GIBBERELLIN-INDUCED GENE EXPRESSION ASSOCIATED WITH CYTOPLASMIC MALE STERILITY IN SUNFLOWER

M. Duca[1], A. Port[1], M. Orozco-Cardenas[2], C. Lovatt[3]
Moldova State University, Chisinau, Republic of Moldova[1]
University of California, Plant Transformation Research Center - Department of Botany and Plant Sciences, Riverside, California, USA[2]
University of California, Department of Botany and Plant Sciences, Riverside, California, USA[3]
Correspondence to: Duca Maria
Email: mduca2000@yahoo.com

ABSTRACT

Gibberellins (GAs) are plant hormones with diverse roles in plant growth and development. Male sterility is one of several GA responses. In an effort to understand the involvements of GAs in sunflower male reproductive development, physiological, biochemical and molecular studies of GA-induced and cytoplasmic male sterility were performed. Male sterility was induced by exogenous application of gibberellic acid (GA_3) to the fertile inflorescence apex in early reproductive development. Using different experimental approaches: hormonal quantitative analyses (gas-liquid chromatography), protein profiles (SDS PAGE) and transcripts assays (RT-PCR) revealed a similarity in expression of cytoplasmic (CMS) and induced (IMS) male sterility. Our results provide the first evidence that expression of CMS-specific orfH522 gene can be induced by GA_3 in plant with original fertile cytoplasm.

Keywords: complimentary DNA (cDNA), cytoplasmic male sterility (CMS), gene expression, gibberellic acid (GA_3), *Helianthus annuus* L., indole acetic acid (IAA), induced male sterility (IMS), proteins

Introduction

Reproductive development is gibberellins (GAs) dependent in a range of plant species (20, 32, 35, 37, 38, 56). Evidence of the involvement of GAs in flower development has resulted from studies of mutants that are affected in GA biosynthesis or signal transduction. For example, severely GA-deficient *Arabidopsis* mutants (such as *ga1-3)* in addition to the dwarf stature have impaired development of floral organs, especially petals and stamens, and retarded anther development results in male sterility. Application of exogenous GA restores the morphology and fertility of the flowers (10, 24, 57). Similarly, tomato GA-deficient mutants (gib-1 and gib-2) are also male sterile and the fertility can be restored by exogenously applied GAs (20, 23). Interestingly, in some instances GAs can promote either female reproductive development or induce male sterility. Thus, GA treatments stimulate the growth of female reproductive organs in tomato, castor bean, maize and induce male sterility in lettuce, safflower and pepper suggesting a complex role of GA that varies from species to species (3, 33, 35, 42). Although the molecular mechanisms by which GA regulates petal, stamen and anther development are still largely unknown, several GA-signalling components that may modulate these processes have been identified. For example, transgenic expression of wild-type or mutant forms of GAI (GA insensitive, *Arabidopsis* GA-signalling component) can retard stamen elongation and induce male sterility in tobacco and *Arabidopsis* (17, 18). Also the infertility caused by impaired floral development is a characteristic of mutants lacking the rice or barley proteins SLR1 or SLN1 which belong to the DELLA subfamily of conserved proteins in higher plants (5, 19). Fiona Murray et al (31) have demonstrated that HvGAMYB is a MYB transcription factor GA-up-regulated suggesting to be part of a GA signal transduction. Transgenic barley lines with an excess of fourfold levels of endogenous GAMYB protein in their anthers were male sterile. However, additional GA signaling components, downstream cellular and biochemical events and the molecular nature of the GA response are not well understood.

Gibberellins are also involved in sunflower male development as have been demonstrated, by studies of exogenous applications of gibberellic acid (GA_3) that induced male sterility (1, 44). Although the treated plants have been characterized by visible GA-related phenotype suggesting on affected plant responses, there are no reports on the molecular basis of GA-induced male sterility in sunflower. To investigate the GA responsive gene related to male fertility were used three isonuclear line - fertile, GA-induced male sterile (IMS) and cytoplasmic male sterile (CMS) plants.

PET1-CMS in sunflower was identified in an interspecific cross between *Helianthus petiolaris* and *H. annuus* (27) and is associated with the expression of a novel mitochondrial gene, *orfH522*, which encodes a 16-kDa polypeptide (13, 14, 26, 29, 46).

In this study, we employed hormonal analyses by gas-liquid chromatography, SDS PAGE and RT-PCR to examine GA-induced male sterility in comparison with cytoplasmic male sterility known in sunflower. The similarity of physiological and molecular events in expression of GA-induced male sterility and cytoplasmic male sterility are discussed on the basis of our results.

