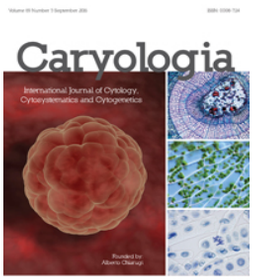

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RETRACTED ARTICLE: Karyotype analysis and identification of extra chromosomes in primary aneuploid stocks of grass pea (*Lathyrus sativus* L.) by fluorescence chromosome banding

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ABSTRACT

Prominent primary aneuploid stocks namely seven primary trisomics, seven primary tetrasomics, and six double trisomic types were earlier developed in grass pea (*Lathyrus sativus* L.), a hardy legume. Despite distinct morphological features, identity and nature of their extra chromosome(s) were elusive, hampering assignment of desirable breeding traits into specific linkage groups. The present study aims to analyze the banding pattern and to reveal the identity of extra chromosome(s) involved in these 20 aneuploid types in grass pea. Conventional orcein banding was first done using the root-tip squash technique in all aneuploids along with disomic ($2n = 2x = 14$) parent, and karyomorphological features were noted. Chromosomes were classified following the total length of individual chromosomes and arranged in order of decreasing sizes, keeping their centromeres in a straight line. DNA-base specific chromomycin A3 (CMA) and 4,6′diamidino-2-phenylindole (DAPI) banding pattern were finally employed to convincingly distinguish the chromosomes from each other. Molecular banding and idiograms revealed unique CMA and DAPI banding pattern in each of the seven chromosome pairs and identified extra chromosome(s) involved in aneuploidy. Fluorescence banding also showed differential CMA-specific GC-rich repeat region and DAPI-specific AT-rich repeat regions in chromosomes with the longest chromosome bearing GC-rich nucleolar organizing region. No polymorphism within homologous pairs was found. The result confirmed the primary nature of present aneuploids and identified the additional chromosome(s) in aneuploids. Based on the extra chromosome(s) involved, new designation of grass pea aneuploids has now been proposed. The results have immense significance in location of desired traits or mutations on specific linkage groups of grass pea.

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Aneuploids; fluorescence chromosome banding; NOR chromosome; grass pea

Introduction

Aneuploidy is defined as a chromosome number that is not an exact multiple of the usual haploid number. The biological consequences of aneuploidy are often related to genomic instability, anomalous cell division processes, karyomorphological polymorphisms, and alterations in metabolic processes, resulting in reproductive anomaly, sterility, and cell proliferation or tumorigenesis in both plants and animals (Torres et al. 2008; Holland and Cleveland 2009). Primary trisomics, tetrasomics and double trisomics are valuable aneuploid stocks in plants. Primary trisomics ($2n = 2x + 1$) contain a normal somatic complement plus an extra (not translocated) chromosome, identical to two homologs. Therefore, the number of possible trisomics in a species will be equal to its haploid (n) number of chromosomes. Primary tetrasomics ($2n = 2x + 2$) have two homologous chromosomes extra to the entire diploid complement. Therefore, an individual with primary tetrasomy for a particular chromosome will have homology with a pair of chromosomes of the same set. Double trisomics ($2n = 2x + 1 + 1$) gain two additional chromosomes (non-homologous to each other) to the existing diploid set (Gwyn and Palmer 1989; Singh 2003; Henry et al. 2005; Talukdar 2013). Using modified segregation ratios, these cytogenetic stocks are being used in classical and molecular gene mapping in *Arabidopsis*, cereals, legumes, vegetables, and in other models and commercial crop plants (Singh et al. 2007; Henry et al. 2010).

Grass pea (*Lathyrus sativus* L.), belonging to the tribe Vicieae (Adan.) de Candolle of the economically important family Fabaceae is the only cultivated member under the genus *Lathyrus*. The crop is grown in the Indian sub-continent, West Asia and, to a lesser extent, in Europe, the Middle East, Australia, northern Africa, Canada, Chile and Brazil for both food and forage with

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a very low input condition (Kumar et al. 2011; Hillocks and Maruthi 2012). This crop is strictly diploid having a chromosome number $2n = 2x = 14$ (Battistin and Fernández 1994; Seijo and Fernández 2003; Arzani 2006). A complete set of primary trisomics, tetrasomics, and several double trisomics have been isolated in different mutant lines of grass pea (Talukdar and Biswas 2007; Talukdar 2008, 2015). Several desirable agronomic traits such as dwarf habit, pod indehiscence, high branching, erect stem and fasciated internode, distichous to polystichous pedicels, bold seed, and several seed coat color mutants have been successfully mapped in different extra chromosomes of grass pea primary trisomics (Talukdar 2009). Origin, morphological and meiotic manifestations, and transmission of these primary aneuploids in grass pea have been detailed earlier (Talukdar 2008; Talukdar and Biswas 2007). However, identity of their extra chromosomes remains elusive in mitotic karyotype. Despite distinct morphological manifestations, the identity of the linkage group involved in aneuploidy could not be elucidated by conventional cytological screening. Therefore, the identity of extra chromosomes on which desirable agronomic and biochemical traits have been mapped is still not known in grass pea. Conventional orcein-banded karyotype analysis is not effective to identify chromosomal polymorphism in symmetrical homogeneous karyotypes. Moreover, occurrence of chromosomal structural rearrangements is highly difficult to trace by this method. Conventional karyotype analysis is also not suitable for detecting minute alterations in GC and AT-rich repeats in the karyotype, and deletion of heterochromatic regions may change the karyotype without affecting its overall morphology (Ghasem et al. 2011). In this backdrop DNA based fluorescent chromosome banding has been introduced for molecular karyotyping, marking of individual chromosomes and phylogenetic studies in different plant species (Alam and Kondo 1995; Akter and Alam 2005; Khandaker et al. 2007; Nath et al. 2015). In *Luthyrus*, varietal identification has been successfully done through fluorescence based techniques (Ali et al. 2000; Talukdar 2010). However, despite the immense importance in future classical and molecular breeding programs, no studies have been carried out to identify and characterize the extra chromosomes involved in aneuploid stocks of grass pea. Thus, our main objectives were to (i) characterize and compare the chromosome banding pattern; and (ii) discriminate individual chromosomes including nucleolar organizing (NOR) chromosomes in seven primary trisomics, seven primary tetrasomics and six different double trisomics in grass pea through DNA-base specific fluorescence chromosome banding. We took tetrasomics and double trisomics to further confirm the fidelity of their extra chromosomes in relation to primary trisomics and disomics. In this paper, we (i) report the root-tip karyotype of grass pea chromosomes through conventional staining; and then (ii) confirm the distinct identity and nature of seven pairs of chromosomes and additional chromosome(s) involved in the origin of 20 different aneuploids through molecular karyotyping in grass pea.

Materials and methods

Orcein staining

Fresh root tips of trisomic, tetrasomic and double trisomic plants along with disomic parent BioR-231 (a total of 21 genotypes) were cut and pretreated with 2 mM 8-hydroxyquinoline for 2 h 40 min at room temperature. This was followed by fixation in 45% acetic acid for 15 min at 4°C and this was then hydrolyzed in a mixture of 1 N HCl and 45% acetic acid (2:1) at 60°C for 10 s. The root tips were stained and squashed in 1% aceto-orcein. For each genotype, the chromosome length was determined from the mean length value ± standard error (SE) of the chromosomes in 10 well-spread metaphase plates. Chromosomes were classified and numbered according to the order of total length of individual chromosomes, as earlier standardized in grass pea (Biswas 1998; Talukdar 2010). Chromosomes were arranged in order of their decreasing size, keeping their centromeres in a straight line as already standardized for grass pea. The longest chromosome was numbered chromosome 1 and the shortest chromosome was designated as 7. Arm ratios (length of short arm:length of long arm) of 0.50–0.75 for submetacentric and > 0.75 for metacentric (m) chromosomes were considered (Biswas 1998; Talukdar 2010).

