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Research article

Optimization of protein extraction and proteomic studies in *Cenchrus polystachion* (L.) Schult

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ABSTRACT

Apomicts have been studied at their genetic levels, but there are no any direct evidence of its mechanism. In order to understand the mechanism involved, a close relative of *Pennisetum*, *Cenchrus polystachion*, an apomictic species was explored for more insights into protein expression in reproductive structures. Optimization of protein extraction was studied with the leaf tissue and optimized protocol was extrapolated to other five tissues. The phenol-based protein extraction emerged as the best method for plant leaf tissue providing a better protein yield, separation of bands, removal of non-protein components like polyphenolic compounds and nucleic acids. The proteome analysis of leaf, stigma, immature ovary, seed, anther sac and pollen tissues of *Cenchrus polystachion* were carried out identifying a total of 135407 proteins against the Poaceae database from UNIPROT/TrEMBL. The target candidate proteins found in all the tissues were identified and mainly comprised of Actin Protein, PIP, Starch Synthase, ATP Synthase, Glutathione S Transferase, Dehydroascorbate reductase, Ascorbate peroxidase and heat shock proteins. Visualization and descriptive statistics conveyed all the necessary information to understand the differential expression of proteins in *Cenchrus polystachion*. This study forms a base to understand the role of tissue specific expressed proteins in an apomictic plant.

1. Introduction

In the era of commercial cultivars, a process like apomixis will have a big impact if its basis is elucidated. Apomixis will be economical for industry and farmers [1]. The use of omics approach can help identify the key to this mechanism. *Cenchrus polystachion*, an apomictic grass of Poaceae family is a close relative to *Pennisetum* [2] and the structural similarity of the ASGR region between *Pennisetum* & *Cenchrus* is been shown by FISH [3]. The apomixes in *Pennisetum* and *Cenchrus* is of obligate type, and even though if it shows minute level of facultative nature of reproduction, that is equivalent to negligible [4, 5]. Even though apomixis is found in many species, its study becomes complicated owing to its absence in major crop species [6]. Therefore wild grass species can be explored as the best apomictic species. In modern agriculture apomixis can serve as a reproductive strategy for cloning through seeds and producing maternal replicas [7]. Study of proteome of a system can be a key to understand its minimal details. Protein preparation & extraction serves as the first step in the proteomics study. As the principle behind extraction focuses on obtaining the highest quality of protein & the best yield, therefore selecting a good extraction method based on the type of sample should be the foremost step and plays an important role. The aim of the best extraction method should be: Removal of interfering polyphenols, lipids, and polysaccharides [8], giving an easily solubilized proteins after precipitation and controlling the loss of some labile PTMs. A good method combines good lysis buffer and a precipitation method [9]. Even though various methods are available for protein extraction & precipitation, the advantage of using few of them like TCA extraction or phenol extraction is there compatibility with mass spectrometric analysis as compared to methods like acetone precipitation or methanol/chloroform method which can be applicable to only small volume of samples. Emergence of sophisticated mass spectrometers have made the characterization easy even for Gel-free proteomics strategies [19,10]. Apomixis can have a broad impact on agriculture if it is engineered into sexual crops. Therefore, this research focuses on identification of those proteins playing a role in apomixis in *Cenchrus polystachion*.

2. Materials and methods

Cenchrus polystachion plant was considered for the study. The identified plant material was grown in green house of the university in

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different pots. At the time of flowering, the following tissues were collected namely leaf, stigma, pollen, anther, immature ovary & mature seed respectively from the plants. Immature flower was emasculated for the collection of stigma and immature ovary while the anthers and pollens were collected at 4.00 am before the onset of anthesis. The seeds were collected from the same plant after the flowers were matured. The six tissue samples were collected as biological triplicates totalling up to a total of 18 samples, sorted and preserved at -20^{O}c till further use. All the chemicals used for this work were of HPLC grade.

2.1. Protein extraction

2.1.1. TCA (Trichloro acetic acid) extraction

TCA method for plant protein extraction from leaf tissue was optimized for pH and TCA concentration. The pH range selected for TCA was 7, 7.5, 8, 8.5,9 and three different concentrations were selected as 10%, 50% and 100%. 100mg of leaf powder was weighed and grinded with 1.5ml of lysis buffer B with a composition of 50 mM Tris HCl, 25 mM Sucrose, 0.1 M EDTA pH 8,1% Triton X 100, 10% Glycerol, 10mM DTT (Dithiotritol) and 0.05% β mercaptoethanol for 5 min at 4 °C in chilled mortar pestle. It was incubated for 30 min at 4 °C and centrifuged for 10 min at 10000 rpm. The supernatant was collected and transferred to a new Eppendorf tube. The Precipitation was done as: For TCA 10% method, 1 volume of 10% TCA in acetone with 0.07% β-mercaptoethanol solution was added to the supernatant, For TCA 50% method, 1/4 amount of TCA 50% solution prepared in distilled water was added to the supernatant and For TCA 100% method, the supernatant was precipitated with 1 volume of TCA stock (5 g in 2.27 ml of distilled water) to 4 volumes of supernatant i.e in a 1.5 ml tube the ratio approx. 250 μl: 1 ml. The solution was incubated overnight at -20 °C. The mixture was centrifuged for 10 min at 10000 rpm and the pellet was collected. For washing for TCA 10%, same volume of chilled acetone was added to the pellet and vortexed, For TCA 50% ice cold acetone containing 0.002 g DTT was used and for TCA 100% ice cold acetone was used. The mixture was incubated at -20 °C for 10–15 min. Again, it was centrifuged for 5 min at 10000 rpm, the supernatant was discarded and pellet was preserved.

