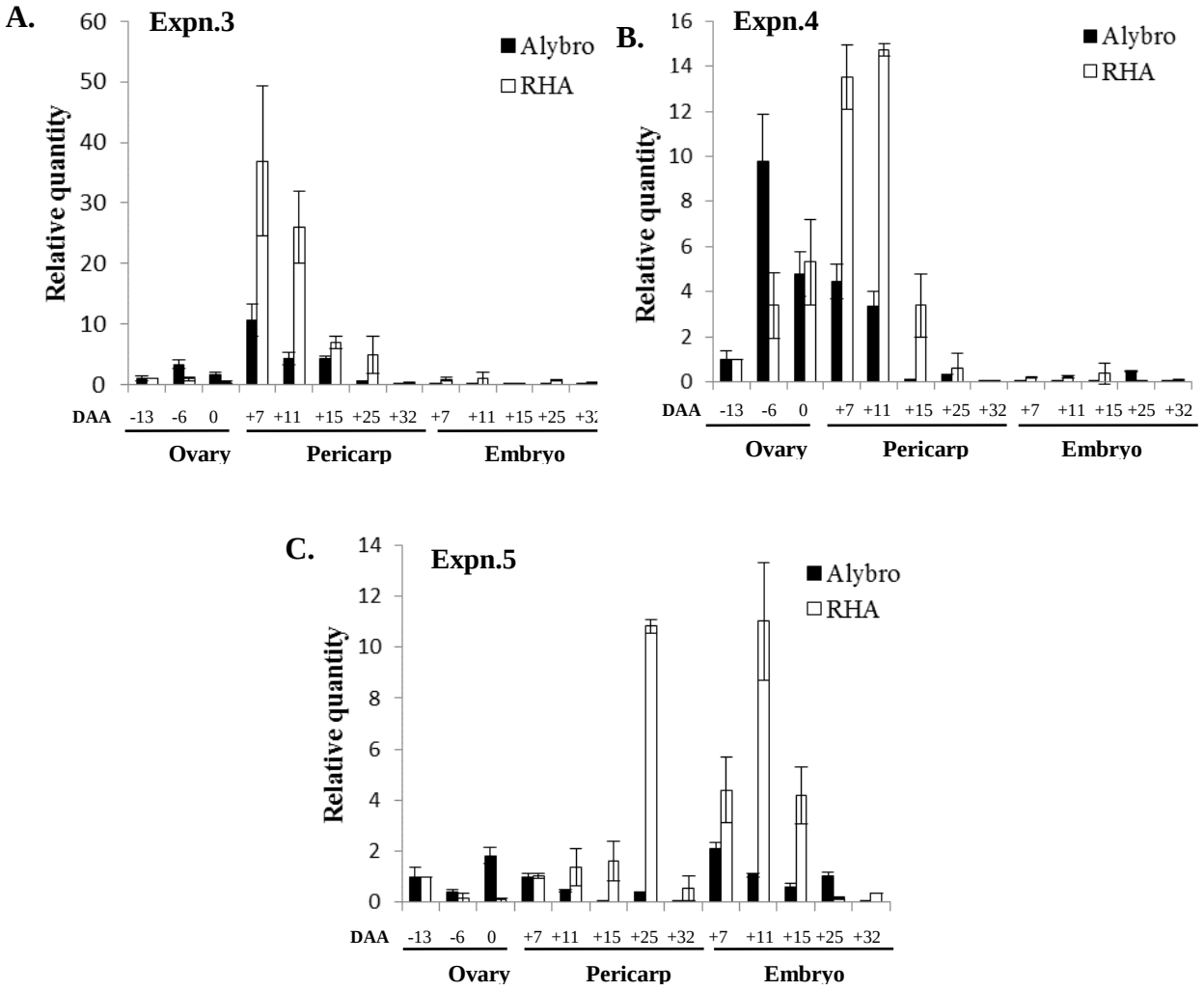


Figure 2. Seasonal time-course of expansin expression in ovary, pericarp and embryo of sunflower. A, B, C. Relative quantity analysis of three expansins genes, this target genes were quantified and normalized to β Tubulina using the Livak method (n=3).