Materials and Methods

Plant material, growth conditions and chemical treatments

Sunflower (*Helianthus annuus L.*) seeds were kindly provided by Center for Scientific Research "Magroselect" (Soroca, R. Moldova). We used fertile SW501 and male sterile SW501CMS lines that are near-isonuclear. The fertile (maintainer) line carried the sunflower (*H. annuus L.*) cytoplasm and the sterile line contained the *H. petiolaris Nutt* cytoplasm. Both lines were treated with GA_3 in order to induce male sterility (in case of SW501) and to restore male fertility (in case of the SW 501CMS).

Greenhouse and field experiments were carried out. Sunflower plants were cultivated in experimental field according to conventional technologies (54, 55) and in pots at standard greenhouse conditions during the spring. The inflorescence tissues were analyzed in early stage of flower development R_1 and R_2 according to Schneiter and Miller (45). The R_1 stage refers to the time when the inflorescence begins to enlarge and is visible as a bud with the bracts closed forming a star-like structure. In the R_2 stage, the inflorescence was further enlarged and separated 0.5 to 2 cm from the youngest leaves.

Treatment with exogenous gibberellic acid. GA_3 (Sigma) solution was prepared by dissolving GA_3 in the minimal amount of 96% ethanol and bringing up to remaining volume in distilled water to make a final concentration of 0.005% (1). The treatment with GA_3 solution was carried out by spraying plant at developing inflorescence buds period (R_1 and R_2). At this stage, prior to the opening of the inflorescence, male meiosis occurs in disc flower anthers (50). Non-GA_3 treated control plants were sprayed with distilled water. Plant tissue was collected 24 hours after a single GA_3 application for RNA and protein isolation and after 24, 48, 72 and 96 hours for plant hormones extraction.

Analysis of total proteins

Total proteins were extracted by homogenisation of 1g plant tissue (inflorescence and leaves at R_1 stage) with 3 ml of the 62.5 mM Tris-HC1 extraction buffer, pH 6.8 at room temperature with magnetic stirring. After a series of organic extractions and purifications the protein extracts were reduced to dryness. For one-dimensional SDS-polyacrylamide gel electrophoresis the samples were solubilized in Laemmli sample buffer in reducing conditions, 6% (v/v) of 2-ME and thereafter were heated at 100°C for 5 min before migration. The samples were separated on 12.5% SDS-polyacrylamide gels and visualized with Coomassie Briliant Blue staining (25). Molecular weights were estimated using standard proteins (Pharmacia). Protein electrophoresis was performed three times using independent plant materials.

Analysis of endogenous GA_3 and IAA content

Quantitative analysis of phytohormones was performed by gas-liquid chromatographic method using indole-3-acetic acid (IAA) and gibberellic acid (GA_3) as internal standards (4, 7).

Samples (100g fresh weight at R_1 stage) were homogenized in cold (-20^{0}C) 80% acetone and extracted over-night at -10 °C. After a series of organic extractions and purifications the extracts were reduced to dryness in vacuum at 40^{0}C. The residue was dissolved in 0.1 ml N,O-bis(trimethylsilyl)-acetamid with addition of 0.05 ml of trimethylclorosilan (1%) and then subjected to chromatography.

The chromatograph FRACTOVAP 4200 equipped with a detector of flame ionization, line programs for temperature MOD 410, integrator MEGA SERIES SP 4270, rustproof column (2m x 4mm) with 5% SE-30 DMCS Cromoton W, 60/80 mesh (0.15-0.2 mm) was used for analysis with gas carrier N_2 - 25 ml/min. Air flow was maintained at 300 ml/min, while hydrogen flow was 25 ml/min. The injector temperature was + 210^{0}C and the detector temperature was +220^{0}C. The phytohormones were determined in the following temperature regime: after the injection the temperature was maintained at 60^{0}C for 4 min, further on the temperature increase rate was 12^{0}C/min until the temperature of + 220^{0}C was achieved. This temperature was maintained until the end of chromatography cycle. The phytohormone contents were expressed in ng per gram of fresh weight, (ng/g fwt).

Data are presented as means ± SE (standard errors) of three separate experiments (n = 6 for each experiment) and Student's t test ($P < 0.05$) was used to determine the statistical significance of differences between genotypes.

RNA isolation

Tissue was isolated from plants (at R1 and R2 stage) and immediately frozen in liquid nitrogen. To examine expression patterns, the total RNAs were extracted from developing inflorescence buds using Trizol reagent (GIBCO BRL) according to manufacture's protocol. Yield and purity was monitored by denaturing 1.44% agarose gel electrophoresis (41) and by UV absorbance (A260/A280) using Genesis 10UV/VIS spectrophotometer (Fisher). A yield ranging 2-3µg/µl RNA extract from sunflower tissues has obtained, the ratio λ260/280 was between 2 and 2.1.

