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Karyotype and Sex Expression in *Coccinia indica,* a Dioecious Cucurbit

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Abstract: Cytological and immunochemical studies were carried out on dioecious *Coccinia indica* to understand the genetic control of sex expression. The somatic chromosome number of both the sex forms of *C. indica* was found to be $2n = 24$ and the karyotype shows high homogeneity, though a distinct heteromorphic pair of sex chromosomes is found in male sex forms of *C. Indica*. Soluble protein profile from the tuberous roots of the male and female plants of *C. indica* did not show any marked distinction, only a variation in the staining pattern was observed. However, in the immunoblot assay the cross reactivity of anti-p-22 (antibody raised against a female biased 22 kD protein of *Momordica dioica*) at 29 kD and 32 kD regions of the protein profiles in both sex forms establishes p-22 as a sex linked marker. The ratio of the intensity of 29/32 kD polypeptide is also an indicator of sex expression and the data has been analysed as well.

Key words: Karyotype · Immunoblot · Dioecious · *Coccinia indica*

INTRODUCTION

In many sexually reproducing plant species all individuals are essentially identical in the gender condition i.e. Hermaphroditic, less than 4% of plant species are dioecious in nature and strictly maintain their sexual phenotypes [1]. The vast majority of the dioecious plant species have no visibly different sex chromosomes and only some species show distinct X and Y chromosomes in relation to sex [2-5]. Again several kinds of evidences suggest that the sex ratio in dioecious plant species is controlled by the expression of allele in one to several loci [6]. The chromosomal sex determination system in flowering plants also indicates that plant sex chromosomes have evolved recently through replicated independent events [7].

Coccinia indica Wight and Arn. is the dioecious vegetatively propagated species of cucurbitaceae which shows wide spread distribution in the tropical and sub-tropical regions of the world [8]. Earlier workers have found that a series of polyploidy (=24,36,48) exists in *C. indica* with distinct heteromorphic pair of sex chromosomes [9-12] in which Y chromosome plays a decisive role in the differentiation of the male sex [13]. Due to meagre information of sex determination in angiospermic plants, sex linked genetic marker could be also useful in understanding the genetics of dioecism in this taxon. In our recent work on the vegetative reproductive structure of *Momordica dioica,* we have detected a female biased 22 kD polypeptide and through immunoblot assay it has been noted that antibody raised against this 22 kD protein (p-22) not only reacts with 22 kD protein in female plant but also with 29 kD and 32 kD polypeptides of both the sex forms of *M. dioica* [14]. The importance of this genetic marker has prompted the authors to assess the role of anti-p-22 in other dioecious system as well. The present investigation has therefore been aimed at karyotype and immunochemical analysis to resolve the differences between the sexes of *C. indica*.

MATERIALS AND METHOD

The tuberous roots of the sex forms of *C. indica* grown in wild condition collected from West Tripura were grown in the experimental garden of Women's College, Agartala.

Study of Chromosome: To analyse the somatic chromosome shoot tips were pre-treated in saturated solution of paradichloro benzene for 4h at 10-15°C followed by overnight fixation in 1 : 3 acetic acid-ethanol

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mixture. The shoot tips were stained overnight in 2% aceto orcein after hydrolysis in 5N Hcl at 10°C for 15 min and finally squashed in 45% acetic acid.

To analyse the meiotic chromosomes young flower buds of male sex form of C. *indica* was fixed in 1: 2 glacial acetic acid - ethyl alcohol mixture for 3h, then in 1:3 acetic-ethanol mixture for overnight and finally transferred to 70% ethanol for 3-4 days. Then the buds were treated with 45% acetic acid for 15 min, followed by warm hydrolysis in 1 N Hcl for 20 min at 55°C. Finally after thorough washing in distilled water flower buds were stained in 2% aceto-carmine for 2-3h and smeared in 45% acetic acid. As the cytoplasm takes colour, smearing in 45% acetic acid gives desired result.