Fluorescence chromosome banding

Fluorescent chromosome banding was carried out following the methods of Alam and Kondo (1995) with some modifications (Talukdar 2010). Root tips of aneuploid plants along with disomics were carefully dissected and squashed with 45% acetic acid on glass slides. The cover slips were removed quickly on dry ice and the slides were allowed to air-dry for at least 48 h before study. The air dried slides were first pre-incubated in McIlvaine's buffer (citric acid-disodium hydrogen phosphate, pH 7.0) for 30 min followed by distamycin A (0.1 mg ml^{-1}) for 10 min. The slides were then rinsed in McIlvaine's buffer supplemented with 5 mM $MgSO_4$ for 12 min. One drop of chromomycin A3 (CMA, product no-C2659, Sigma-Aldrich, Bangalore, India) (0.1 mg ml^{-1}) was added to the materials. After waiting for 12 min, slides were rinsed with McIlvaine's buffer with $MgSO_4$ for 8 min, then mounted in 50% glycerol and kept at 4°C overnight before study. The CMA-banded chromosome was observed under a fluorescent microscope (Olympus BX51, Olympus America, USA [Indian Supplier: Techno Lab & Instruments, Kolkata, India]) using a Blue Violet (BV) filter cassette. For DAPI (PureBlue DAPI, BIORAD, Mumbai, India) staining, slides were first rinsed with

distilled water and destained using 45% acetic acid for 15 min. Slides were again washed in distilled water, and air-dried overnight. Destained preparations were then treated with 0.2 mg ml^{-1} actinomycin D in McIlvaine's buffer at pH 7.0 and stained in 0.1 µg ml^{-1} DAPI solution for 15 min. Slides were then mounted in 50% glycerol, and were observed under a fluorescence microscope using a UV filter cassette. All the experiments were carried out in triplicate, and a total of 10 slides both per concentration tested and per treatment duration were prepared. CMA-banded karyotype was classified (Alam and Mahbub 2007; Talukdar 2010) with slight modifications for the present *Lathyrus* aneuploids. CMA/DAPI region per chromosome was calculated from 10 metaphase plates using the following formula:

$$\frac{\text{Mean length of CMA/DAPI band}(\mu m)}{\text{Total (mean) length of respective chromosome}(\mu m)} \times 100$$

For CMA karyotype (Table 2) θ = entirely fluorescing chromosomes, α = chromosomes having bands in and around centromeric regions, β = chromosomes having bands in terminal/sub-terminal regions, δ = chromosomes having bands in one full arm + centromere, and φ = chromosomes having no bands (Alam and Mahbub 2007; Talukdar 2010). CMA and DAPI % was then calculated on the basis of total chromatin length for each genotype.

For DAPI, the designations for chromosomes were: A, one pair of dots; B, two pairs of dots; C, one pair of dots and one band; D, two bands; E, one band; and F, no bands/dots (Alam and Mahbub 2007; Talukdar 2010). A combined idiogram involving both CMA and DAPI bands was prepared for different aneuploids in comparison to disomics. Following earlier classification based on distinctive morphology, the trisomics will be designated as Tr I to Tr VII, tetrasomics as Tetra I to Tetra VII, and double trisomics as their combinations (Talukdar 2008, 2015; Talukdar and Biswas 2007).

Statistical analysis

Data are means ± SE of at least three replicates. Multiple comparisons of means were carried out by ANOVA followed by Duncan's multiple range test using SPSS software (SPSS Inc., Chicago, USA v. 10.0) with a significance level at $p < 0.05$.

Results

Orcein-banded karyotype in disomics and trisomics

In the disomic parent, orcein-banded karyotype analysis revealed its diploid nature ($2n = 2x = 14$ chromosomes) showing five pairs of submetacentric and two pairs of metacentric chromosomes (Figure 1A, Table 1). Chromosome length ranged from 4.86 to 7.66 µm with mean length of 6.35 µm. The ratio of longest:shortest chromosome was 1.58:1 with mean arm ratio of 0.69 (Table 1). Pair 1 possessed secondary constriction regions and was the longest of all (Figure 1A).

In all trisomics, apart from the normal diploid complement one extra chromosome was consistently present in the somatic cells. Considering seven different trisomics, chromosome mean length varied between 4.84 µm and 7.65 µm with mean chromosome length of 6.39 µm (Table 1). With a mean arm ratio of 0.70, the ratio of the longest:shortest chromosome was nearly 1.6:1 (Table 1). In Tr I, five pairs of submetacentric + one extra submetacentric chromosome and two pairs of metacentric chromosomes consistently appeared in squash preparation (Figure 1B, Table 1). The extra chromosome was the longest (7.65 µm ± 0.07) and contained secondary constriction (Figure 1B), and was distinctively identified as chromosome pair 1 in Tr I. Somatic cells consistently showed five pairs of submetacentric and two pairs of metacentric chromosomes + one extra chromosome in each of Tr II and Tr IV. Based on arm ratio, it was possibly chromosome 3 (0.92) triplicated in Tr II and chromosome pair 5 (0.83) triplicated in Tr IV. In Tr III, apart from five pairs of submetacentric and two pairs of metacentric chromosomes, one additional submetacentric chromosome appeared consistently. This chromosome was the second largest (6.76 µm) with arm ratio of 0.56, similar to its homolog pair 2 (Table 1). The extra chromosome of Tr V exhibited a submetacentric nature with an arm ratio of 0.53. According to total length, this was very close to its homologous pair 4 (6.06 µm) but arm ratio was very close to submetacentric pair 2 also. A similar condition was encountered in the case of both Tr VI and Tr VII, in which the extra chromosome in each case exhibited very close arm ratio (0.64–0.66) but following the total length, pair 6 (5.30 µm) was possibly triplicated in Tr VI and pair 7 (4.84 µm) in Tr VII (Table 1). Total chromatin length in all trisomics increased significantly ($p < 0.05$) over that of the disomic parent (Table 1).

Orcein-banded karyotype in tetrasomics and double trisomics

The identity of extra chromosomes was assumed in Tetra I to Tetra VII to be identical to Tr I to Tr VII, but in tetrasomics one extra chromosome additional to trisomic was consistently observed. The occurrence of 16 chromosomes was uniformly observed in root tip squash preparations of seven different types of tetrasomics ($2n = 16$; $2x + 2$) and six double trisomics ($2n = 16$; $2x + 1 + 1$). Pair 1 in Tetra I, pair 3 in Tetra II, pair 2 in Tetra III, pair 5 in Tetra IV, pair 4 in Tetra V, pair 6 in Tetra VI, and pair 7 in Tetra VII were duplicated but the other pairs were apparently not disturbed; exhibiting identical length, arm ratio and total length to their homologous partners (Figure 1C, Table 1). Total chromatin length increased significantly