2.1.2. Phenol extraction (M1)

This is a modified method of Liu et al., 2015 [10]. Proteins were extracted by grinding 100mg tissue for 2 min using a motar and pestle by the method given by Liu et al., 2015 with just a modification of the lysis buffer A with a composition of Phenol 50% v/v, Sucrose 0.45 M, EDTA 5 mM, β mercaptoethanol 0.2% v/v and 50 mM Tris HCl pH 8.5.

2.1.3. Ammonium acetate extraction (M3)

This method is a modified method of Ghatak et al., 2016 [11]. Proteins were extracted by grinding 100mg tissue for 2 min using a motar and pestle by the method given by Ghatak et al., 2016 with a modification of the lysis buffer used which was Lysis buffer B (Refer 2.1.1).

Protein concentration for all the methods was determined by Bradford Assay by Jones et al., 1989.

2.2. Electrophoretic profile of the proteins

Tissue specific proteins were subjected to electrophoresis to study their electrophoretic profiles in a BIORAD PAGE system. Sample volume enough to provide >30 μg of protein from each sample was precipitated using acetone precipitation method. Briefly, 4 volumes of chilled acetone were added to 1 volume of sample and kept at -20 degrees centigrade for 2 h. Samples were then centrifuged for 10 mins at 10,000 rpm. The supernatant was discarded and protein concentration in the precipitates was re-measured by Bradford Assay. 30 μg protein from the precipitate was dissolved in sample buffer containing 5 mM DTT. Proteins were then separated on a 4–12% gradient PAGE at 80–90 V. Gels fixation was done in 100 ml solution of 46% Methanol and 7% Acetic Acid for 1 h and stained in 100 ml solution of 46 % Methanol, 7% Acetic acid and 0.1 % Coomassie Brilliant Blue for 1 h. Gels were destained with 100 ml solution of 5 % Methanol and 7.5 % Acetic acid for 24 h and the destaining solution was replaced every 1 h. The gel was divided into 7 lanes and each lane of the gel was subjected to densitometric analysis. The gels were stored in 1% Acetic Acid solution at 4 °C.

2.3. Protein precipitation and trypsinization

Protein pellet were precipitated by acetone precipitation method. The precipitated pellet were dissolved in 10 μL of 8 M urea and the total volume was made to 15 μL with water. 20 mM DTT was added to samples for reducing them and heated for 15 min at 60 °C. Sample were cooled and 50 mM mM IAA (Iodoacetamide) was added to the samples to alkylate them and incubated for 15mins in the dark at RT. 82 μL of Ammonium Bicarbonate was added and proteins were digested by adding trypsin protease in the ratio of 1:30 and were incubated at 37 °C for 16 h. 1–2 μL of concentrated TFA (Trifluoro Acetic acid) was added after the incubation period was over to stop the reaction. The digested peptides were acidified by dissolving in 20ul of 0.1% formic acid before desalting.

The desalting was performed with C 18 membrane tips. The samples were again subjected to drying in Speed Vacc (Thermo Fischer Scientific) and dissolved in 20 μl of 0.1% FA (Formic Acid) in water [12]. The concentration of peptide determined using Bradford and subjected to ORBI TRAP analysis.

2.4. Protein identification by Mass Spectrometry

1 μg of digested peptides were injected into Q-Exactive plus Biopharma-High Resolution Orbitrap from Thermo Fischer Scientific equipped with nano HPLC with ESI and APCI mode (positive and negative mode ionization). An Asectis C 18 HPLC column was used for elution of peptides during a 90 min gradient from 5% to 50% (v/v) acetonitrile, 0.1 % (v/v) formic acid. A controlled flow rate of 500 nL per minute was used for MS analysis. The column used was Analytical Column: PepMap RSLC C18 2um, 100 A × 50 cm (Thermo Scientific), Pre-column: Acclaim PepMap 100, 100um x 2cm nanoviper (Thermo Scientific) with the mobile Phase as solvent A as 0.1% FA in milliq water and solvent B as 80:20 (ACN:milliq water) + 0.1% FA. The tune settings for the MS were chosen as follows - spray voltage was 1.8 kV and temperature of the heated transfer capillary was set to 180 °C. The resolution setting for MS1 is 70000 and for MS2 it is 17500. Every one full MS scan was further followed by 10 MS/MS scans. Out of these 10 most abundant peptide molecular ions were selected with a dynamic exclusion window set to 90s [11]. Ions with an unidentified or +1 charge state in the full MS were omitted from MS/MS analysis. The peptides were quantified by intensity-based quantification.