In dicots, such as the model plant *Arabidopsis thaliana*, the control of GW by the growth of the inner integument during ovary and early phases of seed development has been demonstrated,

supporting the hypothesis that potential GW is controlled by the outer seed tissues (Adamski et al., 2009; Fang et al., 2012; Xia et al., 2013; Du et al., 2014). Remarkably, ovary mass at anthesis associate with final pericarp mass across both sunflower genotypes (see Table 2 in Lindström and Hernández, 2015). The present study support the hypothesis that the Expns. play an important role in the extension of grain maternal tissues. The importance of the pericarp on early grain development of wheat was stated many years ago (Rijven and Banbury, 1960), but the physiological processes through which the pericarp controls the final size of the grain is only now beginning to be understood (e.g., Garcia et al., 2005; Léon-Kloosterziel et al., 1994; Schruff et al., 2006; Song et al., 2007).

In sunflower and other crops, such as wheat, sorghum and coffee tree, maternal tissues (ovary/pericarp) have been proposed to control the potential grain size (Calderini and Reynolds, 2000; Yang et al., 2009; Budzinski et al., 2011; Lindström et al., 2006). In these crop species, the maternal tissues undergo rapid cell wall division and expansion, which in turn might determine the physical limit of the endosperm or embryo of the fruit (Budzinski et al., 2011). During the rapid cell wall expansion is where the Expns. proteins are acting.

In a previous study, transcripts of CaExpA2 were detected only in the pericarp of coffea fruit during the later stages of fruit maturation and ripening, highlighting the participation of these isoforms in the regulation of fruit size (Budzinski et al., 2011). Another study addressing fruit elongation showed two Expns. genes (ExpA4-a and ExpA5) inducing fruit length in cucumber, suggesting that the cell-wall related genes are required for fruit elongation in these species (Jiang et al., 2015). Studies by Harrison et al. (2001) found two Expns. in strawberry highly expressed during the process of rapid expansion of immature fruit, while four were expressed mainly during fruit ripening. In agreement, similar results were found in peach (Hayama et al. 2003), pear (Hiwasa et al. 2003) and banana (Asha et al. 2007), showing that high levels of mRNA coding for Expns. were observed in rapidly growing tissues, and multi-gene family of Expns. exhibited differential expression patterns for the different phases of fruit development. In the light of results shown in the present and previous studies, we propose Expn. 4 as a driver of grain growth in sunflower.

CONCLUSIONS

The analysis of results showed in this study supports different expression of Expns. genes between genotypes and across the developmental stages of flower and grain growth. The expression of Expns. variable in ovary, pericap and embryo tissues.

Expns. genes associated with grain dynamics would contribute to a better understanding of mechanisms controlling grain size in sunflower, especially Expn. 4, it is a good candidate for future research.

ACKNOWLEDGEMENTS

Staff of EEAA. Laboratory of crop physiology and molecular biology, Universidad Austral de Chile. Fondecyt 1141048. Conicyt Ph.D scholarship.

LITERATURE

Adamski, N.M., E. Anastasiou, S. Eriksson, C.M. O'Neill, and M. Lenhard. 2009. Local maternal control of seed size by KLUH/CYP78A5-dependent growth signaling. Proceedings of the National Academy of Sciences 106:20115-20120.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 25, 3389–3402.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol*. 215: 403–410.

Asha, V.A., Sane, A.P. & Sane, P.N. 2007. Multiple forms of α-expansin genes are expressed during banana fruit ripening and development. Postharvest Biology and Technology. 45, 184-192

Brummell, D.A., M-H. Harpster, P. Dunsmuir, 1999. Differential expression of expansin gene family members during growth and ripening of tomato fruit. *Plant Mol Biol*. 39:161-169.

Brunner, A. M., Yakovlev, I. a, & Strauss, S. H. 2004. Validating internal controls for quantitative plant gene expression studies. BMC plant biology, 4, 14.

Budzinski, I. G. F., Santos, T. B., Sera, T., Pot, D., Vieira, L.G. E., & Pereira, L. F. P. 2011. Expression patterns of three α-expansin isoforms in Coffea arabica during fruit development. Plant Biology, 13(3), 462–471.

Cantagallo, J.E., Medan, D., Hall, A.J., 2004. Grain number in sunflower as affected by shading during floret growth, anthesis and grain setting, Field Crops Res. 85, 191–202.