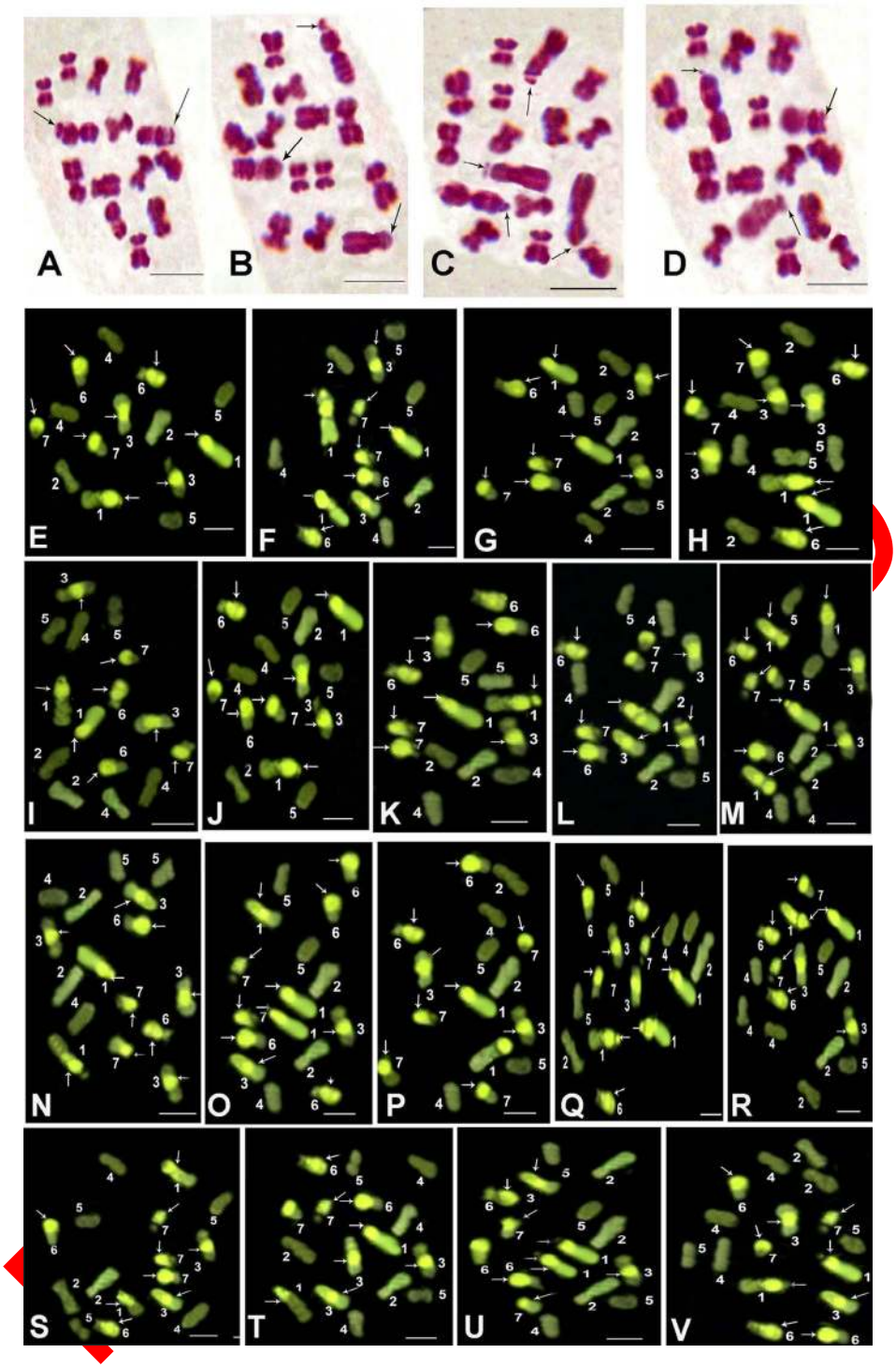

Figure 1. Orcein-stained root-tip mitotic squash in (A) disomic parent with $2n$ =14 chromosomes, and (B) Triplo I, (C) Tet I, and (D) double trisomic Triplo 1-6 showing $2n = 2x + 1 = 15$, $2n = 2x + 2 = 16$, and $2n = 2x + 1 + 1 = 16$ chromosomes, respectively. Arrowhead (→) in each case denotes secondary constriction region in pair 1 chromosome. CMA banding with chromosome numbering in somatic complements of (E) disomic, (F) Triplo I, (G) Triplo II, (H) Triplo III, (I) Triplo IV, (J) Triplo V, (K) Triplo VI, (L) Triplo VII, (M) Tet I, (N) Tet III, (O) Tet VI, (P) Tet VII, and six double trisomics (Q) Triplo 1-6, (R) Triplo 2-4, (S) Triplo 5-7, (T) Triplo 3-4, (U) Triplo 2-5, and (V) Triplo 4-6 of grass pea. Arrowhead (→) in each case denotes CMA-stained region only. At least 10 metaphase plates randomly taken from three replicates per genotype were analyzed. 1 SD = 10 µm. Tet II, IV, and V did not show any CMA^+ bands, and thus are not shown.

($p < 0.05$) over that of the disomic and trisomic parent but the ratio of longest to shortest chromosome remained about 1.55:1 (Table 1).

In double trisomics, combinations of two trisomic chromosomes were noticed. In general, the suspected extra chromosomes were tentatively identified based on the total length and arm ratio in relation to their homologous counterparts in somatic complement. Type I or *acicular-revolute* type possessed two extra chromosomes; three copies of pair 1 with identical morphological features in relation to their homologs consistently occurred in squash preparation. However, the identity of three copies of chromosome 6 was assumed based on their similarities with each other (Figure 1D, Table 1).

Table 1. Overall comparative accounts of chromosomes in disomic parent BioR-231, primary trisomics (Tr I-VII), primary tetrasomics (Tetra I-VII) and different double trisomics of grass pea (*Lathyrus sativus* L.) in orcein banding.

Genotypes		Diploid chromosome number (2*n*)	Range of chromosome length (µm)	Total 2*n* chromatin length (µm)	Ratio of longest to shortest chromosome	Range of short arm length (µm)	Range of long arm length (µm)	Karyotype in diploid complement + extra(s)
Old designation[a]	New designation[b]							
Disomic	Disomic	2*n* = 2*x* = 14	4.86–7.66	84.72 ± 0.10c	1.58:1	1.96–2.98	2.94–4.80	4m + 10sm
Tr I	Triplo I	2*n* = 15; 2*x* + 1	4.88–7.65	92. 33 ± 0.12b	1.56:1	1.96–2.99	2.93–4.79	(4m + 10sm) + 1sm
Tr II	Triplo III	2*n* = 15; 2*x* + 1	4.90–7.63	90.95 ± 0.10b	1.56:1	1.96–2.98	2.94–4.79	(4m + 10sm) + 1m
Tr III	Triplo II	2*n* = 15; 2*x* + 1	4.87–7.64	91.48 ± 0.11b	1.57:1	1.95–3.01	2.94–4.80	(4m + 10sm) + 1sm
Tr IV	Triplo V	2*n* = 15; 2*x* + 1	4.89–7.60	90.23 ± 0.09b	1.55:1	1.96–2.98	2.93–4.81	(4m + 10sm) + 1m
Tr V	Triplo IV	2*n* = 15; 2*x* + 1	4.90–7.59	90.78 ± 0.09b	1.55:1	1.97–2.99	2.93–4.79	(4m + 10sm) + 1sm
Tr VI	Triplo VI	2*n* = 15; 2*x* + 1	4.84–7.63	90.02 ± 0.08b	1.58:1	1.95–2.97	2.94–4.81	(4m + 10sm) + 1sm
Tr VII	Triplo VII	2*n* = 15; 2*x* + 1	4.91–7.60	89.59 ± 0.08b	1.55:1	1.96 –2.99	2.95–4.78	(4m + 10sm) + 1sm
Tetra I	Tet I	2*n* = 16; 2*x* + 2	4.89–7.61	99.92 ± 0.16a	1.56: 1	1.96–2.99	2.92–4.83	(4m + 10sm) + 2sm
Tetra II	Tet III	2*n* = 16; 2*x* + 2	4.91–7.64	97.18 ± 0.12a	1.56:1	1.96–2.97	2.94–4.80	(4m + 10sm) + 2m
Tetra III	Tet II	2*n* = 16; 2*x* + 2	4.88–7.64	98.23 ± 0.14a	1.57:1	1.95–3.00	2.94–4.80	(4m + 10sm) + 2sm
Tetra IV	Tet V	2*n* = 16; 2*x* + 2	4.89–7.61	95.72 ± 0.11a	1.56:1	1.96–2.99	2.93–4.81	(4m + 10sm) + 2m
Tetra V	Tet IV	2*n* = 16; 2*x* + 2	4.90–7.60	96.84 ± 0.11a	1.55:1	1.93–2.99	2.93–4.79	(4m + 10sm) + 2sm
Tetra VI	Tet VI	2*n* = 16; 2*x* + 2	4.84–7.62	95.32 ± 0.09a	1.58:1	1.96–2.98	2.94–4.80	(4m + 10sm) + 2sm
Tetra VII	Tet VII	2*n* = 16; 2*x* + 2	4.91–7.61	94.51 ± 0.09a	1.55:1	1.96–2.99	2.95–4.79	(4m + 10sm) + 2sm
Dt type I	Triplo 1-6	2*n* = 16; 2*x* + 1 + 1	4.89–7.63	97.21 ± 0.13a	1.56:1	1.95–2.98	2.94–4.81	(4m + 10sm) + 2sm
Dt type II	Triplo 2-4	2*n* = 16; 2*x* + 1 + 1	4.90–7.60	97.54 ± 0.13a	1.55:1	1.94–2.98	2.95–4.79	(4m + 10sm) + 2sm
Dt type III	Triplo 5-7	2*n* = 16; 2*x* + 1 + 1	4.88–7.64	95.11 ± 0.10a	1.57:1	1.93–2.99	2.93–4.82	(4m + 10sm) + 1m + 1sm
Dt type IV	Triplo 3-4	2*n* = 16; 2*x* + 1 + 1	4.89–7.61	97.01 ± 0.13a	1.56:1	1.96–2.98	2.94–4.80	(4m + 10sm) + 1m + 1sm
Dt type V	Triplo 2-5	2*n* = 16; 2*x* + 1 + 1	4.91–7.62	96.98 ± 0.11a	1.55:1	1.97–2.97	2.93–4.81	(4m + 10sm) + 1sm + 1m
Dt type VI	Triplo 4-6	2*n* = 16; 2*x* + 1 + 1	4.89–7.62	96.08 ± 0.10a	1.56:1	1.95–2.97	2.94–4.79	(4m + 10sm) + 2sm

[a]Old designation was based on chronological appearance and morphological discrimination of aneuploids.
[b]New designation is based on extra chromosome(s) identified by fluorescence chromosome banding in respective genotypes.
Data are means ± SE of at least three replicates. Means followed by different superscript letters are significantly different at $p < 0.05$ in ANOVA followed by Duncan's multiple range test. Dt, double trisomics.

In type II or *bifid-alternate* type, one additional copy each of chromosomes 2 and 4 was found similar to their respective homologous partners. Both the chromosomes possessed submetacentric natures with arm ratio of 0.53–0.56. Type III or *linear-lanceolate-round* type possessed three copies of chromosome 5 (metacentric) and chromosome 7 (submetacentric) along with other somatic complements (Table 1). In type IV or *alternate-paired lateral* type, chromosome 3 (metacentric) and chromosome 4 (submetacentric) with arm ratio of 0.92 and 0.53, respectively, were possibly triplicated. The rest of the complement was not disturbed. Three copies of chromosome 2 (submetacentric) and chromosome 5 (metacentric) along with normal diploid complement were likely involved in the origin of type V or *bifid-ternate-verticillate* type. Type VI or *alternate-revolute* type possessed two submetacentric chromosomes with arm ratio and total length similar to pair 4 and 6 in triplicate along with other somatic complement (Table 1). The ratio of longest to shortest chromosome hovered around 1.55:1 which was close to the disomic parent but chromatin length increased significantly ($p < 0.05$) in all double trisomic types over that of disomic and trisomics (Table 1).

