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Androgenesis in chickpea: Anther culture and expressed sequence tags derived annotation

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Double haploid technique is not routinely used in legume breeding programs, though recent publications report haploid plants via anther culture in chickpea (*Cicer arietinum* L.). The focus of this study was to develop an efficient and reproducible protocol for the production of double haploids with the application of multiple stress pre-treatments such as centrifugation and osmotic shock for genotypes of interest in chickpea for their direct use in breeding programs. Four genotypes, ICC 4958, WR315, ICCV 95423 and Arearti were tested for anther culture experiments. The yield was shown to be consistent with 3-5 nucleate microspores and 2-7 celled structures with no further growth. To gain a further insight into the molecular mechanism underlying the switch from microsporogenesis to androgenesis, bioinformatics tools were employed. The challenges on the roles of such genes were reviewed while an attempt was made to find putative candidates for androgenesis using Expressed Sequenced Tags (EST) and interolog based protein interaction analyses.

Keywords: Androgenesis, Anther culture, Chickpea, EST, Protein - protein interaction

Chickpea (*Cicer arietinum* L.) the world's second most important food legume is a self-pollinating diploid (2n) with a genome size of 738 Mbp[1]. Grown in subtropical and semi-arid regions, it is known to be a rich source of dietary protein and fibre, and is low in glycemic value which makes it a primary source of human dietary protein in the developing world. However, breeding efforts, both by traditional or molecular methods are hampered by narrow genetic diversity in elite gene pool[2] and a paucity of biotechnological methods[3,4]. Recently, draft whole genome shotgun sequence of CDC Frontier, a chickpea *kabuli* variety has been reported which highlighted candidate genes for disease resistance and agronomic traits[1]. Despite the availability of vast genomic data and markers for various agronomically important traits, gene regulatory networks for specific developmental pathways like androgenesis, somatic embryogenesis, apomixes etc. are not yet available. Prediction of putative candidates for such traits based on interolog mapping and protein-protein interactions reveal valuable insight into the functional repertoire and help to identify novel molecular factors responsible for modulating these pathways under specific conditions.

Double haploids (DH) are important tools for plant breeding and molecular genetics. They are produced by doubling the chromosomes of haploid plants either spontaneously *via* endomitosis or by chemical means leading to homozygous DH individuals with two identical copies of each chromosome[5]. Haploids and DH can be produced by various mechanisms such as androgenesis[6], apogamy[7] or wide crosses[8]. The production of DH is the fastest route to homozygosity in plants[9]. The current method of obtaining homozygosity in chickpea by recombinant inbred lines (RILs) is not only time consuming but also not feasible considering the fact that certain regions in the genome may retain heterozygosity despite repeated selfing of F_2 populations[10]. On the other hand, legume species are considered recalcitrant to DH production[11] wherein successful induction of androgenesis and regeneration of haploid plants is limited to a few legumes species[12-15]. Despite a number of efforts, DH technology is not used as a routine tool for breeding in any grain or pasture legume and is at technology development stage in chickpea[16-18]. Inducing cell

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division and cell differentiation to produce androgenic embryos is modulated by several factors, including genotype, growth conditions of donor plants, stage of microspores at culture, pre-treatment of flower beds etc. Abiotic stress pre-treatments such as centrifugation, electroporation and osmotic shock were shown to have a positive effect on induction of androgenesis in a number of species including legumes[19,20].