Reverse transcription reactions

Total RNA (2µg) was treated with RQ1 RNase-Free DNase - 1u/µg RNA (Promega) at 37°C for 30 min. according to manufacturer's instructions. The treated RNA was used for reverse transcription in a reaction which contained: 1 x amplification buffer, 250 µM of each dNTP, pH 8, 0.25 µg primers oligo dT_{12-18} and 2.5 µg random hexanucleotides, 1 units/ µl RNase inhibitor, 2.5 mM $MgCl_2$ in a total volume of 20 µl in the presence of 4 units/µl of M-MLV reverse transcriptase (Promega). The mixture was incubated for 60 minutes at 37°C. The reaction was stopped by an incubation of 5 minutes at 100°C (41).

PCR amplification, cloning and sequencing

The reaction mixture used for PCR amplification contained 2µl of reverse transcription reaction, 20-30 pmol of the sense and antisense primers, 200 µM of each dNTP, 2,5 mM $MgCl_2$, 10 µl

of the 10 x PCR buffer supplied with the Tag DNA polymerase (Qiagen) which was used at a concentration of 2.5 U per reaction, in a total volume of 100 µl.

The PCR reaction was performed in PTC 100 thermocycler (MJ Research) programmed: 2 min. at 95 °C; followed by 40 cycles: 30 s at 95 °C, 1 min at 60° C, and 2 min and 30 s at 72 °C.

Primers for RT-PCR (*orfH522 gene)* were the following: *A* (5`-GGCGCACTCTCTTTTTCTGT-3`) and *B* (5`-CTTGAATGGCAGTGGTGATG-3`).

The amplified PCR products were separated and detected using agarose gels (1%) electrophoresis and ethidium bromide staining according to standard protocols (41). The molecular size of the amplified products was evaluated using 1kb and 100 bp DNA ladder for comparison. RT-PCR was performed three times using samples collected from three distinct biological replicates. Database searches and primer design was performed using Genbank/EMBL and Primer 3 program.

Sequence analysis. PCR products, purified from agarose gels with QIAquick Gel Extraction kit (QIAGEN) and cloned into pGEM-T vector (Promega), were sequenced to confirm gene-specific amplification. The BLAST program (2) was used for homology search with data banks.

Results and Discussion

GA_3 treated sunflower plants display male-sterile phenotype. The work of Anashchenko (1) and our previous data with exogenous GA_3 application on sunflower had led to the conclusion that the male sterility induction effect by GA_3 is restricted to specific stage (R_1 and R_2) of blossoming. In agreement with this, GA_3 application at the beginning of inflorescence bud formation induced only male sterility in the fertile line. No additional effects were observed.

The monitoring of the anther and pistil development in the GA_3 treated fertile plants in comparison with non-treated fertile (30 plants of each) implied that the resulting phenotype as the CMS plants have arrested microsporogenezis and no pollen. However, the female reproductive tissues of these plants appeared phenotypically normal. Some morphological differences of male reproductive organs were observed between wild-type (fertile), CMS and GA_3 treated fertile plants. The anthers of CMS plant were poorly developed, smaller in size and paler in comparison with the fertile plants. The anthers of IMS plants were morphologically indistinguishable having an aberrant structure with complete atrophy of the androceum.

Although the reversibility of the male sterile plants was observed under exogenous GAs application, resulting viable pollen (20, 23) the CMS conferred by Pet1 cytoplasm cannot be reversed by the GA_3 in the used concentrations at this stage of development, again demonstrating that GA_3 induce male sterility or rescue male fertility in a dose and time dependent manner.

Phytohormones analysis of CMS and GA_3-induced male sterility in sunflower. Our interest in plant responses to GAs on the one hand and the results of phenotypic analyses of IMS plants on the other hand, prompted us to examine initially the changes of hormonal levels associated with male sterility prior to molecular aspect of this type of male sterility (IMS).

As expected, quantification of endogenous GA_3 content from fertile and CMS inflorescence buds revealed lower levels in the sterile line (approximately 70%) than in the fertile plants, (**Fig. 1**). But our main goal was to compare the sensitivity (responsiveness) degree to exogenous GA_3 between CMS and IMS plant. For this reason gibberellin's content measurements were performed after 24, 48, 72, 96 hours post-treatment.

Our results indicated that a single GA_3 application (0.005%) at early stage of generative organ development induced hormonal concentration changes. Increases in the GA_3 levels, 28% for CMS and 26% for IMS, were observed in the first 24 hours post-treatment. However, this enhancement effect of endogenous hormone amount in response to GA_3 was transient, because GA_3 levels started to decline soon after. The following 48, 72 and 96h after treatment showed a significant reduction of gibberellins level in CMS and IMS plants.