CMA banding in disomic parent

The disomic parent exhibited a uniform presence of 2*n* = 14 chromosomes and a complete absence of polymorphism within any homologous pair in the diploid complement (Figures 1E, 3A). Eight CMA^+ (CMA-positive) bands were recorded in the haploid complement; two in terminal regions of the long arm of pair 1 (the nucleolar organizing region (NOR) pair with 44.45% CMA-banded region/chromosome), two in intercalary regions of long arm of pair 3 and two bands each in the whole short arms of pairs 6 and 7 (Figures 1(E), 3(A)). CMA-banded regions were conspicuously absent in pairs 2, 4 and 5. Total CMA-positive

banded regions indicating GC-rich repeat sequences were calculated as 27.49% of total chromatin length in diploid genomes of the disomic parent (Table 2). Bright CMA bandings were found at the secondary constriction regions which were associated with NOR in pair 1 (Figure 1E). For aneuploids, results have been presented based on old designations of trisomic, tetrasomics and double trisomics.

CMA banding in trisomics

In Tr I, association of pair 1 as an extra chromosome was confirmed by the identical CMA banding pattern with its two homologous partners (Figures 1(F), 3(B)). One CMA$^+$ thin band followed by one thick band was uniformly visualized in the terminal position of the long arm of all three copies of pair 1. Banding pattern was identical to the disomic parent in rest of the cases. Total % CMA-positive banded regions were increased by about 1.3-fold over those of disomics (Table 2). In Tr II, chromosome 3 triplicated, uniformly exhibiting two thick bands in intercalary regions of long arm and short arm (Figures 1(H), 3(D)). Percentage CMA-positive banded regions of total chromatin were increased by about 1.2-fold over those of disomics (Table 2). CMA$^+$ bands could not be detected in a total of four chromosomes each in Tr III, IV and V (Figures 1G–J, 3C–F). However, considering the length and metacentric/submetacentric nature, it seems likely that chromosomes 2, 4 and 5 were the extra chromosomes of Tr III, Tr V and Tr IV, respectively. Two bands each in the whole short arms and interstitial region of pairs 6 and 7 were found in three copies in Tr VI and VII, respectively (Figures 1K, L, 3G, H). Percentage CMA-positive banded regions of total chromatin were increased by 1.2-fold over those of disomics (Table 2). CMA-banded karyotype formulas changed accordingly in each of the seven trisomics (Table 2).

DAPI banding in disomic

Ten DAPI$^+$ bands and two DAPI$^+$ pairs of dots were visualized in the disomic parent consisting of about 3.81 µm (9.00%) of total chromatin length (Figures 2A, 3A, Table 1). Out of a total of 10 bands, eight bands were found in terminal/sub-terminal regions of pairs 2, 3, 5, and 7. The other two were visualized in interstitial regions of pair 4. Two pairs of dots were distinctive in terminal positions of short arms of pair 6. DAPI staining was conspicuously absent in chromosome pair 1 in this parent (Figures 2(A), 3(A)).

DAPI banding in trisomics

DAPI banding pattern fully supported chromosomes 1, 3, 6 and 7 as extra chromosomes of Tr I, Tr II, Tr VI and Tr VII, respectively, and confirmed the involvement of chromosomes 2, 4 and 5 as additional primary chromosome in the rest of the trisomics. A total of 10 DAPI$^+$ bands and four DAPI$^+$ dots were observed in Tr I which was identical with disomic parent. All three copies of pair 1 exhibited uniform absence of DAPI bands or dots in Tr I (Figures 2(B), 3(B)). In Tr II, one terminal DAPI band was observed in chromosome 3 which was present in three copies. The number of DAPI dots, however, was still four (Figures 2(D), 3(D)). In Tr III, one interstitial band was conspicuously present in three copies and the chromosome was identified as 2 (Figures 2(C), 3(C)). One medial band in the short arm of chromosome 5 and one intercalary band near the centromere in the long arm of chromosome 4 was found in three copies in Tr IV and Tr V, respectively (Figures 2E, F, 3E, F). The banding patterns in the rest of the chromosomes were in duplicate and identical to the disomic parent. In Tr VI, one pair of DAPI dots was uniformly visualized in three copies and the chromosome was identified as 6 while one DAPI band in the sub-terminal region of the long arm of chromosome 7 was identified in triplicate condition in Tr VII (Figures 2G, H, 3G, H). Except for Tr I and Tr VI, the total number of DAPI bands increased to 11 in the other trisomics (Table 2). In Tr VI, three pairs of DAPI dots were observed due to triplication of chromosome 6 (Figures 3G, Table 2). Barring Tr I, the % DAPI-stained region of total chromatin length increased by about 1.1-fold in the rest of the trisomics in comparison to the disomic parent (Table 2). DAPI-banded karyotype formulae changed accordingly in each of the seven trisomics (Table 2).

CMA and DAPI banding in tetrasomics

The specific chromosome which was confirmed as extra in seven different trisomics by CMA and DAPI banding was found to be duplicated in seven tetrasomics, with identical banding pattern to their respective homologs; thus they are not repeated here. However, due to the increase in chromosome number, the total number of CMA and DAPI band/dots as well as % of CMA/DAPI region of total chromatin length was also increased in tetrasomics (Table 2). Considering all pairs, the number of CMA bands in Tetra I increased to 20 and CMA-positive banded regions were increased over disomics by about 1.8-fold (Figure 1M, Table 2). Similarly, in Tetra II, the number of CMA bands increased to 20 and DAPI to 12 (Figures 1(N), 2(J), Table 2). The CMA-banded region was enhanced by about 1.3-fold in relation to the disomic parent (Table 2). Chromosomes 2 and 5 were identified in four copies with one terminal DAPI band on the long arm in Tetra III and at the medial position of the short arm of Tetra IV, respectively (Figures 2I, L, Table 2). The DAPI-stained region increased by about 1.1-fold in these two tetrasomics. In Tetra V, chromosome 4 was characterized by one intercalary DAPI band (near the centromere) in two pairs (Figure 2K). Similar

Table 2. Fluorescence banding pattern of seven pairs of chromosomes along with additional chromosome(s) in seven primary trisomics (Triplo I-VII), primary tetrasomics (Tet I-VII) and six different double trisomics of grass pea (*Lathyrus sativus* L.) in DNA base specific CMA/DAPI banding.

Chromosome pair[a]	CMA$^+$ band number in diploid complement	Length of individual chromosome (µm) in haploid[b]	% CMA-band of total length[c]	Number of DAPI positive bands + pair of dots in 2*n*	% DAPI-band of total length[c]	CMA-banded karyotype formulae (2*n*)[d]	DAPI-banded karyotype formulae (2*n*)[e]
Disomic						2α+2β+4δ+6φ	2A+10E+2F
Pair 1	04	7.61 ± 0.08	21.62	0	0		
Pair 2	0	6.76 ± 0.06	0	02	5.71		
Pair 3	04	6.23 ± 0.06	57.58	02	12.5		
Pair 4	0	6.06 ± 0.06	0	02	9.38		
Pair 5	0	5.50 ± 0.05	0	02	6.90		
Pair 6	04	5.30 ± 0.05	72	04 (dots)	1.26		
Pair 7	04	4.90 ± 0.04	65	02	8.0		
Total 1–7	16	42.36 ± 0.12	27.49[d]	10 + 4 dots	9.00[d]		
Triplo I (Tr I))	18	7.61 ± 0.08	35.38[c]	10 + 4 dots	9.00[d]	2α+3β+4δ+6φ	2A+10E+3F
Triplo II (Tr III)	16	6.76 ± 0.06	27.49[d]	11 + 4 dots	9.48 [c]	2α+2β+4δ+7φ	2A+11E+2F
Triplo III (Tr II)	18	6.24 ± 0.07	33.00[c]	11 + 4 dots	9.50[c]	3α+2β+4δ+6φ	2A+11E+2F
Triplo IV (Tr V)	16	6.06 ± 0.06	27.49[d]	11 + 4 dots	9.95[c]	2α+2β+4δ+7φ	2A+11E+2F
Triplo V (Tr IV)	16	5.51 ± 0.04	27.49[d]	11 + 4 dots	9.47[c]	2α+2β+4δ+7φ	2A+11E+2F
Triplo VI (Tr VI)	18	5.30 ± 0.03	32.99[c]	10 + 6 dots	9.10[d]	2α+2β+5δ+6φ	3A+10E+2F
Triplo VII (Tr VII)	18	4.90 ± 0.03	32.57[c]	11 + 4 dots	9.95[c]	2α+2β+5δ+6φ	2A+11E+2F
Tet I (Tetra I)	20	7.61 ± 0.09	43.28[a]	10 + 4 dots	9.00[d]	2α+4β+4δ+6φ	2A+10E+4F
Tet II (Tetra III)	16	6.76 ± 0.09	27.49[d]	12 + 4 dots	10.00[c]	2α+2β+4δ+8φ	2A+12E+2F
Tet III (Tetra II)	20	6.23 ± 0.08	38.51[b]	12 + 4 dots	10.01[c]	4α+2β+4δ+6φ	2A+12E+2F
Tet IV (Tetra V)	16	6.06 ± 0.07	27.49[d]	12 + 4 dots	10.90[a]	2α+2β+4δ+8φ	2A+12E+2F
Tet V (Tetra IV)	16	5.51 ± 0.06	27.49[d]	12 + 4 dots	9.94[c]	2α+2β+4δ+8φ	2A+12E+2F
Tet VI (Tetra VI)	20	5.30 ± 0.06	38.49[b]	10 + 8 dots	9.20[d]	2α+2β+6δ+6φ	4A+10E+2F
Tet VII (Tetra VII)	20	4.89 ± 0.03	38.65[b]	12 + 4 dots	10.90[a]	2α+2β+6δ+6φ	2A+12E+2F
Triplo 1-6 (Type I)	20	7.63 ± 0.08 / 4.89 ± 0.05	43.67[a]	10 + 4 dots	10.10[b]	2α+3β+5δ+6φ	3A+10E+3F
Triplo 2-4 (Type II)	16	6.76 ± 0.06 / 6.06 ± 0.06	27.50[d]	12+ 4 dots	10.43[b]	2α+2β+4δ+8φ	2A+12E+2F
Triplo 5-7 (Type III)	18	5.50 ± 0.06/ 4.88 ± 0.04	30.55[c]	12 + 4 dots	10.56[b]	2α+2β+6δ+6φ	2A+12E+2F
Triplo 3-4 (Type IV)	18	6.23 ± 0.07/ 6.06 ± 0.06	36.95[b]	12 + 4 dots	10.55[b]	3α+2β+4δ+7φ	2A+12E+2F
Triplo 2-5 (Type V)	16	6.76 ± 0.06/ 5.51 ± 0.05	27.50[d]	12 + 4 dots	10.28[b]	2α+2β+4δ+8φ	2A+12E+2F
Triplo 4-6 (Type VI)	18	6.06 ± 0.06 / 5.30 ± 0.05	36.25[b]	11 + 6 dots	11.00[a]	2α+2β+5δ+7φ	2A+12E+2F