Sodium Dodecyl Sulphate Gel Electrophoresis

Quantitative Estimation of Protein: 2 gm of fresh tuberous tissue were homogenised in 5 ml of extraction buffer (EB) containing 0.25 M sucrose and 1 mM EDTA in 0.1 M Tris-Hcl buffer (pH 6.8). The homogenates were then centrifuged at 18000 rpm for 45 min at cold. The supernatants were collected and immediately used for electrophoresis. Protein concentration was determined by the method of Lowry [15] using BSA as a standard.

Molecular Analysis of Extracted Samples: The soluble protein obtained in EB was boiled with equal amount of 1x strength electrophoresis sample buffer (12.5% glycerol, 1.25% SDS, 178 mM 2-mercaptoethanol, 0.005% bromophenol blue, 62.5 mM Tris-Hcl and pH 6.8) for 5 min and was allowed to cool at room temperature before proceeding. Electrophoresis in 12% polyacrylamide slab gel, containing 0.1% SDS was carried out by the method of Laemmli [16] and approximately 40µg protein was loaded onto each lane. The protein pattern was visualised by staining the gel for overnight with 0.2% Coomassie brilliant blue R-250 in methanol: acetic acid: DDH2O (9: 2: 9) mixture and destained with several changes of mixture of isopropanol: acetic acid: DDH_2O (2: 1: 7). The positions of the bands were expressed as relative mobilities (R_m) by measuring the distance migrated by the particular bands to that of the indicator bromophenol blue. The different R_m values of the protein bands were numbered serially. Using two cycle semi log graph paper, their molecular weights were determined from the standard curve.

Immunoblot Assay Study

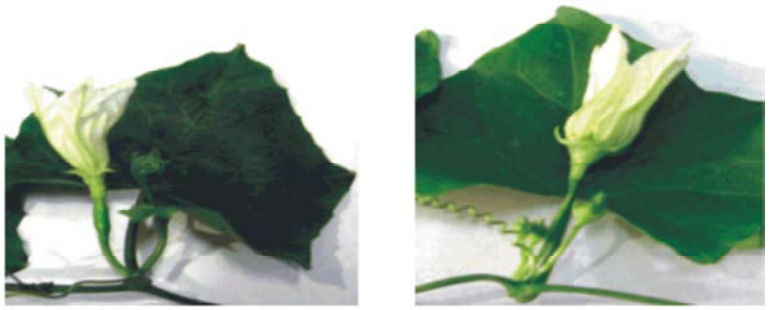
Raising of Antibody Against p-22: From the stained gel strips, 22 kD polypeptide of the protein profile of female sex of *M. dioica* were taken out and homogenised in EB. The suspension containing 80-100µg proteins were injected into rabbits subcutaneously at 2-3 places on the thigh and back on day 0. The step was repeated on 8th, 15th and 22nd day from day 0. After a rest of 10 days blood was collected and allowed to stand at room temp for 2h. It was then centrifuged at 1000 rpm for 10 min. The serum was collected, decomplemented at 56°C for 30 min, mixed with 0.3% Na-azide solution, aliquoted and stored in deep freeze until use.

Immunoblot Experiment: After electrophoresis in 12% polyacrylamide mini slab gel, the resolved polypeptides were transferred electrophoretically onto nitrocellulose paper with 0.1 Amp. Current for 90 min at 4°C [17]. The nitrocellulose sheet was then blocked with 0.5% Tween 20 in TBS buffer (20 mM Tris and 50 mM NaCl, pH 7.5) for 2 h at 37°C. After blocking the sheet was transferred to a fresh solution of TTBS (20 mM Tris, 50 mM NaCl and 0.05% Tween 20) containing anti p-22 at 1: 7500 dilution and again incubated at room temperature for 90 min with shaking. Then the sheet was washed with TTBS for a period of 2 h, then incubated with 1 : 5000 dilution of anti-IgG alkaline phosphatase conjugate (Cat-A 8025, Sigma) in TBS for 1 h with gentle agitation. The paper was then washed with TTBS containing Tween 20 (0.05%) for 3 times each with duration of 30 min. The bands were developed with 5 bromo-4-chloroindonyl phosphate and nitroblue tetrazolium [18].