SEQUEST algorithm present in Proteome Discoverer Version 2.2 was used for searching the raw data. The identification confidence was set to a 5% FDR at the protein level [11] and the variable modification were set to acetylation of N-terminus and oxidation of methionine. The mass tolerance of 10 ppm was set for the parent ion and 0.8 Da for the fragment ion. A Poaceae database from UNIPROT/TrEMBL(release 2018_09; 1568432 entries) was used for protein identification. A Consensus run was then done for all the six tissues with all three biological replicates using a consensus workflow CWF_Comprehensive_Enhanced Annotation_LFQ_and_Precursor_Quan & processing workflow used was Bsic Sequest HT.

2.5. Statistical analysis

The obtained data was normalized and was assessed for the significance of differential expression by calculating the p values & adjusted p values for the ratios selected on the grouping & quantification. The ratio calculation was done by pair wise ratio-based method and the maximum

allowed fold change was set to 100. The p value threshold was selected as 0.05 and the data was subjected to ANOVA (background based) method of proteome discoverer 2.2. The error rate was managed by adjusting the p value by Benjamini-Hochberg correction. The obtained data was log transformed and then the Principal component analysis (PCA) was done using the proteome discoverer version 2.2.

3. Results and discussion

The objective of this study was to optimize a plant-based protein extraction method and to study the proteome of *Cenchrus polystachion* tissues. The plant was identified as *Pennisetum polystachion,* now officially known as *Cenchrus polystachion*. It was deposited in the herbarium of Savitribai Phule Pune University, Pune, Maharashtra, India. The deposition was given the herbarium accession no. 486.

3.1. Protein extraction

The protein extraction method was optimized with the leaf tissue. The best method was further used for protein profiling of all the tissues. TCA 50% method at pH 8 gave better protein yield which can be due to inhibition of protease activity at higher pH and ionization of phenolic compounds (Figure 1). From the TCA based methods, three methods were chosen for further optimization based on their protein yield, TCA 10% with β Mercaptoethanol (M2) at pH 7.5 with yield of 1.447 mg/gm, TCA 50% at pH 8 (M4) with yield of 1.947 mg/gm and TCA 50% at pH 9 (M5) with yield of 1.497 mg/gm(Table 1 & Table 2). The leaf protein yield was less than 3.080 mg/gm obtained from maize leaf by Wang et al., 2016 [6], 263 mg/gm obtained from leaves of *Ficus deltodia* [14] and more than the leaf protein yield of 1.450 mg/gm for *Vitis vinifera* [15]. These methods were then studied along with the Phenol Extraction methods (M1) and ammonium acetate extraction (M3). TCA based extraction serves advantageous as it inactivates proteases and precipitates proteins. It also easily releases membrane proteins by delipidating the membranes. The TCA/acetone method can also lead to extraction of phenolic contaminants.

These TCA methods were then compared with the other methods and are referred as shown in Table S2. The method M1 yielded an amount of 2.363 mg/gm of protein, which can be attributed to the sucrose gradient forming a good phase separation as compared to 0.820 mg/gm obtained with M3 (Figure 2) (Table 3). The phenol extraction gave a better yield than what Wang et al., obtained a protein yield of 1.640 mg/gm for maize leaves [9] and was in comparison to protein yield of *Vitis vinifera* of 2.230 mg/gm [10,14,15]. The good yield of M1 was due to the removal of interfering substances like non protein compounds, polyphenolic compounds and nucleic acids, due to its high clean-up capacity and its ability to decrease the associations between proteins with other molecules [16].

Though TCA methods also gave a good protein yield from leaf but the difficulty observed with a TCA pellet is that they are difficult to redissolve in the suspension buffer which may result into precipitation of some proteins in the washing solutions causing protein loss [17]. The M1 method was chosen as the best method for the tissues of *Cenchrus polystachion* and was further used for extraction with all other five tissues. The tissue specific protein yield was found to be the highest for seed at 84.453 mg/gm and low for leaf at 1.946 mg/gm. The yield for immature ovary, stigma, seed and anther sac were 20.429 mg/gm, 39.428 mg/gm and 53.299 mg/gm respectively (Table 4).

3.2. Electrophoretic profile of the proteins

Tissue specific protein extracts were analysed for the protein separation and band intensities. Proteins were run with 12% resolving gel and 4% stacking gel on a BIORAD SDS system. After the protein separation, the gels were stained by the coomassie brilliant blue staining. Even though the Coomassie Brilliant Blue is less sensitive, it is more compatible if the peptides have to further subjected to LC-ESI-MS/MS

Table 1. Protein Yield (mg/gm of fresh weight) by different TCA concentrations with five pH range.

TCA Conc.	pH				
	pH 7 (mean ± SD)	pH 7.5 (mean ± SD)	pH 8 (mean ± SD)	pH 8.5 (mean ± SD)	pH 9 (mean ± SD)
	(mg/gm)	(mg/gm)	(mg/gm)	(mg/gm)	(mg/gm)
TCA 50%	0.6533 ± 0.1	1.0167 ± 0.01	1.9467 + 0.07	1.1967 ± 0.01	1.4967 ± 0.1
TCA 100%	0.7467 ± 0.09	0.4001 ± 0.1	0.2003 ± 0.07	1.1567 ± 0.01	0.8833 ± 0.05
TCA 10%B	1.2133 ± 0.05	1.4466 ± 0.09	0.7 ± 0.07	1.2033 ± 0.03	1.0433 ± 0.01

Table 2. Nomenclature used for different methods.