[a]Length, CMA/DAPI banded region did not change significantly between disomics and aneuploids, thus description of individual chromosomes of trisomics, tetrasomics and double trisomics is not presented here; old designation mentioned within bracket.
[b]Characteristics of individual extra chromosome given only for disomics. Length of only extra chromosome(s) of aneuploids has been mentioned. The rest of the chromosomes were identical to disomics, and thus not repeated.
[c]CMA-band % of total length, and % DAPI-banded region was presented per chromosome in disomics and per total chromatin length in aneuploids. Per pair % share of the CMA/DAPI region is identical between disomics & aneuploids, and thus not shown. Means followed by different lowercase letters are significantly ($p < 0.05$) different from each other by DMRT.
[d]For CMA, θ = entirely fluorescing chromosomes, α = chromosomes having bands in and around centromeric regions, β = chromosomes having bands in terminal/sub-terminal regions, δ = chromosomes having bands in one full arm + centromere, φ = chromosomes having no CMA bands.
[e]For DAPI, A = one pair of dots, B = two pairs of dots, C = one pair of dots and one band, D = two bands, E = one band, and F = no bands/dots.

to their respective trisomic parents, no CMA banding was conspicuous in chromosomes 2, 4 and 5 (photo not shown). The number of DAPI bands thus increased to 12 in all these three tetrasomics. CMA/DAPI banding also identified chromosome 6 and chromosome 7 in four copies in Tetra VI and Tetra VII, respectively (Figures 1O, P, 2M, N). The number of CMA bands and DAPI dots in Tetra VI therefore increased to 20 and 8, respectively (Table 2). The total number of CMA bands and DAPI bands were 20 and 12, respectively, in the somatic complement of Tetra VII (Figures 1P, 2N, Table 2). The CMA as well as GC-rich repeat region was increased by about 1.4-fold in Tetra VI and by about 1.5-fold in Tetra VII. Moderate increase in DAPI/AT-rich region was also observed in Tetra II–VII (Table 2).

CMA and DAPI banding in double trisomics

Combinations of banding patterns of two non-homologous extra chromosomes were observed in six different types of double trisomics. Extra chromosomes and the remaining diploid complements in all double trisomics exhibited identical fluorescence banding patterns in relation to their trisomic parents. However, addition of two non-homologous chromosomes in double trisomics changed the number and share of the CMA and DAPI-stained region. Type I possessed three copies each of chromosome 1 (NOR chromosome) and 6 along with normal diploid counterpart. The number of CMA bands was enhanced to 20 while DAPI dots increased to six (Figures 1(Q), 2(O), 4B). Total CMA-positive banded regions and GC-rich repeats were increased by about

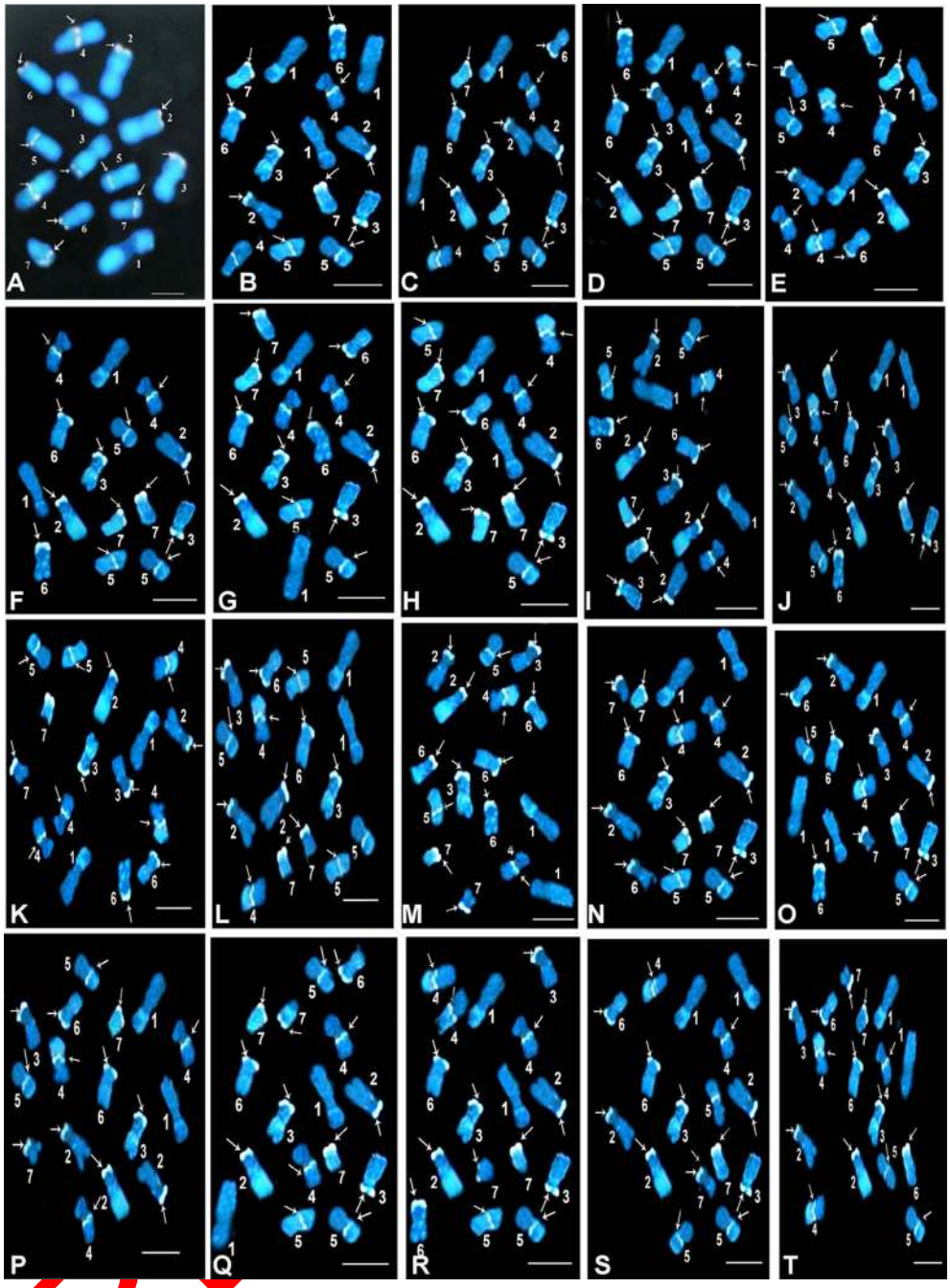

Figure 2. DAPI-stained mitotic metaphase of (A) disomic parent with $2n = 14$ chromosomes, and (B) Triplo I, (C) Triplo II, (D) Triplo III, (E) Triplo IV, (F) Triplo V, (G) Triplo VI, and (H) Triplo VII with $2n = 2x + 1 = 15$ chromosomes, (I) Tet II, (J) Tet III, (K) Tet IV, (L) Tet V, (M) Tet VI, and (N) Tet VII with $2n = 2x + 2 = 16$ chromosomes, and (O) Triplo 1-6, (P) Triplo 2-4, (Q) Triplo 5-7, (R) Triplo 3-4, (S) Triplo 2-5, and (T) Triplo 4-6 showing $2n = 2x + 1 + 1 = 16$ chromosomes in somatic complement of grass pea root tip. Arrowhead (→) in each case denotes CMA-stained region only. At least 10 metaphase plates randomly taken from three replicates per genotype were analyzed. Scale bar 1 SD= 10 µm. Tet I did not show any $DAPI^+$ bands, and thus is not shown.