Calderini, D.F., L.G. Abeledo, R. Savin, and G.A. Slafer. 1999. Final grain weight in wheat as affected by short periods of high temperature during pre- and post-anthesis under field conditions. Australian Journal of Plant Physiology. 26: 453-458.

Calderini, D.F., and M.P. Reynolds. 2000. Changes in grain weight as a consequence of de-graining treatments at pre- and post-anthesis in synthetic hexaploid lines of wheat (Triticum durum x T-tauschii). Aust. J. Plant Physiol. 27:183-191.

Calderini D.F., M.P. Reynolds, G.A. Slafer, 2006. Source-sink effects on grain weight of bread wheat, durum wheat and triticale at different locations. Aust. J. Agric. Res. 57: 227-233.

Cosgrove, D. J. 2015. Plant expansins: diversity and interactions with plant cell walls. Current Opinion in Plant Biology, 25, 162–172.

Cho, H., H. Kende, 1998. Tissue localization of expansins in deepwater rice. Plant J 15:805-812.

Du L, Li N, Chen L, Xu Y, Li Y, Zhang Y, Li C. 2014. The ubiquitin receptor DA1 regulates seed and organ size by modulating the stability of the ubiquitin-specific protease UBP15/SOD2 in Arabidopsis. The Plant Cell 26, 665–677.

Fang W, Wang Z, Cui R, Li J, Li Y. 2012. Maternal control of seed size by EOD3/CYP78A6 in Arabidopsis thaliana. The Plant Journal 70, 929–939.

Fleming AJ, McQueen-Mason S, Mandel T, Kuhlemeier, C. 1997. Induction of leaf primordia by the cell wall protein expansin. Science 276: 1415–1418.

Fredslund, J., & Lange, M. 2007. Primique: automatic design of specific PCR primers for each sequence in a family. *BMC bioinformatics*, *8*, 369.

Garcia, D., J.N. Fitz Gerald, and F. Berger. 2005. Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in Arabidopsis. Plant Cell 17:52-60.

Harrison, E. P., McQueen-Mason, S. J. and Manning, K. 2001. Expression of six expansin genes in relation to extension activity in developing strawberry fruit. Journal of Experimental Botany 52: 1437-1446.

Hasan, A.K., J. Herrera, C.X. Lizana and D.F. Calderini. 2011. Carpel weight, grain length and stabilized grain water content are physiological drivers of grain weight determination of wheat. Field Crops Research. 123: 241-247.

Hayama, H., Ito, A., Moriguchi, T., & Kashimura, Y. 2003. Identification of a new expansin gene closely associated with peach fruit softening. Postharvest Biology and Technology, 29(1), 1–10.

Hiwasa, K., Rose, J.K., Nakano, R., Inaba, A. & Kubo, Y. 2003. Differential expression of seven_α-expansin genes during growth and ripening of pear fruit. Physiology Plantarum 117, 564-572.

Jandel, 1991. Table Curve v. 3.0. User's manual for version 3.0 AISN software. JandelScientific, Corte Madera, CA.

Layat, E., Leymarie, J., El-Maarouf-Bouteau, H., Caius, J., Langlade, N., & Bailly, C. 2014. Translatome profiling in dormant and nondormant sunflower (*Helianthus annuus*) seeds highlights post-transcriptional regulation of germination. *The New phytologist*, *204*(4), 864–72.

Léon-Kloosterziel, K.M., C.J. Keijzer, and M. Koornneef. 1994. A seed shape mutant of arabidopsis that is affected in integument development. Plant Cell 6:385-392.

Lindström, L. I., & Hernández, L. F. 2015. Developmental morphology and anatomy of the reproductive structures in sunflower (Helianthus annuus): a unified temporal scale, 10(November 2014), 1–10.

Liu, Y., Liu, D., Zhang, H., Gao, H., Guo, X., Wang, D., Zhang, X., Zhang, A., 2007. The α and β-expansin and xyloglucanendotransglucosylase/hydrolase gene families of wheat: molecular cloning, gene expression, and EST data mining. Genomics. 90, 516–529.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using realtime quantitative PCR and the 2ΔΔC(T) Method. Methods 25(4): 402–408.

Lizana, C.X., R. Riegel, L.D. Gomez, J. Herrera, A. Isla, S.J. McQueen-Mason and D.F. Calderini. 2010. Expansins expression is associated with grain size dynamics in wheat (*Triticum aestivum* L.). Journal of Experimental Botany. 61: 1147-1157.