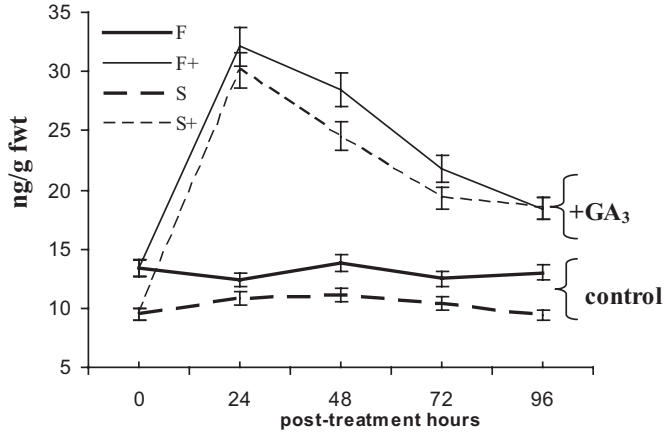

Fig. 1. Effect of GA_3 treatment on endogenous hormone (GA_3) level of fertile SW501 (F) and sterile SW501 CMS (S) inflorescence

GAs from 100 mg fresh weight plant tissue were purified and analyzed by gas-liquid chromatographic methods using GA_3 as internal standards. The values represent the mean ± SE of three independent experiments with six samples each. The data are significant according to Student's test analysis ($P \leq 0.05$)

It is known that the bioactive gibberellins act synergetic with auxins in many physiological processes including reproductive development (8, 37, 38, 38, 58, 59). Based on this information we expected also increases in a quantity correlation among the bioactive GAs and auxins. Additionally, the auxins amount (IAA) assaying under our experimental conditions may confirm the responsiveness (or the capacity to respond) to GA exogenously applied in special for CMS line that have not show any visible morphological changes under treatment. Thus, inflorescence samples were assayed for the concentration of endogenous IAA in parallel with the GA_3 content.

The inflorescence apex of sunflower was found to contain approximately 4-fold and 6–fold more IAA than GA_3 content for fertile line and those with CMS respectively (**Fig. 2**).

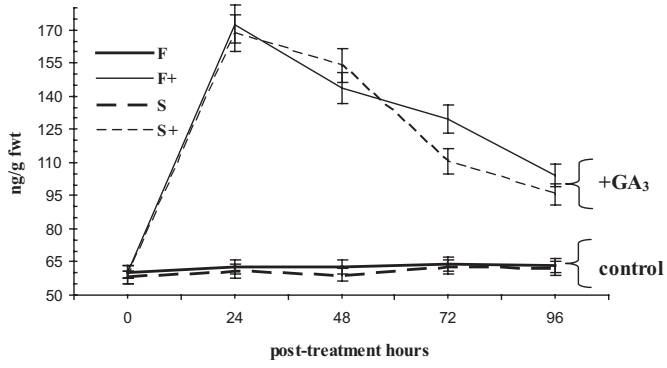

Fig. 2. GA_3 treatment effect on IAA endogenous level of fertile SW501 (F) and sterile SW501 CMS (S) inflorescence

Auxins from 100 mg fresh weight plant tissue were purified and analyzed by gas-liquid chromatographic methods using indole-3-acetic acid (IAA) acid as internal standards. The data represent the mean ± SE of three independent experiments with six samples each.

Student's t test analysis of the significance of differences ($P \leq 0.05$) between lines showed a similar IAA endogen content in untreated fertile and male sterile plants. GA_3 treatment increased IAA levels in both lines CMS and IMS to approximately the same level. But this initial enhancement of hormone concentration by exogenous GA_3 occurs for only a short time (~24h) similar to the endogen gibberellins level (GA_3), and is then followed by large reductions in the next 72 h post treatment. Although the hormones level 24 h up to 96 h after treatment was diminished, this remain still elevated in comparison with the control, at least during analyzed period.

In summary, the above results denote both a synergistic quantitative relationship among active GAs and auxins in response to exposure to GA_3 and a sensitive homeostatic mechanism whereby plants regulate their endogenous hormone levels.

Additionally, for better interpretation of these data the ratio of IAA/GA_3 was analyzed next. As a result of hormone level changes by GA_3, the IAA/GA_3 ratio 24 h after treatment demonstrated increased values in male-fertile inflorescence that are close to those found for untreated CMS plants (**Fig. 3**). This finding has not been attested for treated male sterile genotype, as the hormonal ratio remained unchanged, despite of substantial changes in hormonal level induced by gibberellic acid. It is important to accentuate, although the AIA/GA_3 ratio is changeable in dependence on specific hormonal requirements, this ratio in control remain on the whole experimental period less than those of male sterile phenotype (CMS treated and untreated, and IMS).