Lack of understanding of the molecular and cellular mechanisms of microspore embryogenesis impedes the efficient use of DH technology. Functional genomic approaches that determine genes associated with androgenic induction have revealed several hundreds of up-regulated and down regulated genes while the functions of some have been investigated[21,22]. Due to their high regeneration efficiencies, barley, rapeseed, tobacco[23], maize[24] and wheat have been considered model species to study the mechanisms of stress-induced androgenesis. *In vitro* embryo cultures derived from both somatic and gametophytic tissues have been used successfully as tools to identify genes such as ECA1 (early-culture abundant 1), AGPs (arabinogalactan proteins), ECGST (glutathione S-transferase), ECLTP (early-culture lipid transfer protein), BBM (babyboom) etc., expressed during early embryo development[25-30]. Despite the considerable amount of information available regarding the microspore embryogenesis, little is known about this process in legumes[31]. Holistic approaches such as the integration of genomics, proteomics and metabolomics, from the perspective of systems biology, have a great potential in revealing the interaction between different signaling cascades involved in triggering androgenesis. Keeping in view of the aforementioned reasons, the aim of the present study is to develop an efficient protocol for the production of DH in chickpea based on the anther culture protocol reported by Grewal *et al*.[32]. While trying to reproduce this protocol, parameters like pre-treatments and media were modified in order to achieve higher rates of induction.

Materials and Methods

Donor plant—Experiments were carried out with four genotypes ICC 4958, WR-315, ICCV 95423 and Arearti obtained from Genetic Resources Unit, ICRISAT (Patancheru, India). Plants were grown in the green house maintained at 25/20 °C (day/night) under natural light conditions with the intensity varying from 450-600 µmol m^{-2} s^{-1}. A 6:2:1 ratio of black soil, vermin-compost and sand mixture was autoclaved and filled in 30 cm pots with three plants per pot and labelled appropriately.

Bud selection, cold pre-treatment and sterilization—Buds 2-3 mm in length, containing mid–late uni-nucleate microspores (confirmed with acetocaramine staining) were harvested and stored in a Petri plate lined with a moistened filter paper. The buds were cold pre-treated at 4 °C for 2-4 days before processing them for culture. The cold pre-treated buds were sterilized in 2% buffered bleach for 20 min with occasional stirring and then rinsed three times with sterile distilled water to remove all traces of bleach.

Pre-treatments applied to elicit/enhance androgenesis—Stress pre-treatments were applied individually and in combination in order to determine their effect on the response of microspores. While applying multiple stresses, the parameters of each stress treatment were individually varied keeping others constant. Anthers were aseptically removed from the buds and centrifuged (fixed rotor microcentrifuge, GeNei) in a 1.5 mL sterile eppendorf centrifuge tube with 1 mL of liquid medium (high osmotic pressure, RM-IK) at varying speeds (168, 200, 500, 671 and 1000 *g* for 3, 5, 10 and 15 min). Anthers were finally subjected to an osmotic stress for 4 days in induction media (high osmotic pressure liquid media, RM-IK, RM-D or CH1). A maximum of 30 anthers were cultured per 60×12 mm sterile plastic Petri plate with 4 mL media. The cultures were maintained in dark at 24±2 °C and then transferred to a semi solid embryo development medium.

Anther culture—A range of media were tested for all the four genotypes. All the media were derived from Grewal *et al*.[32] and Croser *et al*.[33] media (Table 1). For induction, liquid media RM-IK, RM-D and modified form of CH1 media were used with various combinations of auxins and cytokinins (Table 2). Each experiment had three plates with an average of 30 anthers per plate. Cultures were incubated at 24±2 °C in dark. After four days in liquid induction medium, the anthers were transferred to embryo development medium (EDM) and maintained under same conditions. A few anthers were allowed to remain in liquid medium for over a month. Anthers were squashed in 4% acetocaramine and microspores were observed under light microscope at regular intervals to track changes during culture.