Ma N, Wang Y, Qiu S, Kang Z, Che S, Wang G, Huang J. 2013. Overexpression of OsEXPA8, a root-specific gene, improves rice growth and root system architecture by facilitating cell extension. *PloS One* 8, e75997.

Marowa, P., Ding, A., & Kong, Y. 2016. Expansins: roles in plant growth and potential applications in crop improvement. *Plant Cell Reports.*

McQueen-Mason SJ, Durachko DM, Cosgrove DJ. 1992. Two endogenous proteins that induce cell wall extension in plants. Plant Cell. 4: 1425-1433.

Meimoun, P., Mordret, E., Langlade, N. B., Balzergue, S., Arribat, S., Bailly, C., & El-Maarouf-Bouteau, H. 2014. Is gene transcription involved in seed dry after-ripening? *PloS one*, *9*(1), e86442.

Oracz, K., El-Maarouf-Bouteau, H., Bogatek, R., Corbineau, F., & Bailly, C. 2008. Release of sunflower seed dormancy by cyanide: cross-talk with ethylene signalling pathway. Journal of experimental botany, 59(8), 2241–51.

Pramod, S., Downs, K. E., & Welch, M. E. 2012. Gene expression assays for actin, ubiquitin, and three microsatellite-encoding genes in *Helianthus annuus* (Asteraceae). American journal of botany, 99(9), e350–2.

Rijven, A.H.G.C., and C.A. Banbury. 1960. Role of the grain coat in wheat grain development. Nature. 188: 546-547.

Rondanini, D.P., Mantese, A.I., Savin, R., Hall, A.J. 2009. Water content dynamics of achene, pericarp and embryo in sunflower: Associations with achene potential size and dry-down. Eur. J. Agron. 30: 53–62.

Sampedro, J., Guttman, M., Li, L.-C., & Cosgrove, D. J. 2015. Evolutionary divergence of β-expansin structure and function in grasses parallels emergence of distinctive primary cell wall traits. The Plant journal : for cell and molecular biology, *81*(1), 108–20.

Schneiter and Miller. 1981. Description of Sunflower Growth Stages. Crop Science, Vol. 21.

Schruff, M. C., Spielman, M., Tiwari, S., Adams, S., Fenby, N., & Scott, R. J. 2006. The AUXIN RESPONSE FACTOR 2 gene of Arabidopsis links auxin signalling, cell division, and the size of seeds and other organs. Development (Cambridge, England), 133(2), 251–61.

Song, X.-J., W. Huang, M. Shi, M.-Z. Zhu, and H.-X. Lin. 2007. A QTL for rice grain width and weight encodes a previously unknown RING-type E3 ubiquitin ligase. Nat. Genet. 39:623-630.

Ugarte C., D.F. Calderini and G.A. Slafer. 2007. Grain weigh and grain number responsiveness to pre-anthesis temperature in wheat, barley and triticale. Field Crops Research. 100: 240-248

Xia T, Li N, Dumenil J, Li J, Kamenski A, Bevan MW, Gao F, Li Y. 2013. The ubiquitin receptor DA1 interacts with the E3 ubiquitin ligase DA2 to regulate seed and organ size in Arabidopsis. The Plant Cell 25, 3347–3359.

Yang, Z., Oosterom, E.J.V., Jordan, D.R., Hammer, G.L., 2009. Pre-anthesis ovary development determines genotypic differences in potential kernel weight in sorghum. J. Expt. Bot. 60: 1399–1408.

CHARACTERISATION AND MAPPING OF A LOCUS CONTROLLING LIGHT-YELLOW RAY FLORETS IN SUNFLOWER

Clemence PARIS [1], Bertrand HAQUIN [1], Frantz BOUDET [1], Piquemal JOEL[2]