Sl. No.	Method Name	Nomenclature for the method
1.	P2 (pH 8.5)	M1
2.	TCA 10%B (pH 7.5)	M2
3.	P6 (pH 7)	M3
4.	TCA 50% (pH 8)	M4
5.	TCA 50% (pH9)	M5

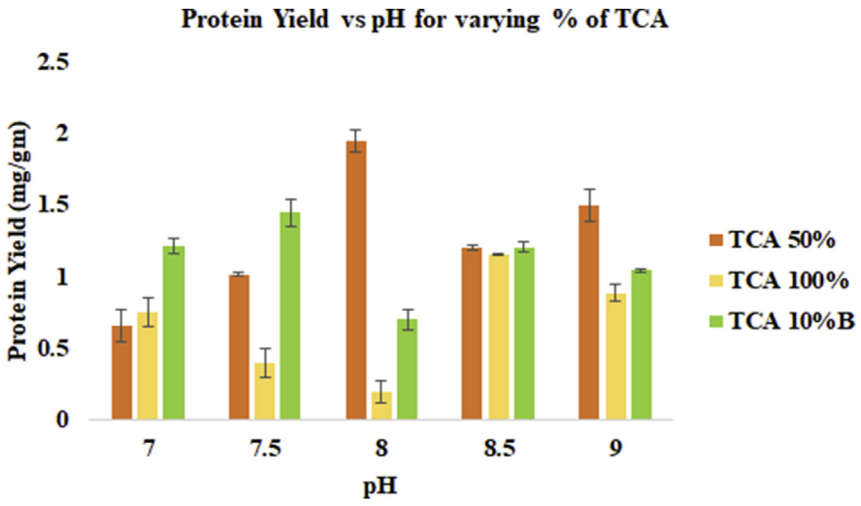

Figure 1. Effect of different pH and TCA concentration on total protein recovery (mg/gm of fresh tissue). Three concentrations of TCA was used 10%, 50% and 100% was studied at five different range.

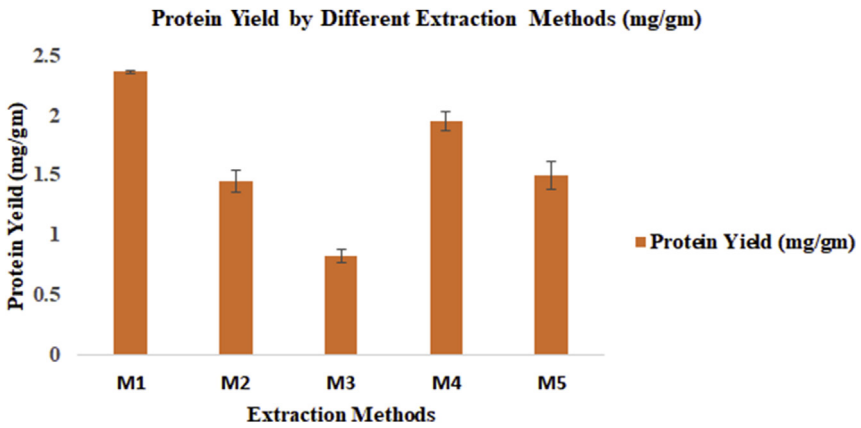

Figure 2. Effect of different extraction methods on Protein Yield. The data clearly indicates M1 as the suitable method for plant tissue protein extraction, also due to its compatibility with Mass Spectrometry.

Table 3. Protein Yield (mg/gm of fresh weight) by TCA and Phenol methods.

Sl.NO.	Extraction Methods	pH
		Protein Yield (mean ± SD) (mg/gm)
1	M1	2.3622 ± 0.01
2	M2	1.4466 ± 0.09
3	M3	0.82 ± 0.05
4	M4	1.9467 + 0.07
5	M5	1.4967 ± 0.1

Table 4. Protein Yield (mg/gm of fresh tissue) of six tissue of *Cenchrus polystachion.*

Sl.No.	Plant Tissue	Protein Yield (mg/gm of fresh tissue)
1	Leaf	1.946 ± 0.03
2	Stigma	39.428 ± 0.2
3	Immature Ovary	20.429 ± 0.2
4	Seed	84.453 ± 0.1
5	Anther Sac	53.299 ± 0.2
6	Pollen	32.149 ± 0.3

Spectrometry [17,18,19]. Efficient protein separation were obtained for all the tissue except for leaf where a smear was observed as shown in Figures 3 and 4. Although in solution digestion approach is applied here, 1 DE is done to ensure better protein separation and to reconfirm the extracted protein (Table 5).