Bioinformatics and Systems Biology analysis—Excepting the model organisms such as barley, maize, wheat and tobacco, it is not known whether or not

specific genes leading to androgenesis do exist. Thwarting the problem, an attempt was made at identifying those genes involved in androgenesis in chickpea. While androgenic development involves understanding embryogenic potential to formation of patterns, it would also be essential to understand the genes which are involved in androgenesis. From preliminary analysis, no specific androgenesis related proteins were found in chickpea. To start with, an approximate 45000 ESTs in chickpea (http://www.ncbi.nlm.nih.gov/nucest/?term=cicer+arietinum) have been employed for a pipeline strategy to check the number of putative genes in this genome. To increase the effectiveness of EST sequences for identification of candidate proteins, clustering procedures such as EG assembler and EST explorer were employed (Fig. 1). The best hits were then chosen for annotation of proteins by searching against *Arabidopsis thaliana* protein sequences. Further, interolog mapping and protein-protein interaction (PPI) studies have been done with a couple of putative genes enlisted to androgenesis. Interolog mapping predicts an interaction between a pair of proteins for example A and B if there exists a known interaction between their orthologs (A', B') in another species. This analysis could be a novel approach to the identification of proteins involved in androgenesis in chickpea from EST data.

Table 1—Composition of media used during centrifugation, induction and for embryo development.

Components (mg/L)	RM-IK	RM-D	EDM	CH1
NH_4NO_3	--	--	1000.0	--
KNO_3	61.0	61.0	2100.0	2500.0
KH_2PO_4	67.0	67.0	325.0	170.0
$NaH_2PO_4.H_2O$	--	--	85.0	--
$CaCl_2.2H_2O$	--	--	600.0	600.0
$Ca(NO_3)_2.4H_2O$	500.0	500	--	--
$MgSO_4.7H_2O$	150.0	150	435.0	370.0
$FeSO_4.7H_2O$	27.8	27.8	27.8	27.8
Na_2 EDTA	37.3	37.3	37.3	37.3
H_3BO_3	10.0	10	5.0	6.2
KI	1.0	1	1.0	0.83
$MnSO_4.H_2O$	20.0	20	15.0	16.9
$ZnSO_4.7H_2O$	15.0	15	5.0	1.4
$CuSO_4.5H_2O$	0.50	0.50	0.10	0.025
$NaMoO_4.2H_2O$	0.25	0.25	0.40	0.25
$CoCl_2.6H_2O$	0.50	0.50	0.10	0.025
Thiamine HCL	0.10	0.10	2.0	--
Pyridoxine HCL	1.00	1.00	0.50	--
Nicotinic acid	0.50	0.50	--	--
Myo-inositol	1000.0	1000.0	250.0	--
L-Glutamine	800.0	800.0	--	--
Glycine	2.00	2.00	--	5.0
Cholecalciferol (vitamin D)	0.10	0.10	0.10	--
Cynacobalamin (vitamin B_{12})	0.01	--	0.01	--
Calcium pantothenate	1.0	1.0	1.0	--
Riboflavin	0.05	0.05	0.05	--
Glutathione	--	1.00	1.0	--
Biotin	--	0.10	0.10	--
Casein hydrolysate	--	---	300.0	200.0
Coconut water (ml)	--	---	20.0	--
Picloram	--	---	1.0	0.265
2-ip	--	----	0.40	--
IAA	4.00	--	--	--
Kinetin	0.40	--	--	--
2,4-D	--	2.0	--	0.530
BAP	--	--	--	0.09
Sucrose	170,000	170,000	40,000	88,998
Maltose	--	--	50,000	--
*p*H	6.60	5.80	5.80	5.8
Agar (%)	--	--	1.60	--

Results and Discussion

The best androgenic response was observed for uninucleate microspores when the buds were 2-3 mm long, with light yellow and translucent anthers. Earlier stages contained tetrads that were unresponsive in culture and resulted in clustering of early uninucleate cells. While at later stages, microspores were unable to shift from gametophytic to sporophytic mode of development and accumulation of starch granules was observed. Some of the late stage microspores enlarged in size with condensed cytoplasm; however no further growth was observed.