One explanation for these results is that the values of IAA/GA_3 ratio of the plants displaying male sterile phenotype show an aberration of optimal balance of these synergist hormones (**Fig. 3**). The hormonal ratio of CMS line is changed due to the gibberellins level because those levels of IAA were similar for both sterile and fertile lines. The increased IAA ratio of treated plants may also be due to active gibberellins levels. It is possible that gibberellins activity may be targeted to enhancement of auxins biosynthesis as well. Although direct evidence for this idea remains to be found, our results suggest that GA activity (high GA_3 level as a response of exogenous GA_3) results in substantial changes in auxins level.

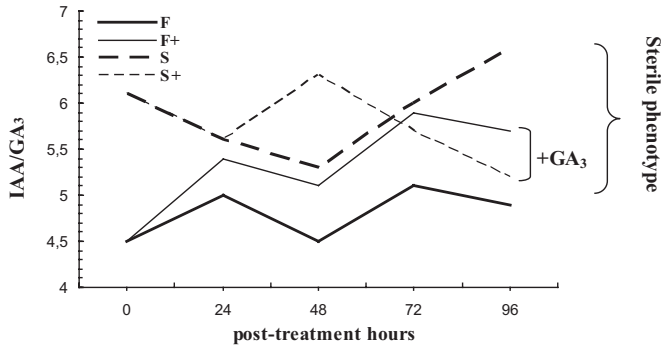

Fig. 3. The IAA/AG_3 ratio of fertile (F) and sterile (S) inflorescense of sunflower

The phenotype assays of CMS plants and exogenous GA effects on fertile and sterile plants of a isonuclear lines, have shown defects in flower development resulting in male sterility in contrast with CMS treated plants that apparently displayed no morphological changes. These observations in conjunction with hormonal quantitative analysis denote that GA-induced responses resulted from the perturbation of the hormone endogen concentration as well as from the ability of cells (inflorescence tissue) to perceive this perturbation and activate the subsequent transduction pathway(s). The fact that endogenous bioactive GA levels in CMS plants is less than those from isonuclear fertile line is not surprising because there is much evidence regarding sterility correlated to deficiencies in GA signalling (10, 20, 32). In contrast to this observation the male sterility was induced in fertile plant as a result of elevated bioactive GA levels in response to exogenous treatment. This finding suggests that too much or not enough GA can prevent the formation and release of viable mature pollen.

Comparative studies of GA-IMS and CMS revealed a transient enhancement of endogen hormone amount in response to GA_3 succeeded by a significant drop in bioactive GA perhaps, due to feedback regulation, in a similar manner for both type of male sterility. The ascertained similarity in response to GA are emphasized and by endogen IAA measurements that showed a synergic increasing of IAA levels after GA_3 treatment in both lines CMS and fertile to approximately the same level. It is important to note that although the endogen AIA and bioactive GA_3 levels 24 h up to 96 h after treatment was in decreasing, these hormonal levels remained elevated in comparison with the control, at least during analyzed period. According to our data it appears that the observed developmental defect correlated with male sterility was probably due to an elevated level of GA signaling, which mimics the effect of GA overdose on flower development. Although GA is required for stamen development, it is known that an overdose of GA on wild-type *Arabidopsis* plants and other species also results in reduced fertility (21). This would explain why exogenous applied GA_3 on CMS plants under our experimental conditions that induced

the similar effect on endogen hormone levels have not restored the fertility. This enhancement effect of endogen GA level for CMS treated plants was much more than those that would be need to compensate the GA-deficiencies. Thus, according to our results the elevated amounts of hormone in treated male sterile plants also mimics the effect of GA overdose on reproductive development. These conclusions were evident from the increased values of IAA/GA_3 ratio in plants with male sterile phenotype that revealed a synergistic quantitative relationship among active GAs and auxins in response to exposure to GA_3. Bioactive GA level may stimulate the biosinthesis of auxins. Alternatively auxins accumulation may enhance GA responses. This finding is in accordance with experiments which have shown that GAs de-repress gene expression by inducing proteolysis of GA signaling repressors - the DELLA proteins (47, 48, 53) and auxin modulate the rate of GA-mediated DELLA protein degradation through decreasing the protein's stability (8, 34). Similar interactions between auxins and GA in male development and fertility were found with GA-reversible male- sterile tomato mutant, *sl-2*, revealing that reduction of indole-3-acetic acid by lowering growing temperatures restores anther fertility without GA applications (49). The authors concluded that GA activity in anther development could be modulated by the relative amounts of auxins. Also, it has been reported that the increase of auxins in tobacco altered male development (52).