3.3. Protein highthroughput orbitrap analysis

Extracted proteins from all the tissues were digested in solution with trypsin and the peptides were analysed with Q-Exactive plus Biopharma-High Resolution (Thermo Fisher Scientific Pte Ltd.). The obtained raw files of all the tissues were searched with the SEQUEST algorithm of Proteome Discoverer 2.2(Thermo) against a Poeacea UNIPROT/TrEMBL database. Proteomic analysis resulted in a total of 135407 proteins, of which 26704, 30955 and 25400 proteins from stigma, immature Ovary and seed respectively as compared to 20547 proteins from leaf and 25679 and 26667 proteins from anther sac and pollen respectively. The proteins present in all the three biological replicates were considered for further analysis. Out of the proteins identified, 45.23 % were involved in metabolic process, 6.06 % were involved in the regulation of biological process, 9.33 % were involved in transportation process and 6.26 % were involved in a response to stimuli. 13.85 % of proteins were membrane proteins and 7.74 % were cytoplasmic proteins. With regards to its molecular function 33.72 %, 8.79 %, 10.34 %, 13.31 % of proteins were involved in catalytic activity, protein binding, metal ion binding, nucleotide binding and 15.61 % for other functions respectively.

3.4. Quantification and statistical analysis

The statistical analysis of the identified proteins was performed using proteome discoverer 2.2 (Thermo Scientific). The PCA analysis was done after log transforming the data (Figure 5). The score plot suggests that the two principal components namely PC1 and PC2, explained 31.9 % (18.8 % and 13.1 % respectively) of the total variance of the dataset, respectively. PCA analysis revealed that the male gametophytic tissue i.e pollen and anther sac are clearly separated from the female gametophytic tissue like immature ovary by PC1 (Principal Component 1). The PCA loadings plot determine unique set of proteins for each tissue (Figure 6).

The study of differential expression study of the tissue specific proteins results in around 2473 unique proteins in *Cenchrus polystachion*

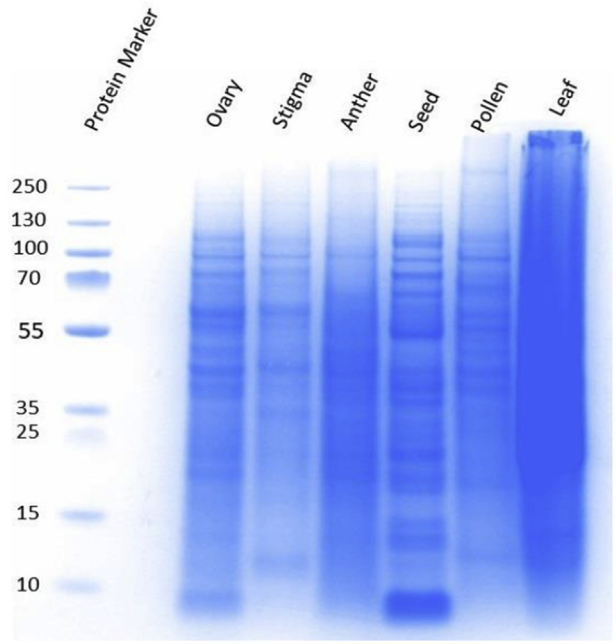

Figure 3. Electrophoretic profile of the different tissue of *Cenchrus polystachion.* The molecular weight marker of the range 250–10 kDa was used as a reference for studying the protein band pattern of the proteome of all the tissues and described the relative changes in band intensities between the six tissues.