Effect of stress pre-treatments—Given the benefit of combined abiotic stress treatments in other species, cold treatment at 4 °C for 4 days followed by centrifugation and induction in high osmotic medium for 4 days resulted in 3-5 nucleate microspores in all the four genotypes tested. Centrifugation at lower

Table 2—Modified CH1 media

Media (mg/L)	Auxin	Concentration (mg/L)	Cytokinin	Concentration (mg/L)
CHPB	Picloram	0.5	BAP	0.05
CHPZ	Picloram	0.5	Zeatin	0.05
CHPT	Picloram	0.5	Thidiazuron	0.05
CHDB	Dicamba	0.5	BAP	0.05
CHDZ	Dicamba	0.5	Zeatin	0.05
CHDT	Dicamba	0.5	Thidiazuron	0.05
CH2B	2,4-D	0.5	BAP	0.05
CH2Z	2,4-D	0.5	Zeatin	0.05
CH2T	2,4-D	0.5	Thidiazuron	0.05

speeds (168-300 *g*) and longer period of time (10 min) or higher speeds (671-1000 *g*) and shorter period of time (3 min) had no significant difference on the response when cultured in CHPB, CH1 and RM-D media. The two *desi* varieties ICC 4958 and WR 315 responded at higher speeds for short period of time giving 5 nucleate microspores. The *kabuli* variety ICCV 95423 was responsive to lower speeds for longer durations giving 2-4 nucleate microspores, while Arearti was the least responsive genotype (Fig. 2).

The induction of the first division of nuclei was observed after 4 days in high osmotic medium; upon transfer to EDM medium, further divisions were observed after 6–10 days in culture. The specific combination of osmotic media and the variety (i.e *desi/kabuli*) of chickpea cultivars also had significant effect on the androgenic response. Induction was most effective when the *desi* varieties ICC 4958 and WR 315 were centrifuged and cultured in RM-D medium, and the *kabuli* varieties ICCV 95423 and Arearti in RM-IK medium.

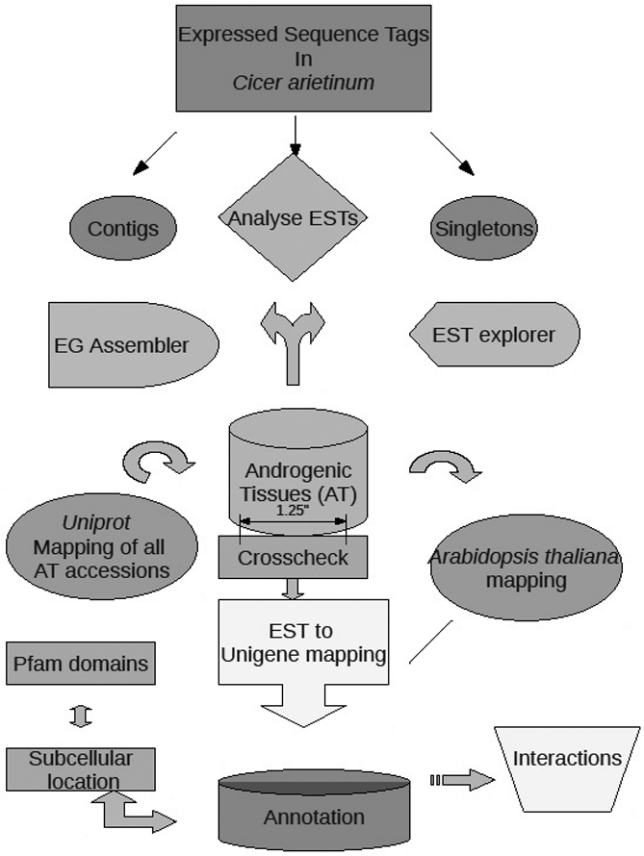

Fig. 1—Schematic diagram of pipeline used to find putative AD related proteins in chickpea. All ESTs were subjected to Uniprot mapping and annotated for potential domains using Pfam.org and TargetP for subcellular location.