1.8-fold, whereas total DAPI-banded region/AT-rich repeats were increased over disomics by about 1.12-fold (Table 2). In type II, triplication of chromosomes 2 and 4 resulted in increase in DAPI bands to 12 but number of CMA bands remained at 16 (Figures 1(R), 2(P), 4C, Table 2). Type III exhibited characteristic banding pattern of chromosome 5 and 7 in triplicate apart from normal diploid complement. The CMA band number increased to 18, while DAPI enhanced to 12 in type III (Figures 1(S), 2(Q), 4E, Table 2). Total CMA/DAPI-positive banded regions were increased by approximately 1.2–1.4-fold (Table 2). Type IV showed addition of two chromosomes whose banding pattern was identical to chromosomes 3 and 4 (Figures 1(T), 2(R), 4D). The number of CMA bands increased to 18 while the DAPI region increased to 12 in type IV. Total CMA-positive and DAPI-stained banded regions were increased by about 1.3-fold and 1.2-fold, respectively, in comparison to the disomic parent (Table 2). In type V, chromosomes 2 and 5 were distinctly visualized in three copies (Figures 1(U), 2(S),

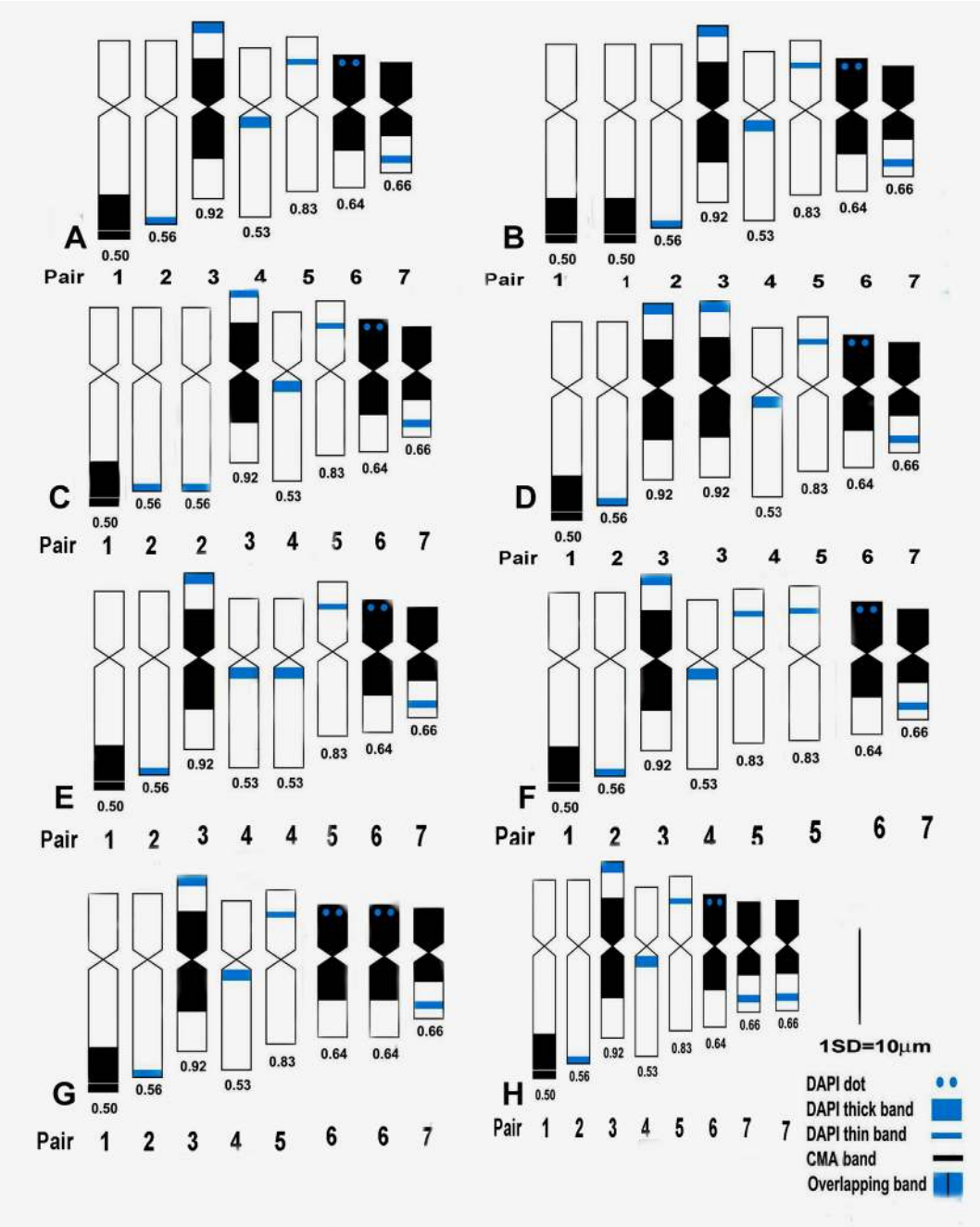

Figure 3. Combined idiograms of both CMA and DAPI-banding pattern in haploid complement of (A) disomic parent and haploid + extra chromosome of seven types of trisomics, (B) Triplo I, (C) Triplo II, (D) Triplo III, (E) Triplo IV, (F) Triplo V, (G) Triplo VI, and (H) Triplo VII in grass pea. Scale bar 1 SD = 10 µm.

4F). Banding pattern was present in duplicate for the rest of the chromosomes. Number of DAPI bands in type V increased to 12 but the CMA region remained unchanged (Table 2). In Type VI, chromosomes 4 and 6 with their characteristic banding patterns triplicated (Figures 1(V), 2(T), 4G. No modification was observed in rest of the somatic pairs. The number of CMA bands was 18, while DAPI bands increased to 11. There were six DAPI dots (Figure 4G). CMA-positive banded regions as well as GC-rich repeats in type VI were increased by about 1.4-fold. DAPI banded region was enhanced by nearly 1.2-fold (Table 2).

Based on the present conventional orcein-banded karyotype and DNA-base specific fluorescence chromosome banding, specific extra chromosomes of each of the seven different primary trisomics, primary tetrasomics and six double trisomic types have been confirmed. It was also confirmed that preliminary designation based on their chronological appearance in progeny and morphological features did not correspond to their extra chromosome involvement. Considering seven chromosome types (from longest to shortest) in the haploid complement as corresponding to seven linkage groups in grass pea, Tr I, II, III, IV, V, VI and VII are now newly designated as Triplo I, Triplo III, Triplo II, Triplo V, Triplo IV, Triplo VI, and Triplo VII, respectively, carrying pairs 1, 3, 2, 5, 4, 6 and 7 as extra in their somatic complement. Accordingly, the tetrasomic progenies of