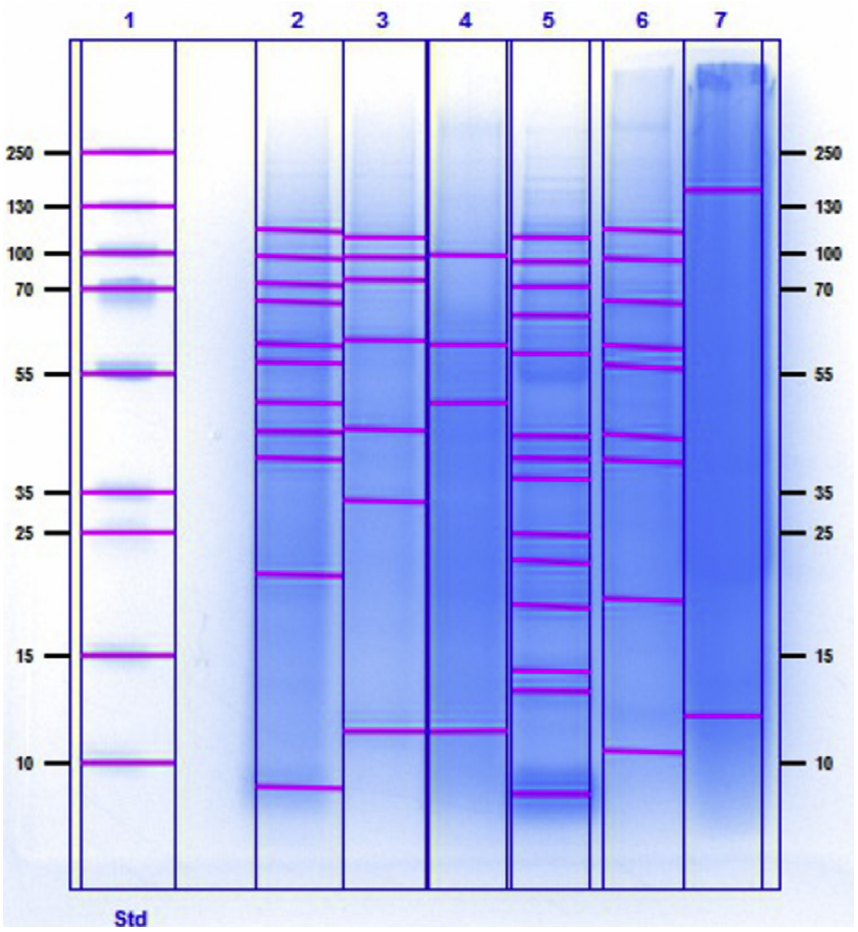

Figure 4. Densitometry Analysis of the SDS Profile of the tissue specific proteins of *Cenchrus polystachion.* 1. Lane 01 Protein Molecular Marker, 2. Lane 2 Immature Ovary, 3. Lane 3 Stigma 4. Lane 4 Anther Sac, 5. Lane 5 Seed, 6. Lane 6 Pollen, 7. Lane 7 Leaf (For Details Ref Table 5).

Table 5. Densitometry analysis of Lane 1–7 of SDS Profile of tissue specific proteins.

Lane 01		Lane 02		Lane 03		Lane 04		Lane 05		Lane 06		Lane 07	
Band No.	Molecular Weight (KDa)	Band No.	Molecular Weight (KDa)	Band No.	Molecular Weight (KDa)	Band No.	Molecular Weight (KDa)	Band No.	Molecular Weight (KDa)	Band No.	Molecular Weight (KDa)	Band No.	Molecular Weight (KDa)
1	250	1	113.3	1	109.1	1	97.8	1	109.1	1	113.3	1	157.3
2	130	2	95.6	2	95.6	2	59.7	2	91.5	2	93.5	2	11.9
3	100	3	73.2	3	76.5	3	49.2	3	71.6	3	67.4		
4	70	4	67.4	4	60.5	4	11.3	4	64.9	4	59.4		
5	55	5	59.7	5	44.4			5	58.2	5	56.1		
6	35	6	56.8	6	32.5			6	43.3	6	43.3		
7	25	7	49.2	7	11.3			7	39.8	7	39.4		
8	15	8	44.1					8	36.8	8	18.9		
9	10	9	39.8					9	24.8	9	10.4		
		10	21.0					10	22.2				
		11	10.0					11	18.4				
								12	14.1				
								13	13.1				
								14	10.0				

(Refer Supplementary File 1) but the proteins close to *Pennisetum* and *Cenchrus* species were targeted as shown in Table 6 to study their apomictic character, their role in plant seed production and pollen fertility.

In the monocot Poaceae family (grasses), the maternal plant significantly contributes to embryo and seed formation by a range of zygotic, sporophytic, and environmental factors [19]. Low levels of actin protein has been said to have a role in reduced fertility in plants and termination of ARP protein may impair many developmental processes [20]. Seed germination is a process involving hormonal signals of auxins, abscisic acid and gibberellic acid. Auxin transport and signalling depend on actin organization. [20,21] The aleurone layer of the seed, the seed coat, and

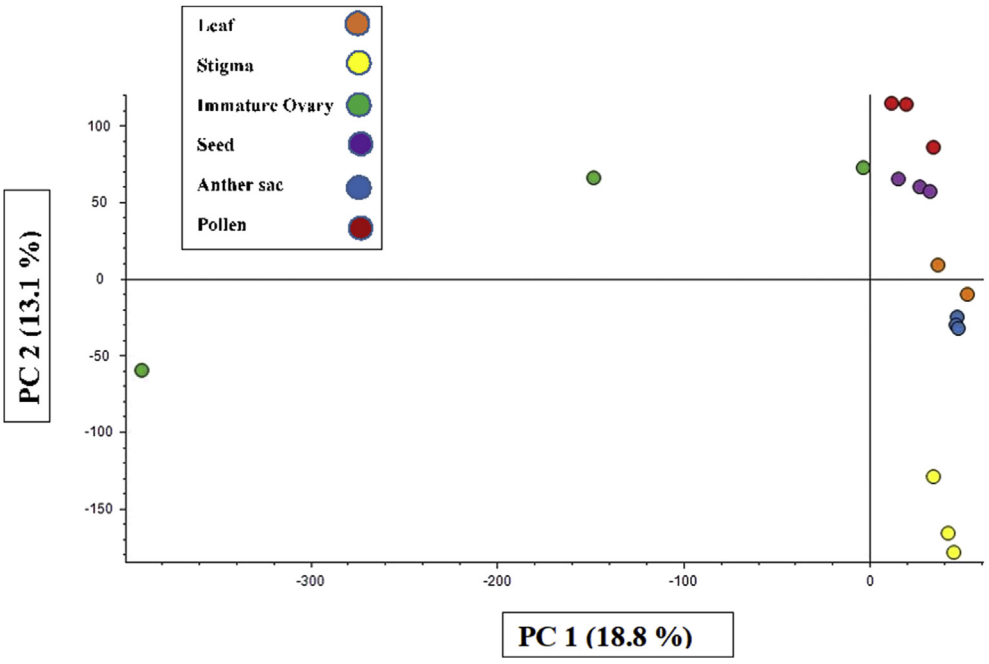

Figure 5. Principal component analysis. The strong variance is seen on PC1 and PC2 and correlates with tissue specific proteome functionality. Plot of the statistically significant proteins of all the tissues of *Cenchrus polystachion.*