Effect of media composition—Composition of media had significant effect on the induction and further divisions of microspores. Of the 12 different induction media tested, the most effective was CHPB followed by RM-D. Cold pre-treated anthers, when centrifuged and cultured in these media for 30 days resulted in 4-8 celled microspores in three of the cultivars tested except for Arearti (Fig. 3). Multi cellular microspores were observed after 30 days in continuous culture but the number of such microspores was as low as 1–3 per anther. Centrifugation did not seem to be an important factor when continuous cultures were maintained in RM-D medium for a prolonged period (~30 days). While percent response per anther was found to be poor in all the genotypes tested (Fig. 4), androgenesis was induced in low percentages. Vegetative tissue was also found to be dividing in CH1 and RMD media.

EST analysis and interolog mapping—A range of media and pre-treatments were tested for induction of androgenesis in chickpea based on recent successful reports by Grewal *et al*.[32] and Croser *et al*.[33], which yielded insignificant response. This calls for modified and well informed approach towards developing DH protocol in chickpea. In this direction, a preliminary attempt was made to identify genes involved in androgenesis. Analysis of several thousand ESTs resulted in very few proteins with plausible role in androgenesis. Sequence similarity searches employing FASTA and tFASTx between query sequences from *Arabidopsis thaliana* known to be related to androgenesis[29] (text mining), and chickpea EST databases showed that AGL16 and AGL6 are best matches (Table 3). Although it is less likely that the DNA matches complementary with the ESTs (cDNA)

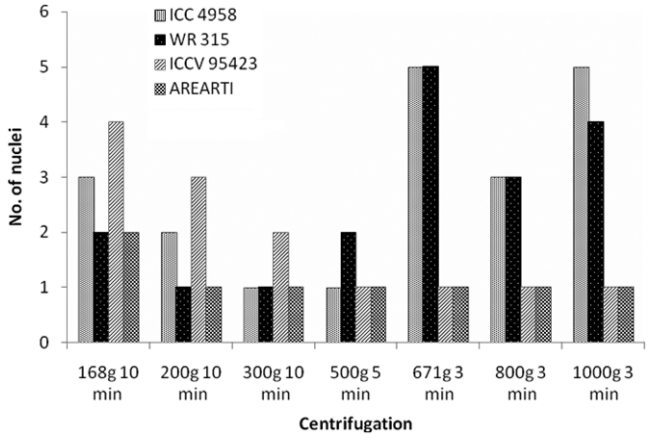

Fig. 2—Effect of centrifugation on genotype and response. Divisions of nuclei observed after 10 days in culture.

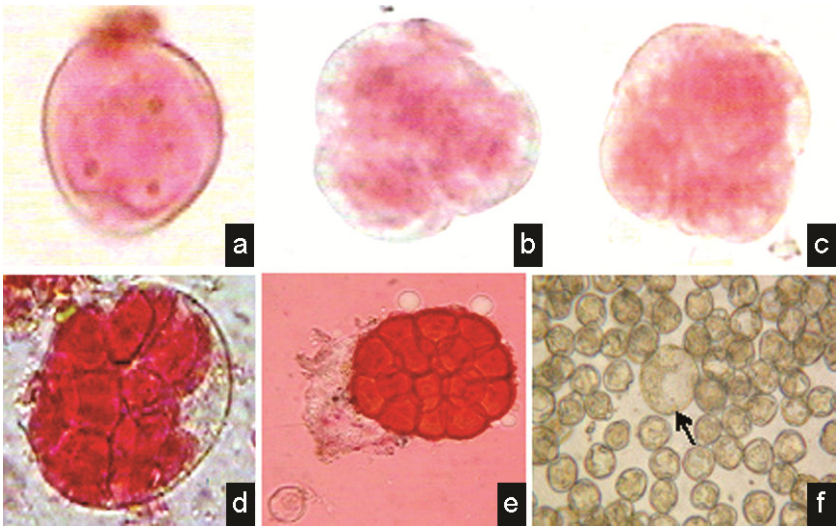

Fig. 3—Androgenic response of microspores observed under bright field light microscope. ICC 95423 microspore division in CHPB medium, (a) 3 nucleated microspore (b) 3 celled and (c) 4 celled. (d) ICC 4958 in RM-D medium, multicellular microspore. (e) WR 315 in CHPB medium, multicellular microspore. (f) Difference between responsive enlarged (indicated by arrow) and non-responsive microspores.