Taken together, these data suggest similarity between CMS and IMS lines and leads us to explore the possibility that GA induced male sterility in sunflower could have the same molecular basis as cytoplasmic male sterility of sunflower. To address this question we have been performed two experiments. In the first, we attempted determined whether the proteins profile displays some differences between CMS and IMS and specially the presence of 16 kDa protein that in CMS sunflower is associated with the expression of a novel mitochondrial gene, *orfH522* responsible for cytoplasmic male sterility (14, 15, 22). In the second experiment we included the transcripts analyses with the specific *orfH522*gene sequence.

Protein analysis. CMS associated protein in sunflower as the most CMS-specific proteins is expressed in mitochondria of vegetative and reproductive tissues and may influence their respiratory characteristics (26, 50). *Phaseolus vulgaris* represents the one reported exception where the CMS-associated protein encoded by *orf239* is only detectable in the anthers due to post-translational regulation (39).

Thus, according to this finding to perform SDS-polyacrylamide gel electrophoresis (one-dimensional procedures) was chosen the protein extracts from inflorescence buds and leaves.−Comparative studies on molecular weight (*Mr*) polymorphism of the polypeptide constituents revealed the presence of certain polypeptides with Mr: 90, 80, 38, 26 and 17 kDa only for leaves and the polypeptides Mr: 30, 29, 10 kDa specific for inflorescence tissues (**Fig. 4 A and B**).

The other polypeptides from leaves and inflorescence showed a *Mr* homology, although it cannot be excluded that organ-specific proteins which were not detected by our analyses might also be present.

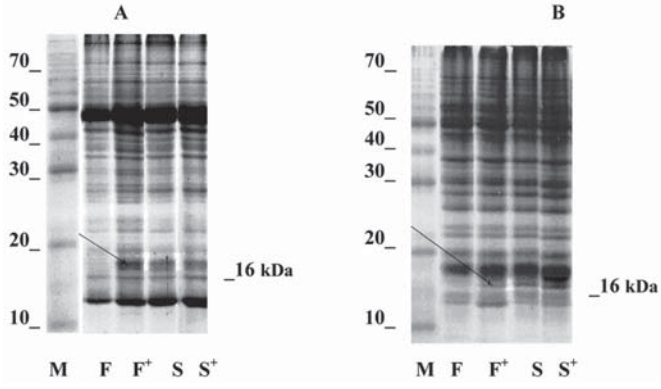

Fig. 4. SDS-PAGE patterns of total protein extracts from leaves (A) and inflorescence tissue (B) of fertile SW501 (F) and sterile SW501ASC (S) untreated and treated with GA_3 (+)

The total protein extracts were separated on a 12.5 % SDS-polyacrylamide gel and analysed after Coomassie blue-staining. Protein size is shown in kilodaltons. The expected 16 kDa protein is marked by an arrowhead; M, molecular weight marker (10-100 kDa). Similar results were observed in three independent experiments

The patterns of the total proteins obtained for the investigated lines showed a high similarity, except the polypeptide with Mr ~16 kDa that was detected only for CMS and GA_3-treated fertile leaves and inflorescence samples. This approach did not specify origin of protein and 16 kDa product could be not

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                                  ↓orfH522
tctgtatggg gggaatcctt tattctcgat caagatgcct caactggata aattcactta tttcacacaa
ttcttctggt catgcctttt cgacttgcgg cgcactctct ttttctgtct ttttattttt ttgatattag
                               A >>>>>>>>>>>>>>>>>>>>>>>>>>>>
tagcccgttc cgtgtttatg gaacagatca cgccctataa aggccgaagt atttctggtc caaatgctca
aagcattctc cccctccctg gtggatccgg cgatgaccca aatagaaaga aagtccctgt gtctaaggac
actgcaaccg cagccgtgag tttactccgg caactcgttc ttgaaatttt agcccgagcg cgggatccgt
ccttgcgtga gggtttgcac aacccaacca cccaagcgtg gaatagggcc atcaccactg ccattcaaga
                                                  <<<<<<<<<<<<<<<<<<<<<<<<<<<< B
gcagtccggg cactactcta tatccacctt gggtgcaatc caaggaacca ttgcgctcgc gggagaatta
gtattttcga gggagcaaag tccttttttt ttacgagtac ttcaactcgt gagagaacgg tactcataga
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Fig. 5. Nucleotide sequence of sunflower *orfH522*

those expected. However, it cannot be excluded that the16 kDa polypeptide represent the only single visible difference that could be detected between the CMS and the fertile lines. This finding aimed us to carry out the transcripts analyses with specific *orfH522* gene sequence.