[1] SYNGENTA, FRANCE
[2] SYNGENTA, FRANCE

Joel.Piquemal@syngenta.com

ABSTRACT

In Sunflower, Ray Florets (RF) color may be yellowish-white, light-yellow, medium-yellow, orange-yellow, orange, purple or reddish brown. However among this high variability of colors, the most widespread encountered in Elite germplasm is medium-yellow. In Syngenta germplasm, some lines display a light-yellow coloration of RF. This light-yellow trait is referenced as a DUS (Distinctiveness, Uniformity, and Stability) trait by official instances for registration. It has been found by Fick (1976) and confirmed by Sharypina *et al*. (2008) that a lemon-yellow RF coloration is inherited according to the principle of recessive epistasis with respect to yellow coloration. Whether or not light yellow and lemon yellow colors are similar is not clearly established. Yue *et al*. (2008) proposed that two recessive genes Yf1 and Yf2 control the occurrence of lemon-yellow RF. The authors were able to map one of the genes controlling the lemon-yellow RF trait Yf1 on linkage group 11. In the present work, an F2 population segregating for light-yellow RF was developed. The observed ratio in our F2 population is in accordance with the hypothesis of one single gene. We were able to establish its position on linkage group 3. A co-dominant SNP molecular marker linked to the gene was developed and is disclosed to the community.

Key Words : Ray Florets color, Genetic Mapping, Single Nucleotide Polymorphism

EXPRESSION PROFILES OF DROUGHT INDUCED WRKY TRANSCRIPTION FACTORS IN SOME SUNFLOWER CULTIVARS; MICROARRAY DATA ANALYSIS

Mehmet EMIN URAS [1], ***Recep VATANSEVER*** [1], ***Uğur ŞEN*** [1], ***İbrahim İlker ÖZYİĞİT*** [1], ***Ertugrul FILIZ*** [2]

[1] *Marmara University, TURKEY*
[2] *Duzce University, TURKEY*

mehmeteminuras@gmail.com

ABSTRACT

Drought or water scarcity is an unbalanced state between the water availability in soil and its physiological demand by plant. Drought stress significantly inhibits the plant growth and development, and reduces the crop productivity as well as it is predicted to become more severe in years to come. Sunflower is mainly regarded as an oil crop able to grow under water-scarce conditions. However, not all sunflower genotypes are homogeneous in water usage regimes under water available and scarce conditions. Thus, this study aimed to analyze the gene expression profiles of drought induced *WRKY* transcription factors (TFs) in eight sunflower (*Helianthus annuus*) cultivars such as Inedi, Melody, Tekny, SF028, SF107, SF109, SF193 and SF326. Two different drought stress scenarios such as Fixed Duration Stress (FDS; 7days/0-0.57 FTSW) and Fixed Intensity Stress (FIS; ≥7days/0-0.09 FTSW) were implemented to understand the *WRKY*s-drought relationship in sunflower plants. Sunflower Affymetrix microarray with 96 samples, including 32423 probesets were retrieved from NCBI GEO Data Sets (access. GSE25719). For array analyses, sunflower cultivars were grown in pots filled with 15 L of substrate, including 50% clay loam, 40% potting soil and 10% sand in greenhouse at 17oC/night and 20-25oC/daylight temperatures. 96 pots were arranged in six blocks, each containing 16 plants with eight treatment and eight control groups. All pots were daily watered and three times fertilized for 25 days before drought stress treatment. A total of 18 putative *WRKY* TFs were analyzed in eight drought-induced sunflower cultivars. Hierarchical clustering analysis demonstrated that expression profiles of *WRKY4* (Heli013712 and Heli000574) and *WRKY30*(Heli009222) TF genes show more divergence from others. Besides, SF028 (FIS), SF107 (FIS), SF326 (FIS) and Tekny (FDS) cultivars demonstrated more similar clustering pattern. Revealing the gene expression profiles of*WRKY* TFs in various sunflower cultivars under different water regimens will provide valuable insights to elucidate the drought-induced transcriptional regulatory elements or mechanisms in plants, with purposes of enhancing the crop productivity and yield.