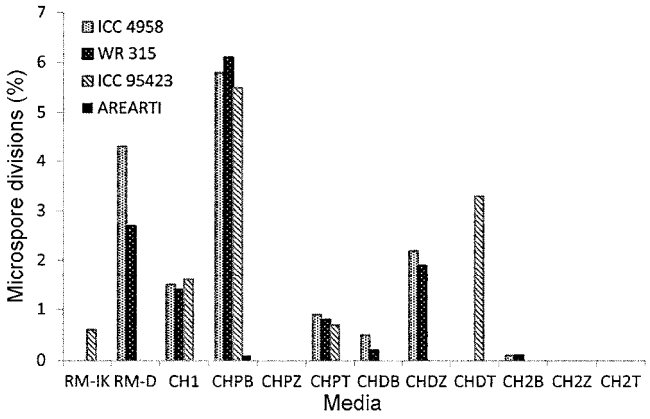

Fig. 4—Microspore division (%) observed for the genotypes tested on different media. Data collected after 30 days in culture.

Table 3—Consensus reached after sequence similarity search between query sequences from Arabidopsis against Chickpea EST datasets.

Arabidopsis thaliana protein	Chickpea		
	Tfastx	FASTA	Consensus
PHE1	1	0	Not a good candidate
SVP	1	0	Not a good candidate
AGL16	1	1	**Possibly a good candidate**
SHP2	0	0	Not a good candidate
AGL6	1	1	**Possibly a good candidate**
AGL24	1	0	Not a good candidate
AGL21	1	0	Not a good candidate

of another organism, as a standard at least 60% of similarity is considered as a candidate. Further functional annotation of all proteins of chickpea when searched against *Arabidopsis thaliana* protein sequences, resulted in few plausible proteins, *viz.* PHE1, SVP, AGL16, SHP2, AGL6, AGL24 and AGL21 which are involved in androgenesis (Table 4). It would be wise to discern how many of these would be bona fide by understanding whether or not they are orthologous across the EST database of chickpea. For example, from the present annotation, AGL15 (AGAMOUS-LIKE 15); DNA binding / transcription factor (AT5G13790) has a few interacting partners by virtue of text mining but transferring (interolog) the interaction to chickpea is usually not affirmative unless wet lab experiments are complemented. The protein interaction network done with AGL15 (Fig. 5) as query revealed functional partners (SVP, PHE1,AGL21, AGL20, SHP1 etc.) and associated functional partners (SEP1, FLC, MYB, AGL53 etc.) which have validated annotations. Together with erstwhile protein interaction networks, done with AGL15 (Fig. 5), similarity searches and functional annotation of chickpea proteins, AGL16 and AGL6 are considered as good candidates for a simplest pull down assay experiment which would provide ample evidence in identifying the androgenesis related genes in chickpea. Therefore, it is believed that EST analysis plays an important role where sequences are not known and annotated, and this is a novel approach towards understanding of the proteome of androgenesis in plants.

Double haploid research in legumes has received considerable attention in the last decade[14,15,32] and this

Table 4—AtPID prediction of query proteins.