RESULT AND DISCUSSION

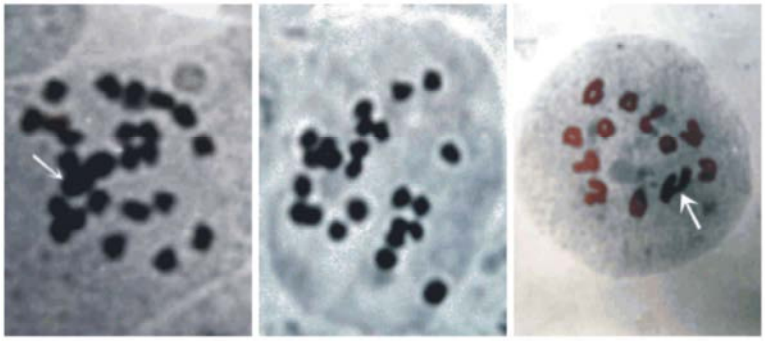
The sexual phenotypes of C. *indica* differ and it has been observed that the male flowers of *C.indica* are in solitary racemes whereas female flowers are solitary (Fig. 1a, b).

In the present investigation the Somatic chromosome number $2n = 24$ (Fig. 2a, b) was found to be constant in the male and female plants of *C. indica* as was reported by earlier researchers [19]. In both sexes there were 11 pairs of metacentric and one pair of sub-metacentric chromosome having secondary constriction. The present cytological investigation also revealed the presence of a distinct heteromorphic pair of sex chromosome in male plants. Diploid males are, therefore, heterogametic with 22 + XY and the females are homogametic with 22 + XX. The male determining Y chromosome is conspicuously large, nearly metacentric and does not bear secondary constriction as reported by Singh [20]. This paradoxical situation can be explained by the fact that in *C. indica* the maleness determining Y chromosome (Fig. 2b) is conspicuously large and is nearly 3-4 times larger than



a b

Fig 1: *C. indica* twigs with male and female flowers respectively.



a b c

Fig 2: Somatic metaphase plates male and female plants of *C. indica* respectively showing 2n=24 chromosomes (Arrow indicates the Y chromosome). Meiotic metaphase-I showing 12 distinct bivalents (Arrow indicates the heteromorphic bivalent)

Table 1: Comparative analysis of karyotypes of *C. indica*

Sex	Somatic Chromosome Number	No. of chromosome with Secondary constriction	Total chromosome Length (μm)	Total F%	Chromosome Symmetry index(SI)
Male	24	2	40.92	44.81	82.15
Female	24	2	38.46	45.14	71.68

majority of the chromosomes present in somatic chromosome complement of the male plant. Detailed karyological studies suggest that apart from the sex chromosomes, the karyotypes of male and female plants exhibit similarity in the types of chromosome present, number of chromosomes with secondary constriction, chromosome arm symmetry index (SI) and TF% (Table 1). Meiotic study from flower buds of male sex form show 12 pairs of distinct bivalents where the heteromorphic bivalent shows homology (Fig. 2c) exists only between the tips of X and Y chromosomes [21, 22].

In our earlier work, we have shown that the somatic chromosome complement (2n=56) of male and female plants of *M. dioica* was more or less homogenous and no XY mechanism operates in controlling sex expression [23]. In the absence of definite X and Y chromosomes it can be assumed that the inherent genetic makeup responsible for the expression of sex forms of both *M. dioice* and *C. indica* perpetuates, through the tuberous roots from which sprouting occurs every year without failing, to reproduce their own kind. We have also reported that the electrophoretic distinction between sexes of *M. dioica* was marked by the presence of 22 kD (p- 22) polypeptide in female sex [24]. But the soluble protein profiles of the tuberous roots of the sex forms of *C. indica* fractionated by SDS-PAGE did not show any such marked distinction. However, a variation in the intensity of bands between the sexes was recorded although equal amount of protein was loaded onto each lane (Fig 3a). It is true that 22 kd protein is absent in the protein profiles of the sex forms of