Transcript analyses with specific gene sequence for cytoplasmic male sterility. Primers for *orfH522* (called *ORFc* by Laver et al., 26) were designed based on the coding region of sunflower CMS Baso (EMBL/GenBank accession X55963). As this *orf* share homology in the 5`-coding region with *orfB* (22) it was important to exclude this region (57 pb) from the sequence to avoid obtaining the undesired product (**Fig. 5**).

To investigate whether the GA_3 treatment have induced the expression of *orfH522* gene in fertile plants and if any influence on the transcripts in CMS and restored hybrid or not were performed RT-PCR using RNA samples (isolated from R_1-inflorescense) and oligonucleotides A and B specific for the *orfH522* sequence (**Fig. 5, 6**).

Two discrete PCR products with very close sizes were amplified from cDNAs prepared from untreated sterile plants (**Fig. 6 A, lanes S**). The first fragment correspond to that with expected size 321 bp and the second additional had the size with 100 bp more than first (~ 400 - 450 bp). As expected, no PCR product was obtained from the line SW501 with the original fertile cytoplasm (control) using these primers (**Fig. 6 A line F**).

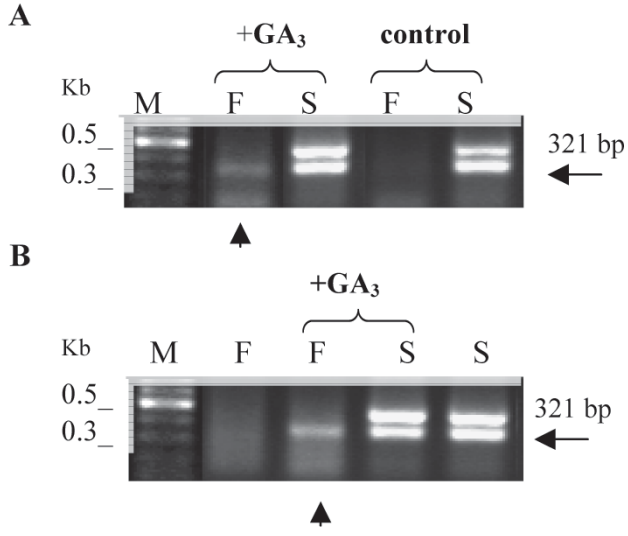

Fig. 6. RT-PCR amplification products from sunflower samples using *orfH522* sequence primer

RT-PCR was performed on the total RNA isolated from R_1 (**A**) and R_2 (**B**) - inflorescence buds of fertile SW501 (F), sterile SW501CMS (S) untreated and treated with GA_3. Lane M contain 100 pb ladder (Takara) and the sizes of the respective bands.

The same two fragments with close sizes were amplified from cDNAs prepared from GA_3- treated sterile plants. For fertile line treated with GA_3 the situation is different, was amplified only 321 bp fragment which is typical for the sterile line, but much weaker in intensity than those of sterile plants (**Fig. 6 A, line F**).

To confirm these results, a similar molecular analysis at RNA level was performed on the treated fertile plant with exogenous GA_3 at older stage of reproductive development (R_2). RT- PCR results revealed patterns identical to those observed for the untreated and treated R_1 inflorescence. As expected, the amount of *orfH522* transcripts was higher using cDNAs prepared from analyzed inflorescence buds of CMS sunflower and treated fertile sunflower at R_2 stage as compared with R_1 stage (**Fig. 6 B**).

In order to verify whether exogenous GA_3- treatment have induced the transcription the same gene as *orfH522* in CMS sunflower, the amplified fragments from cDNA (321 bp) of treated fertile, CMS an restored hybrid plants were cloned and sequenced in both the forward and reverse directions. The sequencing results were compared with the *orfH522* genomic sequence ((EMBL/GenBank accession X 55963). All of the cDNAs sequenced indicate a 99-100 % homology with sequence corresponding to the *orfH522* gene CMS associated.

Due to the present of an additional fragment only in CMS plants we wanted to understand the origin of this ~ 400-500 bp fragment. This amplified fragment was sequenced and Blast search analyses have shown 96-99 % homology to the same *orfH522*. It contained the sequence of the reverse PCR primers followed by the gene sequence up to ATG codon (which marks the start of *orfH522*) and 41 nucleotides more which are present in 265-bp inverted repeated sequence found in the CMS sunflower (26).