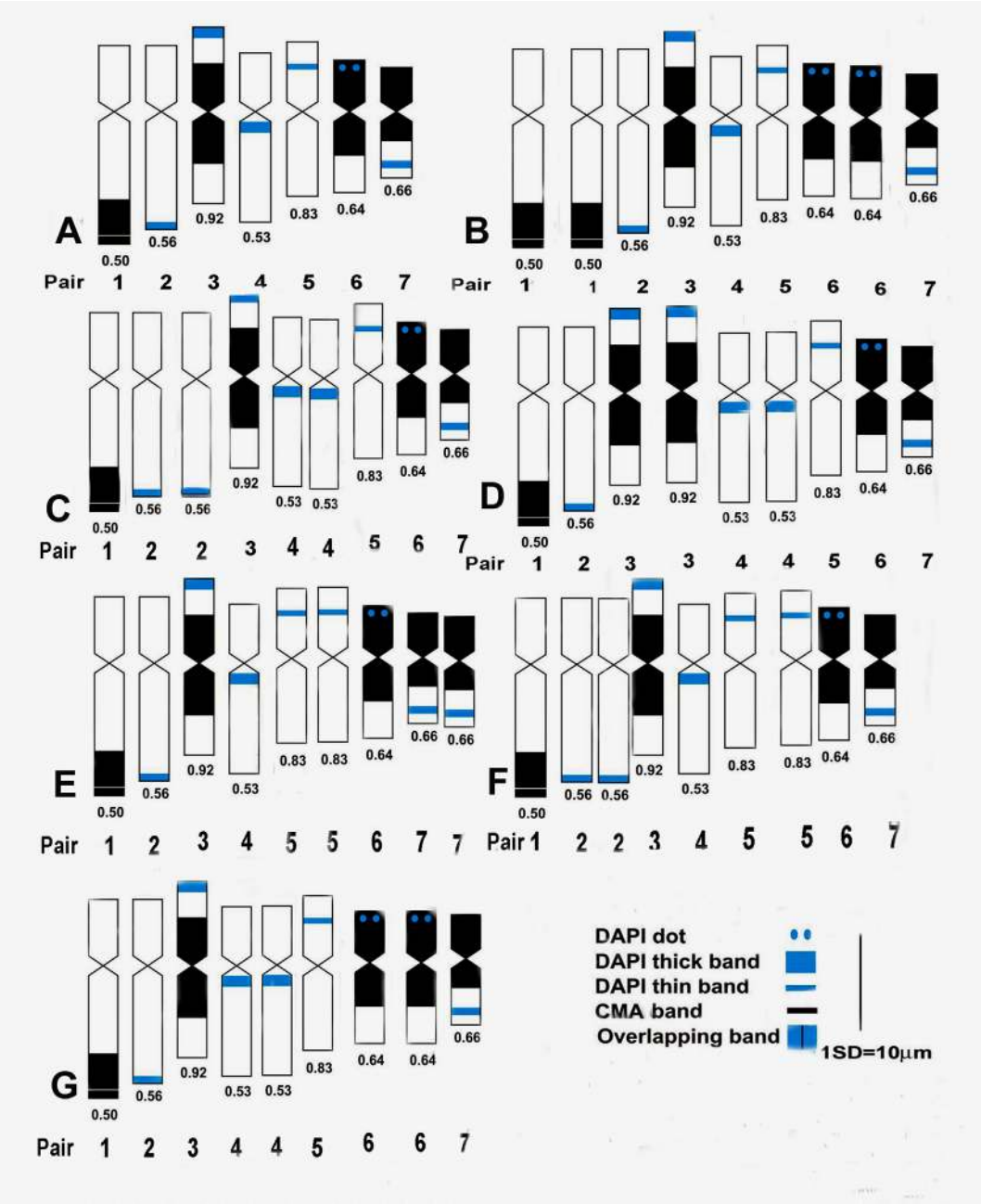

Figure 4. Combined idiograms of both CMA and DAPI-banding pattern in haploid complement of (A) disomic parent and haploid + extra chromosomes of six types of double trisomics, (B) Triplo 1-6, (C) Triplo 2-4, (D) Triplo 3-4, (E) Triplo 5-7, (F) Triplo 2-5, and (G) Triplo 4-6 in grass pea. Scale bar 1 SD = 10 µm.

these seven primary trisomics (Tetra I–Tetra VII) will now be designated as Tet I (pair 1), Tet II (pair 2), Tet III (pair 3), Tet IV (pair 4), Tet V (pair 5), Tet VI (pair 6) and Tet VII (pair 7). Based on the nature of extra chromosomes in the karyotype, the six double trisomic types will now be designated as Triplo 1-6 (pair 1 and 6), Triplo 2-4 (pair 2 and 4), Triplo 3-4 (pair 3 and 4), Triplo 5-7 (pair 5 and 7), Triplo 2-5 (pair 2 and 5), and Triplo 4-6 (pair 4 and 6) (Tables 1, 2).

Discussion

The value of a desirable mutation is enhanced manifold when it is assigned to a particular chromosome or specific linkage group of a crop plant. Primary trisomics, tetrasomics and double trisomics are indispensable biological vehicles to construct linkage maps in different crops. However, identification and characterization of extra chromosomes are extremely important to execute the mapping processes and specific localization of target genes. In the present material (grass pea), conventional orcein-banding revealed the consistent presence of one extra chromosome in seven different trisomics ($2n = 15$; $2x + 1$), and two extra chromosomes in both tetrasomics ($2n = 16$; $2x + 2$) and double trisomics ($2n = 16$; $2x + 1 + 1$) in addition to their normal somatic complement. The diploid nature of the disomic parent was confirmed by uniform occurrence of 14 chromosomes in its somatic complement ($2n = 2x = 14$) with five

pairs of submetacentric and two pairs of metacentric chromosomes. Pair 1 was confirmed as the longest of all pairs and this was the only pair in the present material which carried NOR, although more than one pair of NOR chromosomes was reported earlier in different varieties of grass pea (Seijo and Fernández 2001; Iwata et al. 2013). Based on total chromosome length and arm ratio (short:long), extra chromosomes could not be convincingly resolved in all seven trisomics through orcein karyotyping. It is noteworthy that the present trisomic population was earlier tentatively differentiated into Tr I to Tr VII on the basis of their distinctive morphological traits and chronology of appearance in the progeny (Talukdar and Biswas 2007). However, the revised designation will be used in the rest of the discussion. Despite extensive investigation of their meiotic behaviors, the extra chromosome could not be pinpointed in specific trisomics. In the present study, repeated squash preparation convincingly identified pair 1 as a primary extra chromosome in Tr I (Triplo I), which was duplicated in tetrasomic type (Tetra) I (Tet I), and also involved in double trisomic type I (Triplo 1-6). The shortest chromosome 7 could be identified as an extra chromosome in Tr VII (Triplo VII), duplicated as extra in Tetra VII (Tet VII) and participated as one of the additional chromosomes in type III double trisomic (Triplo 5-7). Based on karyomorphological features as revealed by orcein banding, chromosomes 2 were predicted as extra chromosomes of Tr III (Triplo II), Tetra III (Tet II), and double trisomic type II (Triplo 2-4) and type V (Triplo 2-5). Orcein banding identified pair 2 as the second largest chromosome, after pair 1. However, barring total length, it has close morphological similarity with chromosome 4 (both were submetacentric) which was therefore tentatively assigned as an additional chromosome in Tr V (Triplo IV), Tetra V (Tet IV) and in double trisomic types II (Triplo 2-4), IV (Triplo 3-4) and VI (Triplo 4-6). Pairs 3 and 5 showed a metacentric nature but different total length and arm ratio. While the former was primarily designated as extra chromosomes of Tr II (Triplo III), Tetra II (Tet III) and double trisomic Triplo 3-4, the latter was assigned as extra chromosome of Tr IV (Triplo V), Tetra IV (Tet V), and as one of the extra partners in double trisomic types III (Triplo 5-7) and V (Triplo 2-5). As the shortest chromosome 7 was identified as additional chromosome/s in Tr VII (Triplo VII), Tetra VII (Tet VII) and Triplo 5-7, the rest of pair 6 was primarily assigned to Tr VI (Triplo VI) as an additional chromosome. The duplication of this chromosome in Tetra VI (Tet VI) and as one of the extra partners in double trisomic types I (Triplo 1-6) and Triplo 4-6 was observed. Remarkably, no distortion was observed in karyomorphological features between the diploid complement and extra homologous partners of aneuploid chromosomes. The standard karyotype was prepared according to their total lengths. However, barring nucleolar chromosomes and possibly the shortest one, it was found to be difficult to confirm the rest of the chromosomes due to their metacentric and submetacentric nature with low size differences in the present aneuploids and disomics by conventional staining methods. This was further aggravated with increasing chromosome number in the present aneuploid stocks. Grass pea chromosomes are poorly diversified in their sizes. This was evident from the low ratio (< 2:1) of longest to shortest pair (1.6:1), characterized with graded series of chromosome length in karyotype. The result agrees well with earlier orcein-banded as well as Feulgen stained karyological studies in different diploid genomes of *Lathyrus* spp. (Klamt and Schifino-Wittmann 2000; Seijo and Fernández 2003; Ayaz and Ertekin 2008). Although polyploidy has been studied in *Lathyrus* spp. (Khawaja et al. 1998; Talukdar 2010, 2012), mitotic fidelity in aneuploid genomes was not known in grass pea. Characterization of aneuploid chromosomes through molecular karyotyping for the first time in grass pea thus assumes significance.

In order to clearly confirm the identity and to differentiate the extra chromosomes in different types of aneuploid stocks, DNA base specific fluorescence chromosome banding was employed, and idiograms combining both CMA and DAPI pattern of the Triplo population were constructed. CMA specifically binds to GC-rich repeats while the DAPI stained region denotes AT-rich repeats. Per chromosome sharing of CMA and DAPI-rich regions was calculated in all cases and the banded region presented in Table 2 was considered based on total chromatin length of a particular genotype. CMA and DAPI banding revealed significant differences among seven pairs of chromosomes in the disomic parent. All the chromosomes and their homologous partners could therefore be easily identified and characterized. The seven different additional chromosomes involved in the origin of seven different types of primary trisomy were unambiguously identified by the variation in number, position and types of bands. Each chromosome has its own and unique banding pattern. Interestingly, the characteristic banding pattern unique to each chromosome inherited unmodified to each trisomic and then to seven different tetrasomics and in double trisomics. Both tetrasomics and double trisomics possess higher chromosome number than trisomics. The absence of any intrachromosomal asymmetry or polymorphism within the homologous pair ruled out occurrence of chromosomal rearrangement and confirmed the primary nature of the present grass pea aneuploids. Homology of the extra pair in tetrasomics and the non-homologous nature of extra chromosomes in double trisomics was also confirmed. The

results showed that despite symmetric morphology, the grass pea chromosomes exhibited molecular asymmetry at least in occurrence and position of GC-and AT-rich repeat regions, which was combined in double trisomics and amplified in tetrasomy.