Key Words : Water scarcity, FDS, FIS, genotype, WRKY, cultivar

HIGH THROUGHPUT GENOTYPING TOOLS IN SUNFLOWER

Stephane Munos [1], ***Jerome Gouzy*** [1], ***Ghislain Fievet*** [1], ***Romain Dinis*** [1], ***Nicolas Pouilly*** [1], ***Joel Piquemal*** [2], ***Legrand Ludovic*** [1], ***Sebastien Carrere*** [1], ***Marie-Christine Le Paslier*** [1], ***Marie Coque*** [3], ***Nicolas Langlade*** [1], ***Sunrise Consortium*** [4]

[1] *INRA, FRANCE*
[2] *Syngenta, France*
[3] *Biogemma, France*
[4] *Sunrise Consortium, France*

stephane.munos@toulouse.inra.fr

In the frame of the SUNRISE project (http://www.sunrise-project.fr), 72 sunflower lines have been re-sequenced at an average depth of 10X with paired-end HiSeq sequences. The mapping of the reads on a reference genome sequence (XRQ line), identified 6 348 868 SNPs. We sampled 586 985 SNPs located on 14 129 genomic scaffolds. These SNPs were selected to maximize the diversity and to have at least one polymorphic SNP per genomic scaffold for each of 18 recombinant inbred lines populations designed to perform nested association mapping. All SNPs were used to produce an AXIOM® genotyping array. This tool allows to genotype 96 samples simultaneously. From genotyping data obtained with this 600k array, we sampled a subset of 49 449 SNPs located on 14 021 genomic scaffolds. These 49 449 SNPs were selected for the production of another AXIOM® array that allows the simultaneous genotyping of 384 samples. These 2 high throughput genotyping tools will help to better characterize the sunflower genetic resources and will accelerate sunflower breeding.

Key Words : genotyping, sunflower, High-throughput, SNP, polymorphism

MAS SELECTION ON OLEIC TYPE SUNFLOWER BREEDING

Goksel EVCI [1], Behiye Banu BILGEN [2], Veli PEKCAN [1], Ibrahim Mehmet YILMAZ [1], Sheida DANESHVAR [3], Çağlar ÇOLAK [4], Sami BULUT[5], Yalcin KAYA [4]

[1] Trakya Agricultural Research Institute, Turkey
[2] Namik Kemal University, Turkey, Agricultural Biotechnology Department, Turkey
[4] Trakya University, Genetics and Bioengineering Department, Turkey
[5] Trakya University, Department of Food Engineering, Turkey

yalcinkaya@trakya.edu.tr

Oleic type sunflower is new trend in sunflower production in the world. Its market is increasing year by year recently. On the other hand, new oleic type hybrids are developing and releasing into the market. High Oleic (HO) trait is controlling a gene calling *Ol* utilized and obtained from mostly to a high oleic mutation of variety Pervenets by Soldatov 1976. Although, there are some other sources for high oleic content up to 90% from Bulgaria and Italy, etc. Pervenets mutations are using worldwide as the donor to develop high oleic content inbred lines and hybrids in sunflower breeding programs. However, as a seed trait, oleic type plants could determine after harvest so it needs to wait until seed tests to select oleic types until seed trashing. However, when applied MAS analysis high oleic plants could be determined as much as early stages so it helps extremely to breeders both reducing costs and time wasting and also accurate selection. Different RAPD, SSR (microsatellite) markers were determined until today in different studies. These studies enabled to detect the genetic behavior of high oleic of QTL and linked markers efficiently then leaded to use of molecular tools practically in sunflower breeding programs. On the other hand, PCR analysis with HO specific fragments enabled to amplify either the Pervenets mutation itself or the polymorphism of the SSR locus (TTA repeat variability) located on the Δ12-desaturase gene intron. These markers lead to discriminate genotypes carrying Pervenets mutation and genotypes without mutation. Consequently, the HO PCR specific fragments or SSR markers may be used in selection programs to identify genotypes carrying the Pervenets mutation. However, these markers need further validations in different genetic sources to classify sunflower genotypes accurately based on their oleic acid contents. For example, the length of the SSR depend on the lines that have been used to convert the LO in HO. Therefore, amplified SSR locus should be sequenced from different progenies, because the SSR size estimation may vary depending of the plants and of the PCR reaction. Furthermore, HO PCR specific fragments could not able to distinguish homozygous HO genotypes from Heterozygous HO genotypes so this type primers may be used first selecting HO genotypes (both homozygous and heterozygous) and then extra selection with SSR markers should be done further. As results, further studies need on MAS selection in oleic acid content in sunflower and not dependable to genetic background, practical and widely used molecular markers determining HO in the breeding programs broadly were not released yet for public interest and uses.