GSP	Annotation	Methods	Related
AT4G09960(STK)	STK (SEEDSTICK); protein binding / transcription factor	text mining	15805477 ()
AT4G11880(AGL14)	AGL14 (agamous-like 14); DNA binding / transcription factor	text mining	15805477 (yease two-hybrid assay)
AT3G58780(SHP1)	SHP1 (SHATTERPROOF 1); DNA binding / protein binding / transcription factor	text mining	15805477 ()
AT3G57230(AGL16)	AGL16 (AGAMOUS-LIKE 16); transcription factor	text mining	15805477 ()
AT2G42830(SHP2)	SHP2 (SHATTERPROOF 2); protein binding / transcription factor	text mining	15805477 ()
AT4G18960(AG)	AG (AGAMOUS); DNA binding / transcription factor	text mining	15805477 ()
AT2G22540(SVP)	SVP (SHORT VEGETATIVE PHASE); transcription factor/ translation repressor, nucleic acid binding	text mining	15805477 ()
AT1G65330(PHE1)	PHE1 (PHERES1); DNA binding / transcription factor	text mining	15231736 (NULL)
AT2G45650(AGL6)	AGL6 (AGAMOUS-LIKE 6); DNA binding / transcription factor	text mining	15805477 ()
AT4G24540(AGL24)	AGL24 (AGAMOUS-LIKE 24); protein binding / protein heterodimerization/ protein homodimerization/ sequence-specific DNA binding / transcription factor	text mining	15805477 ()
AT5G41315(GL3)	GL3 (GLABROUS 3); protein binding / transcription factor	text mining	15231736 (NULL)
AT4G37940(AGL21)	AGL21; transcription factor	text mining	15805477 ()
AT1G69120(AP1)	AP1 (APETALA1); DNA binding / protein binding / protein heterodimerization/ transcription activator/ transcription factor	text mining	15805477 ()
AT2G45660(AGL20)	AGL20 (AGAMOUS-LIKE 20); transcription factor	text mining	15805477 ()