In the CMA-banded karyotype, % CMA region of total chromatin length varied significantly ($p < 0.05$) among 21 genotypes (20 aneuploids + one disomic parent). This was due to the addition of different extra chromosome(s) showing a different CMA-banded region. Band number increased to 18 in Triplo I from 16 in the disomic parent, indicating involvement of an extra chromosome. Uniform occurrence of a characteristic CMA band at the terminal-most position at the long arm of three homologous pairs identified the origin of pair 1 as an extra chromosome in Triplo I. This was detected as the longest pair and possessed NOR. This extra chromosome was also confirmed in Tet I which was originally derived from the self-pollinated Triplo I population (Talukdar 2008) and in Triplo 1-6, in which it was triplicated with identical CMA banding. Interestingly, like the disomic parent, DAPI banding was present neither in Triplo I, in Tet I nor in Triplo1-6. The results unraveled presences of GC-rich repeats but complete absence of AT-rich repeat sequence in NOR pair. Fluorescent banding similarly revealed a $CMA^+/DAPI^-$ NOR region in different legumes and clarified species relationships (Akter and Alam 2005; Khandaker et al. 2007). NORs are heterochromatic rich domains and reportedly found to exhibit fragility and mobility, linked with the activation of the transposable elements located near or within rDNA clusters in many plant species (Guera 2000; Huang et al. 2008; Raskina et al. 2008). The presence of one additional NOR chromosome each in the present Triplo I, Triplo 1-6 and its duplication in Tet I considerably increased (1.4–1.8-fold) GC-rich repeats in respective aneuploids. No fragility or mobility of NORs was observed despite the fact that it is consistently carried by the longest chromosome (chromosome 1) in the present grass pea cytotypes, indicating absence of any mobile element induced NOR rearrangements. The enhancement of GC-rich repeats in aneuploids has often been regarded as an ancient mechanism of adaptation in a diploid background (Ho et al. 2008; Ibarra-Laclette et al. 2011). Furthermore, NORs are chromosomal landmarks with tandemly repeated sequences of ribosomal genes and are important constituents of cellular protein synthesis (Iwata et al. 2013). The importance of being GC-rich to tolerate environmental stress has recently been observed in cadmium-exposed grass pea where metal-induced GC→AT transition triggered chromosome breakage and fragility in sensitive genotypes (Talukdar 2014). Additional NOR chromosomes presumably enhanced the antioxidant defense response of the present aneuploids against diverse abiotic stresses (Talukdar 2014). Most importantly, increasing number of NOR-bearing autosomes in the present aneuploids did not lead to polymorphic NORs. The results suggested a conspicuous absence of cryptic transposition and indicated stability of the present grass pea genome, which is not in accordance with the earlier reports on increased fragility of the longest chromosome bearing NOR in grass cells (Huang et al. 2008; Grabowska-Joachimiak et al. 2015).

Identical CMA and DAPI banding patterns confirmed chromosome 3 as extra metacentric chromosomes in Triplo III. Thus, the number of CMA^+ bands and DAPI bands was increased in Triplo III and was further increased in Tet III. In double trisomic Triplo 3-4, involvement of this chromosome as one of the non-homologous extra partners was clearly resolved by identical banding patterns. The location of CMA and DAPI bands in this metacentric chromosome suggested the presence of GC-rich repeats around the centromere and AT-rich repeats at the terminal part of one arm. Per chromosome GC-rich regions constituted about 38% of total chromosomes while AT-rich regions occupied nearly 9.5%. Addition of the chromosome significantly increased the GC/AT rich region by about 1.4-fold in Triplo III in relation to its disomic parent. CMA/DAPI banding also confirmed chromosome 2 as the second largest chromosome which is submetacentric in nature. The presence of a solitary DAPI band at the terminal position of the long arm of this chromosome was unique and was found triplicated in Triplo II, confirming it as the trisomic chromosome in Triplo II. This was further confirmed in Tet II and in double trisomic Triplo 2-4 and Triplo 2-5, as the chromosome was inherited with an unaltered banding pattern in comparison to Triplo II. Complete absence of any CMA^+ region indicated conspicuous absence of GC-rich region of the chromosome. Instead, AT rich repeats are present at the long arm terminal, the % of which increased by about 1.3-fold in Triplo II and by nearly 1.6-fold in Tet II. Similar to Triplo II, the CMA^+ region was not observed in chromosome pairs 4 and 5. However, the positions of the solitary DAPI band are completely different. While pair 4 exhibited one additional copy in Triplo IV, pair 5 was found triplicated in Triplo V with an identical banding pattern in each case. Consistent and uniform occurrence of one medial DAPI band at the short arm of chromosome 5 in Triplo V and one intercalary DAPI band near the centromere at the long arm of chromosome 4 in Triplo IV suggested chromosome 4 is the extra in Triplo IV while chromosome 5 is the additional primary chromosome of Triplo V. This was further confirmed in their respective tetrasomic progenies. Complete absence of any CMA^+ region indicated conspicuous absence of GC-rich regions in both the chromosomes. However, AT-rich repeats increased by about 1.1–1.2-fold in Triplo IV and Triplo V, and by nearly 1.2-fold in both Tet IV and Tet V. The characteristic banding pattern unambiguously detected

involvement of chromosome 4 as extra in Triplo 2-4, Triplo 3-4 and Triplo 4-6, and chromosome 5 in Triplo 5-7 and Triplo 2-5. Complete absence of GC-rich repeats in chromosomes 2, 4 and 5 might be due to either high condensation of heterochromatic regions at metaphase which could prevent CMA binding in these regions, or evolutionary consequences during speciation of a large species complex within *Lathyrus* genus (Mahbub et al. 2007; Storme and Mason 2014).

CMA/DAPI banding unequivocally confirmed the presence of pairs 6 and 7 as extra chromosomes of Triplo VI and Triplo VII, respectively. Two thick CMA bands with two DAPI dots in chromosome 6 triplicated in Triplo VI. Banding patterns revealed CMA rich regions constituted nearly 38% of total chromatin length in chromosome 6. Additional chromosomes in Triplo VI enhanced the GC-rich repeat which was elevated nearly 1.4-fold in Tet VI over the disomic parent. On the other hand, the presence of CMA-stained regions in the whole short arm and nearly 25% of the long arm along with one DAPI band in the sub-terminal position of the long arm of chromosome 7 was consistently resolved in Triplo VII and Tet VII. Thus, chromosome 7 was detected as the extra chromosome with significant enhancement of GC-rich repeats in Triplo VII and Tet VII. Banding patterns confirmed the involvement of chromosome 6 in double trisomic Triplo 1-6 and Triplo 4-6 while chromosome 7 was proved to be one of the extra chromosomes in double trisomic Triplo 5-7.

The unique fluorescence banding pattern and constructed idiograms specific to seven pairs of chromosomes in the present grass pea material was found to be combined in six self-fertile double trisomics in different ways. Accordingly, the number of CMA/DAPI bands and % GC/AT-rich regions changed. Meiotic chromosome associations of more double trisomics in grass pea were recently investigated (Talukdar 2015). In the present study, Triplo 1-6 combined a characteristic banding pattern of chromosomes 1 and 6, resulting in an increase in the number of CMA bands to 20 and DAPI rich regions to six dots. CMA and DAPI-stained regions uniquely combined in Triplo 5-7, Triplo 3-4 and Triplo 4-6. Addition of metacentric chromosome 5 exhibiting only one DAPI$^+$ band along with CMA rich chromosome 7 in Triplo 5-7 balanced the GC/AT level by increasing both GC and AT-rich regions. A similar situation but with different banding position was observed in case of Triplo 3-4 and Triplo 4-6. Two DAPI bands in participating chromosomes balanced the increased CMA rich region to some extent, indicating a more balanced dosage of AT/GC in double trisomics. Combinations of 2 and 4 in Triplo 2-4 and of 2 and 5 in Triplo 2-5 as extra non-homologous chromosomes led to nearly 1.2–1.3-fold increase in AT rich region in these two double trisomics. However, due to the absence of CMA regions there was no increase in GC-rich region in Triplo 2-4 and Triplo 2-5. Therefore, out of seven pairs of chromosomes, pair 4 was involved in three double trisomics as an extra chromosome while pairs 2, 5 and 6 were each identified as extra in two double trisomics. Pairs 1 and 3 was involved as additional chromosomes in one double trisomic each.

Addition of seven different extra chromosomes in genomes of seven morphologically distinct trisomic types confirmed the involvement of an extra dosage in the origin of a complete set of trisomic phenotypes. Fidelity of fluorescence banding in trisomics was confirmed in seven different tetrasomics and six different double trisomics. The message is clear that basic fluorescence DNA-base specific chromosome banding pattern can be utilized successfully in unraveling identity of additional chromosome(s), molecular karyotyping, chromosomal fidelity, nature of aneuploidy and detection of intrinsic genomic/chromosomal stability in an annual crop like grass pea. The identification and molecular characterization of extra chromosomes and revealing of specific linkage groups will be beneficial in genetics, genomics and future breeding programs of this hardy legume crop.

Disclosure statement

No potential conflict of interest was reported by the authors.

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