organs in young seedling had the highest level of expression of ACT7 actin in *Arabidopsis thaliana* prominating its role in germination and its expression levels tend to drastically increase on exposure to auxin [22]. The abundance ratio of 100 for actin protein in *Cenchrus polystachion* provides a strong evidence for its involvement in the seed development and seed production can be enhanced if Actin levels are upregulated.

The PIPs have been reported to have prominent role in seed germination & growth. In *Oryza sativa* seed germination was reduced on silencing PIP genes whereas germination rate increased on PIP overexpression [23]. The abundance ratio of PIP is more in the male gametophytic tissues anther sac and stigma as compared to other tissues which contributes for the male sterile nature of *Cenchrus polystachion*. PIP is responsible for calcium mediated signalling, via calcium uptake it increases the Ca^{2+} concentration of the cell. Increased calcium concentrations are responsible for the stomatal closure resulting in lesser rate of photosynthesis. The elevated levels of CO2 caused due to decrease in rate of photosynthesis can lead to male sterility which is also supported in *Oryza sativa* cultivar Kirara 397, where increase CO2 levels and Increase in the spikelets caused male sterility [24].

Plant GSTs (Glutathione S Transferase) are regarded as the abundant antioxidants. They have a prominent role in defence mechanism [24,25] and in plant growth and development by binding to different hormones & phytohormones [26]. The increased abundance ratio of GST in seed (2.211) and ovary (0.446) contributes to increased seed production.

Starch is the major carbohydrate in plants and starch synthase protein is necessary for its synthesis in seeds [27]. It can serve as a carbon reserve

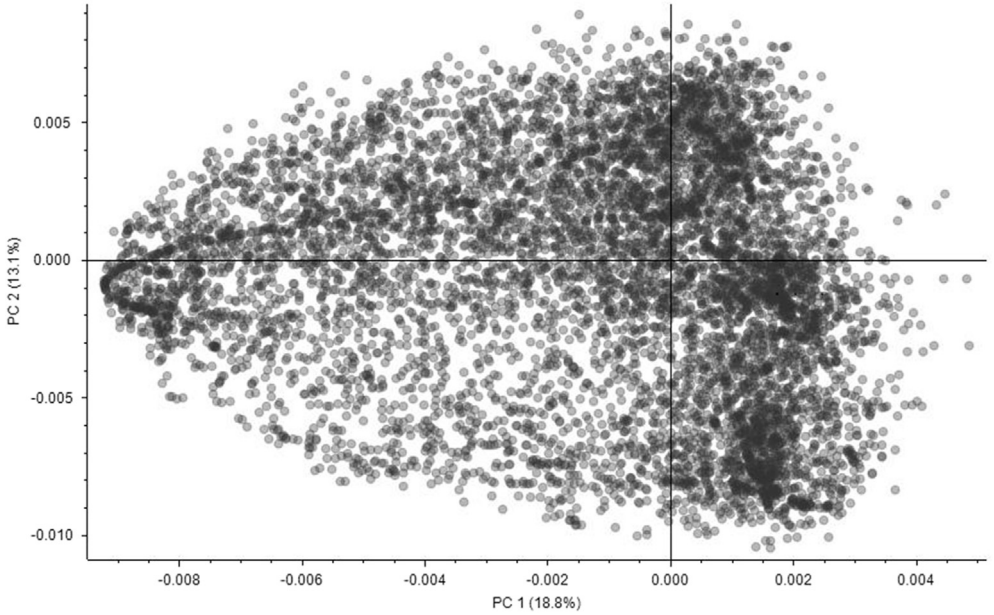

Figure 6. Principal Component Analysis loading plot for all the tissues of *Cenchrus polysatachion*.

Table 6. Abundance Ratios of Differentially expressed Target Proteins of *Cenchrus polystachion.* For the calculation of abundance ratio, values of leaf tissue are taken as source (control) and all other tissues act as sink (samples).