Key Words : Sunflower, MAS, Oleic type, breeding

DNA MARKER DETECTION OF DOWNY MILDEW (PLASMOPARA HALSTEDII) RESISTANCE IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

Nilay YÖNET [1], ***Ezgi ÇABUK ŞAHIN*** [2], ***Yildiz AYDIN*** [2], ***Goksel EVCI*** [3], ***Ahu ALTINKUT UNCUOĞLU*** [4]

[1] *Marmara University-Turkey*
[2] *Marmara University-Turkey*
[3] *Trakya Agricultural Research Institute-Turkey*
[4] *Marmara University, Faculty Of Engineering, Department Of Bioengineering-Turkey*

nilayyonet@gmail.com

ABSTRACT

Downy mildew, caused by *Plasmopara halstedii*, is one of the most destructive diseases in cultivated sunflower (*Helianthus annuus* L.) responsible for significant yield loss. The development and testing of DNA markers of important agronomic traits and in particular markers of resistance to downy mildew is considered to be one of the most priority tasks for breeding resistant sunflower to downy mildew. Resistance for *Plasmopara halstedii* is inherited and provided by dominant genes. Some of downy mildew resistance genes were characterized, but only a few of them were associated with molecular markers. The study was carried out on five different sunflower crosses as parents and their F2 individuals developed by Thrace Agricultural Research Center in Edirne. To perform the molecular genetic analysis, genomic DNA was isolated from leaf tissues. Genotyping of these materials has been currently carried out using 117 SSR of 4 *Pl*-loci including *Pl6*, *Pl8*, *Pl13* and *Plarg* associated with the resistance of sunflower to downy mildew. Further studies with genotyping and validation of resistance will be especially promising for the marker-assisted selection of sunflower with respect to resistance to the downy mildew. This research has been supported by TUBITAK TEYDEB 1501 Program (Project No: 3150030).

Key Words : Sunflower, downy mildew, SSR-markers, marker assisted selection

THE MOLECULAR GENETIC DIVERSITY OF THE BROOMRAPE (*OROBANCHE CUMANA* WALLR.) POPULATIONS OF TURKEY

Sana ZIADI [1], ***Yildiz AYDIN*** [2], ***Goksel EVCI*** [3], ***Ahu ALTINKUT UNCUOĞLU*** [4]

[1] *Marmara University,TURKEY*
[2] *Marmara University, TURKEY*
[3] *Trakya Agricultural Research Institute-Turkey*
[4] *Marmara University, Faculty Of Engineering, Department Of Bioengineering-Turkey*

ziadi.sana@yahoo.fr

ABSTRACT

Broomrape (*Orobanche cumana* Wallr.) which is a holoparasitic plant infecting the sunflower roots is considered as one of the most important constraints for sunflower production in Turkey. The study of natural *Orobanche* populations can help us to understand gene pool dynamics, population size structure and geographical distribution, environmental adaptation and centers of origin. Since the pathogenic composition of broomrape populations has changed over the years, nucleic acid markers provides a clear advantage according to morphological traits due to the stability and the power to identifying new broomrape races. The objective of this research is to study the genetic variation of *Orobanche cumana* Wallr. populations in order to (1) determine the genetic differences between them, (2) infer the inter and intra-population variability and (3) to determine the geographical influence on genetic diversity mainly from Thrace and Adana regions of Turkey by simple sequence repeats. Two field expeditions were conducted along the Thrace region and Adana, where the distribution of *O. cumana* in the wild has been seen, to collect fresh tissue and mature seeds of twenty seven *O. cumana* populations. Orobanche floral buds were used for DNA extraction for SSR analysis. Genetic diversity and population structure are currently being studied with SSR markers in *O. cumana* populations. Obtained results will led to better understand the evolution of parasitic plants and contribute to the establishment of improved crop breeding and management strategies for *O. cumana* control. This research has been supported by TUBITAK BIDEB (Program No: 2216).

Keywords: *Orobanche cumana*, SSR markers, parasitic plants, genetic diversity.

THE DEVELOPMENTAL FEATURES OF THE OVULE AND EMBRYO SAC IN THE HERMAPHRODITE FLOWERS OF *HELIANTHUS ANNUUS* L.

Aslıhan ÇETINBAŞ GENÇ*, *Meral ÜNAL

Marmara University, Department of Biology, 34722, Istanbul, Turkey

aslihan.cetinbas@marmara.edu.tr

INTRODUCTION

The inflorescence of *Helianthus annuus* L. has two types of flowers in a single capitulum; central hermaphrodite disc flowers and peripheral pistillate ray flowers (Herman, 2000; Cvejić et al., 2016). In the present study, the developmental features of the ovule and embryo sac were investigated.