Using CMS system in sunflower and RT-PCR analysis with CMS gene-specific primers confirmed by gene sequencing we found that exogenous GA_3 applied at early reproductive development induce the expression of mitochondrial *orfH522* gene. Its expression as a response to GA_3 treatment was comparatively weaker in fertile R_1-inflorescence than in older stage under the conditions tested. As suggested by our results, the increase in transcript abundance observed in older reproductive stage may be related to a cell/tissue-specific developmental process, perhaps associated with mitochondrial biogenesis. According the published works, most mitochondrial genes show enhanced expression during microsporogenesis in maize (30) rapeseed (9) and, more specifically, in microsporocytes and developing tapetal tissue in sunflower anthers (50). This correlates with the fact that the number of mitochondria per cell increases considerably during flower development. Lee and Warmke (28), using electron microscopy and quantitative analysis, showed that during meiosis in maize the mitochondrial number per cell increases 20- and 40- fold in meiocytes and tapetal cells, respectively. This increasing of mitochondrial numbers per cell during reproductive development have also been observed in *Cosmos bipinnatus* by Dickinson and Li (6).

The significance of these findings has two aspects. **First**, expression induced by *orfH*522 in fertile floral meristems at the beginning of reproductive stage is sufficient to

affect male flower development resulting phenotypic male sterility. Our data support the view that the expression of this mitochondrial gene in specific reproductive cell/tissue early in microsporogenesis is responsible for male infertility. This is in accordance with experiments which have shown that in PET1-CMS sunflower the *orfH522* is expressed in vegetative and reproductive developmental stages in all tissues, but the male sterile phenotype associated with its expression is limited to the anthers tissues only (11, 29). The explanation for this tissue- and developmental - specific phenotype of CMS appears to be caused by the higher demands on mitochondrial ATP production that exist during microsporogenesis (12, 50, 60) and partially defective mitochondria are unable to meet these respiratory demands, thus leading to pollen abortion (40).

Second, CMS associated gene expression in emerging floral meristems is in response to GA signals, thus it is a GA-responsive gene. It is interesting to note that some CMS sources in sunflower (nine from studied twenty eight) originate from different interspecific crosses, spontaneously occurring male-sterile plants in wild sunflower and from induced mutagenesis are reported to have the same organization at the *atp A* locus (15, 16, 43, 51). Such a finding raises the question: how CMS originated from different interspecific crosses, spontaneously occurring in wild population or induced by various factors, seem to have the same CMS-associated region as PET1. As suggested by Laver et al. (26) it is possible that the organization of the *atpA* locus typical for PET1 already exists in a very low concentration as a sublimon in fertile *H. annuus.* This sublimon could have been amplified due to the first cross rather than being the result of a novel recombination event induced by nucleo-cytoplasmic incompatibility. Moneger et al. (29) based on their results suppose that different recombinant molecules generated by recombinations across the 265 bp repeat are present in various cytoplasms and are either maintained at a low level or amplified to normal stoichiometry depending on the nuclear background involved in this control.

On the other hand this idea is in contrast to results obtained by Rieseberg et al. (36). The screening of 1200 plants *H. annuus* and *H. petiolaris* with specific PCR primers no PET1 cytotype was observed in natural populations of either *H. annuus* or *H. petiolaris.*

Induced male sterility reported here seems to sustain the previous hypothesis. Perhaps, gibberellins-induction of male sterility in fertile plant could be the results of amplification and/or transcriptionally activation of CMS *orf* (present as a sublimon according anterior hypothesis) by GA-signalling components via a receptor/signal transduction pathway.

Taken together, the work presented here appears to support the hypothesis based on the current homeostatic model (48), that an increase of the active GAs level derepresses the GA response (feed-forward mechanism). The increased activity of the GA response pathway resulting finally the expression of the gene responsible for male sterility as a GA-responsive genes causes a reduction in the levels of bioactive GAs by inhibiting GA biosynthesis (feedback mechanism) and activating GA catabolism. Also, it is suggested that this enhanced transient GA-signal (induced by a single exogen GA_3 application) in reproductive tissue in a critical stage for microsporogenesis was sufficient to generate changes in GA signal transduction pathway resulting the induction of CMS *orfH522* expression at a given level is able to causes male sterility. Our data thus emphasize that maintaining the levels of the bioactive GAs in appropriate temporal and spatial patterns is a vital requirement for the plant during its growth and development.

Conclusions

In conclusion, the present investigations provide the first evidence that expression of CMS-specific *orfH522* gene can be induced by GA_3 in plant with original fertile cytoplasm. How exogenous applied GA_3 is involved in plant cytoplasmic male sterility induction is still unclear, it is possible that GA-responsive genes affect a subset of GA responses. These are preliminary results and may lead to new investigations to gain further understanding in this field.

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