Sl.No.	Accession	Description	Abundance Ratio: (Anther, Sample)/ (Leaf, Control)	Abundance Ratio: (Ovary, Sample)/ (Leaf, Control)	Abundance Ratio: (Pollen, Sample)/ (Leaf, Control)	Abundance Ratio: (Seed, Sample)/ (Leaf, Control)	Abundance Ratio: (Stigma, Sample)/ (Leaf, Control)
1	E5FQ64	Heat shock protein 90 OS = *Pennisetum americanum*	0.405	0.767	3.229	2.209	0.832
2	A0A2D3HJP1	ATP synthase subunit beta, chloroplastic OS = *Cenchrus purpureus* GN = atpB	0.259	0.045	0.988	2.891	2.083
3	U5XRX7	Small heat shock protein sHsp17.0D OS = *Pennisetum americanum*	100	100	100	100	100
4	A0A1B1SJW5	PIP11 OS = *Pennisetum americanum*	0.174	0.01	0.01	0.05	0.293
5	E0ZQA4	Actin (Fragment) OS = *Pennisetum americanum*	100	100	100	100	100
6	Q5NKR6	Starch synthase, chloroplastic/amyloplastic OS = *Pennisetum americanum*	0.01	3.541	3.104	1.721	0.678
7	U5XYA0	Dehydroascorbate reductase OS = *Pennisetum americanum*	0.01	1.444	31.788	0.01	9.105
8	A4ZYP9	Asorbate peroxidase OS = *Pennisetum americanum*	8.111	1.375	6.934	5.29	12.276
9	F1DI22	Phosphoenolpyruvate carboxylase OS = *Pennisetum americanum*	0.076	0.114	0.084	0.099	0.191
10	A0A1B1SJW5	PIP11 OS = *Pennisetum americanum* GN = PIP1;	0.174	0.01	0.01	0.05	0.293
11	Q5MJ30	Glutathione S-transferase OS = *Pennisetum americanum*	1.084	1.54	0.456	2.211	0.154
12	A0A2I4JE72	Delta-1-pyrroline-5-carboxylate synthase OS = *Pennisetum americanum* GN = P5CS	12.841	100	0.01	100	16.446
13	A0A0K1DBU0	Glutathione S-transferase (Fragment) OS = *Pennisetum americanum*	0.01	0.446	0.01	0.01	0.01
14	A0A0E3H7D1	Putative xylanase inhibitor protein (Fragment) OS = *Pennisetum americanum*	1.168	0.547	0.01	0.48	2.026
15	E0ZQA4	Actin (Fragment) OS = *Pennisetum americanum*	100	100	100	100	100
16	C0JW71	Carbonic anhydrase OS = *Pennisetum americanum*	0.072	0.01	0.057	0.021	0.065
17	A0A2D3HJP1	ATP synthase subunit beta, chloroplastic OS = *Cenchrus purpureus* GN = atpB	0.259	0.045	0.988	2.891	2.083
18	A0A088MTG2	Caffeoyl-CoA-3-O-methyl transferase OS = *Cenchrus purpureus*	0.547	1.236	4.823	0.332	0.239
19	A0A1W6C2J7	Ribulose bisphosphate carboxylase large chain OS = *Cenchrus ciliaris* GN = rbcL	1.221	0.027	0.247	0.026	0.011
20	Q9ATP4	Uncharacterized protein OS = *Cenchrus ciliaris*	0.01	6.158	0.01	0.099	0.642

in the support phase of reproductive growth [28]. High abundance ratio of starch synthase in immature ovary in *Cenchrus polystachion* reflects it requirement in developmental stage i.e from immature ovary to seed. As the seed is formed, it's rich in the starch content due to that starch synthesis in immature ovary and therefore the abundance ratio drops down in seed as compared to ovary. Dehydroascorbate reductase (DHAR) is a naturally occurring enzyme responsible for regenerating ascorbic acid from its oxidised state. Ascorbic acid becomes highly oxidised when it detoxifies reactive oxygen species (ROS) in plants to maintain its photosynthetic activity. This regulation of cellular ascorbic redox state by DHAR affects the cell response towards ROS. The levels of DHAR are downregulated in anthers with an abundance ratio of 0.01 but extremely over expresses in pollen with an abundance ratio of 31.788. This shows that presence of ROS are high in pollen tissue of *Cenchrus polystachion* which may affect the pollen function by making it unresponsive to pollination.

Ascorbate peroxidases (APXs) are enzymes that detoxify peroxides using ascorbate. OsAPx1 is involved in flowering, development of seed and fertilization [28,29]. PEPCase is important for the synthesis of amino acid synthesis in developing cotyledons [30] As Delta-1-pyrroline-5-carboxylate synthase, P5CS, is involved in proline biosynthesis, the negligible abundance ratio of this compound in pollen indicates very less or no proline synthesis in pollens making them immature in nature. Some other proteins include heat shock proteins involved in Abiotic stress tolerance and ATP Synthase for energy were also identified. This study provides some of the key targets which have a prospective apomictic nature associated with them.

4. Conclusion

Apomixis is a process to produce seeds through maternal genotype forming maternal clones even when the plant skips three important steps of fertilization. The combination of best protein extraction strategy and mass spectrometric analysis has made proteome analysis of apomictic *Cenchrus polystachion* possible. In the present investigation for the first time we studied differential tissue specific expression study of proteins in *Cenchrus polystachion*. Based on data obtained and expression levels, the role of key molecules have been identified. The results are suggestive that the role of proteins at different developmental stages is very crucial to understand the mechanism of apomixis. This study will further lead to the identification of key targets for induction of apomixis.

Declarations

Author contribution statement

Deepti Somayajula: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Neetin Desai: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

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