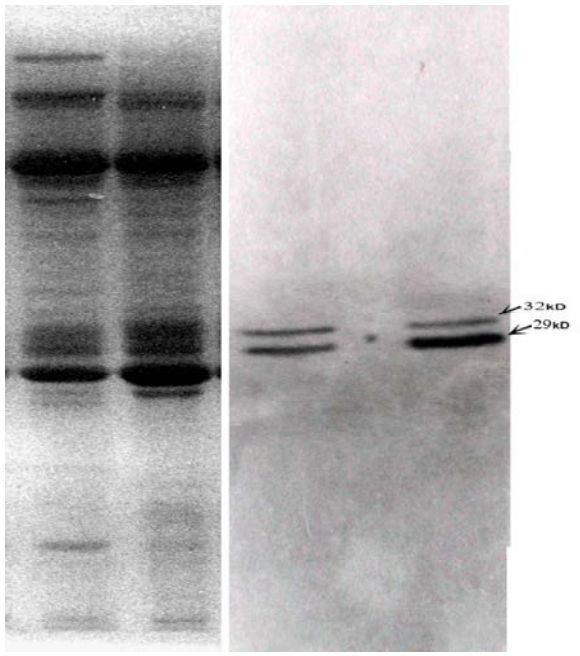


Fig 3: (a) Electrophoregram of protein profile from tuberous roots of male and female plants of *C. indica* respectively.
Fig 3: (b) Immunoblot showing antigen recognition by anti p-22 lane-1 *C. indica* (Male), lane-2 *C. indica* (Female)

diploid *C. indica*, but to understand the role of anti-p-22, the immunoblot experiment was performed to determine the cross reactivity of anti-p-22. To this effect, it has been observed that like in *M. dioica* the antisera also cross reacted at 29 kD and 32 kD region of the protein profiles (Fig 3b) of both the sex forms of *C. indica*. This indicates that two immunologically cross reacting antigens are also present in both the sex forms of *C. indica*. Evidently, such cross reactivity firmly establishes p-22 as a sex linked polypeptide. The immunoblot assay also demonstrates that, in female sex the intensity of, 29 kD polypeptide is more than 32 kD polypeptide, whereas, in male sex, the amount of 29 kD and 32 kD is more or less same (Fig 3b). Thus the ratio of intensity of 29 kD and 32 kD polypeptide differs in male and female sex forms of *C. indica*. It appears that the ratio serves as an indicator of sex expression. We have taken the intensity ratio of 22 kD/32 kD polypeptides as co-efficient of sex expression in this dioecious member which may be..1 or >1. The data also reveals that in the male plant the co-efficient of sex expression is >1, whereas, in female it is...1. Curiously, 22 kD is found only in *M. dioica*, which is a tetraploid plant derived from the diploid form during subsequent course of evolution. This polypeptide probably has evolved under selection pressure and subsequently has been stabilized due to its adaptive value. The homology of 22 kD polypeptide with 29 kD and 32 kD polypeptide of both male and female sex forms of these two dioecious member viz. *M. dioica* and *C. indica* suggests a common ancestry. The difference of antigenic cross reactivity observed between male and female sex forms indicates that the ratio of 29 kD and 32 kD polypeptide might be a contributing factor in controlling the sex mechanism of these dioecious members of cucurbitaceae.

CONCLUSION

The present finding suggests that the p-22 is a sex linked polypeptide. The homology of p-22 with 29kD and 32kD polypeptide of both the sex forms of *Momordica dioica* and *Coccinia indica* indicates a common ancestry. The ratio of intensity of 29/32kD polypeptide could also be used to resolve the mechanism of sex expression in dioecious members of cucurbitaceae.

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