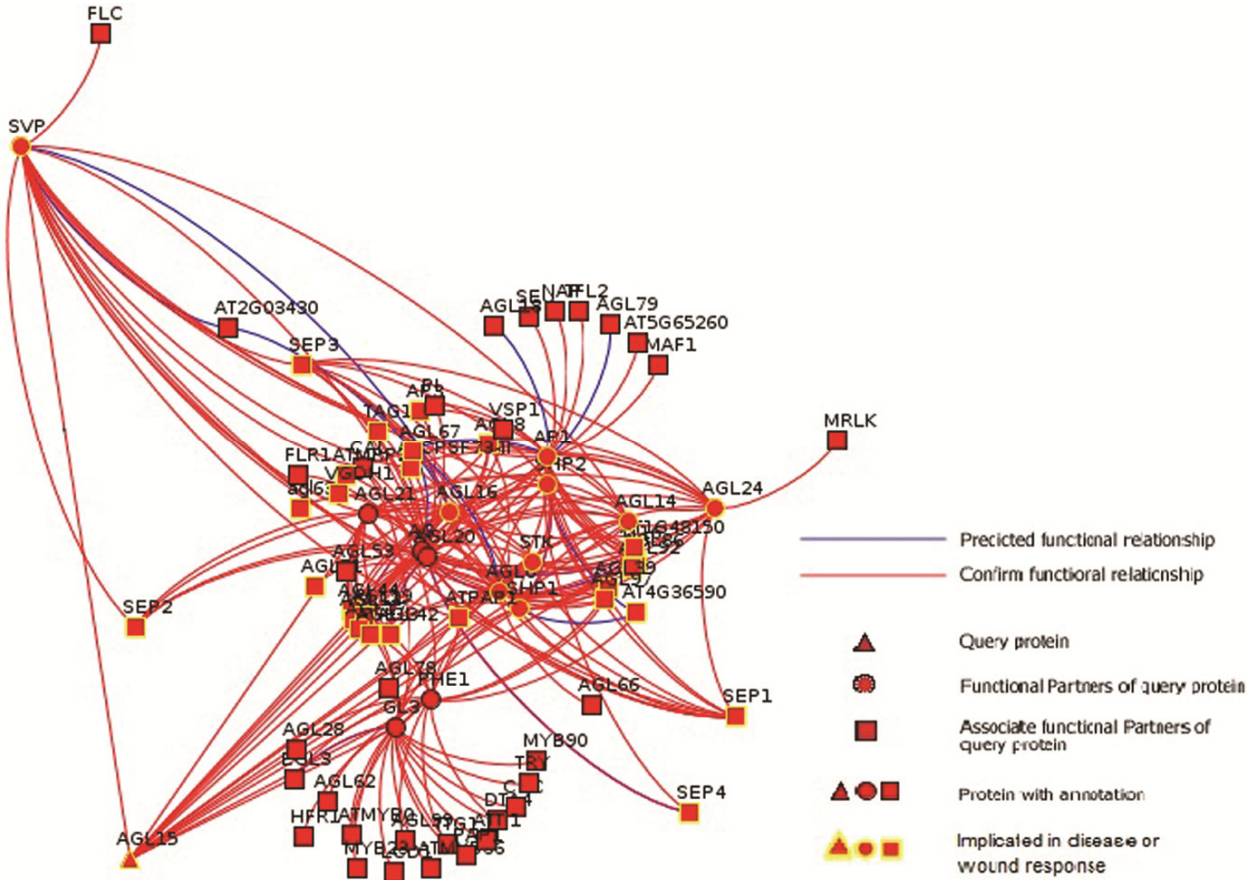

Fig. 5—Protein interaction network of AGL15 which was identified through annotation pipeline as described in Fig. 1.

research was aimed at reproducing the published protocol for the induction of DHs in chickpea. Reproducibility and efficiency are important for any protocol for it to be employed in breeding program. Under certain conditions, microspores can switch from gametophytic to sporophytic mode of development, ultimately leading to regeneration of haploid plants. This switch is modulated by several pre-culture factors such as genotype, growth condition of donor plant, stage of microspore and stress pre-treatments of buds[9,23]. In line with these reports, it was observed that 4 days cold treatment at 4 °C was found to beneficial for induction of androgenesis. A significant difference in responsiveness was observed among genotypes. Contrary to the data from Grewal *et al*.[32], centrifugation did not seem to be an important factor when anthers were maintained in continuous culture in liquid media with high sucrose content (8-17%). Another factor which influenced the microspore divisions is the specific genotype-media combination. This specificity might hinder the development of an efficient media suitable to all genotypes, thus complicating the steps involved. The lack of reproducibility, poor responsiveness, complicated media requirements and need for multiple stress treatments have thus contributed to the difficulty in development of an efficient protocol for DH production in chickpea.

An understanding of the molecular and cellular mechanism underlying the switch from microsporogenesis to androgenesis and the commitment to microspore-embryogenesis may play an important role in identifying key regulators for the production of DHs. Based on the transcriptomic and proteomic studies on model plants[34], a preliminary attempt was made to identify genes related to androgenesis in chickpea, *in silico*, using EST sequences and interolog mapping. Of the 21 genes reported to be involved in androgenesis in model species by Maraschin *et al*.[29], two proteins AGL16 and AGL6 have been identified as putative candidates for androgenesis in chickpea for a simplest pull down assay experiment which would provide ample evidence in identifying the androgenesis genes in chickpea.

Conclusion

Bioinformatics has revolutionized the way an experiment can be designed. Even before the genome is sequenced, the outcome of the number of genes and proteins existing in a genome can be predicted. A putative function can be attributed to a protein derived from EST annotation using protein interaction analysis. Challenges in developing an efficient and reproducible protocol for the production of DHs via androgenesis in chickpea may be attributed to the lack of information regarding these genes. Two proteins AGL16 and AGL6 have been identified as putative candidates for androgenesis in chickpea. Further, prediction of the transcriptional activators or triggers for these candidates would help to build a network of genes and signaling molecules. This analysis could influence the choice of media components and alter the design of the experiment making it a more process and target specific approach. Though this is a beginning, it could put an end to identifying functional genes specific to these processes. This is the first study which reports application of bioinformatics to study androgenesis in legumes in general and chickpea in particular. The methods employed in this study are not specific to androgenesis *per se*, but can be widely employed to ascertain functional relationship of genes that remain to be discovered.

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