MATERIAL AND METHODS

The capitulum at various stages of development were collected from Tekirdağ (Turkey). Firstly, the diameter of capitulum were measured, hermaphrodite flowers were morphologically analysed by stereomicroscope (Olympus 970931). The samples were prepared for light microscope analysis. The material was fixed in acetic-alcohol (1:3, v/v) and embedded in paraffin blocks. The blocks were sectioned at 8-15 μm by Leica RM2235 rotation microtome and sections were stained with hematoxylin. The preparations were photographed with an Evolution LC color camera and an Olympus BH-2 microscope, and the images were analyzed with Image-Pro Express Version 6.0 scientific image processing and analysis software. For SEM analysis, the plant material was fixed in 2.5% glutaraldehyde in 50 mM cacodylate buffer, pH 7.0 (Platt *et al.*, 1983) and then dehydrated with an increasing ethanol gradient: from 70% up to 100%. Then, the material for drying were kept in various percentages of ethanol-HMDS solution at room temperature (Topçuoğlu *et al.*, 2009). Then, coated with 11 nm of gold by using an automated sputter coater and then examined with a SEM (JEOL JMS-59 10LV).

RESULTS

In *Helianthus annuus* L., a hermaphrodite flower contains a pistil with inferior type of ovary which lies below the attachment of other floral parts. The ovule shows basal placentation. It differantiates as small and homogeneous mass on the ovary and consists of the cells with dense cytoplasm and small nuclei. The ovule is unitegmic type, namely have one integument. This situation occurs with the disappearance of one of the integuments or with the merge of the two integuments. Together with the differantiation of the integument and the megaspore mother cell, the funiculi which contacts the ovule to ovary begins to curl. The ovule becomes anatropous at megaspore tetrad stage. The micropyle is long and narrow.

The ovule is *tenuinucellate type. Namely,* megaspore mother cell differantiates just below the nucellar epidermis. The nucellar cells expand and create a loose tissue around the embryo sac. The nucellar tissue can be observe in the mature ovule.

The development of female gametophyte conforms to Polygonum type. Megaspore mother cell produces linear megaspore tetrad by regular meiosis. Functional megaspore which is the the largest one locates at the chalazal part. It undergoes three successive mitosis and forms 2, 4 and 8 nucleated embryo sac, respectively. Two nuclei locate the chalazal part and the other two nuclei locate the micropyle part in 4 nucleated embryo sac. The mature, 8 nucleated embryo sac

contains an egg cell, two polar nuclei, three synergid cells and three antipodal cells. The egg cell and two synergids locate the micropyllar part of the embryo sac and a cell wall is formed around the this cells. This structure is called egg aparture. The egg cell is bigger than the synergids and locate between the synergids. In the egg cell, there is a nucleus in the chalazal part and a big vacuole in the micropyle part of the cell. The cytoplasm is a thin layer and locate only on the periphery. The polarization in the synergid cells is the opposite. Althought the nuclei are found in the base, vacuoles are found in chalazal part. Antipodal cells are smaller than the other cells of the embryo sac. The synergids and the antipodal cells are ephemeral. They become blunt after the fertilization.

LITERATURE

Platt AKA, Oross JW, Thomson WW (1983). Ultrastructural study of the development of oil cells in the mesocarp of avocado fruit. Bot Gaz 144(1):49-55.

Topçuoğlu N, Selvi N, Dokumaci E (2009). The comparison of critical point drying and drying with Hexamethyldisilazane methods for the preparation of mice tissues for scanning electron microscopy II. Lung and stomach Findings. Anad Univ J Sci Techn 1:127-132.

Herman PPJ, Retief E, Koekemoer M, Welman WG (2000). *Asteraceae* (*Compositae*) In: Leistner OA (Ed.). Seed plants of Southern Africa. National Botanical Institute, Pretoria, Strelitzia 10:101-170.

Cvejić S, Jocić S, Mladenović E (2016). Inheritance of floral colour and type in four new inbred lines of ornamental sunflower (*Helianthus annuus* L.). J Hortic Sci Biotechnol 9(1):30-35.