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Autopolyploidy differentially influences body size in plants, but facilitates enhanced accumulation of secondary metabolites, causing increased cytosine methylation

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SUMMARY

Whole genome duplication leads to autopolyploidy and brings about an increase in cell size, concentration of secondary metabolites and enhanced cytosine methylation. The increased cell size offers a positive advantage to polyploids for cell-surface-related activities, but there is a differential response to change in body size across species and taxonomic groups. Although polyploidy has been very extensively studied, having genetic, ecological and evolutionary implications, there is no report that underscores the significance of native secondary metabolites *vis-à-vis* body size with ploidy change. To address this problem we targeted unique diploid–autotetraploid paired sets of eight diverse clones of six species of *Cymbopogon* – a species complex of aromatic grasses that accumulate qualitatively different monoterpene essential oils (secondary metabolite) in their vegetative biomass. Based on the qualitative composition of essential oils and the plant body size relationship between the diploid versus autotetraploid paired sets, we show that polyploidy brings about enhanced accumulation of secondary metabolites in all cases, but exerts differential effects on body size in various species. It is observed that the accumulation of alcohol-type metabolites (e.g. geraniol) does not inhibit increase in body size with ploidy change from 2× to 4× ($r = 0.854$, $P < 0.01$), but aldehyde-type metabolites (e.g. citral) appear to drastically impede body development ($r = -0.895$). Such a differential response may be correlated to the metabolic steps involved in the synthesis of essential oil components. When changed to tetraploidy, the progenitor diploids requiring longer metabolic steps in production of their secondary metabolites are stressed, and those having shorter metabolite routes better utilize their resources for growth and vigour. *In situ* immunodetection of 5-methylcytosine sites reveals enhanced DNA methylation in autopolyploids. It is underpinned that the qualitative composition of secondary metabolites found in the vegetative biomass of the progenitor diploid has a decisive bearing on the body size of the derived autotetraploids and brings about an enhancement in genome-wide cytosine methylation.

Keywords: polyploidy, polyploidy and body size, polyploidy and secondary metabolism, *in situ* cytosine methylation, epigenetics, polyploidy breeding, *Cymbopogon*.

INTRODUCTION

Global duplication seems to be a frequent event in plant evolution. Almost all plant species have doubled their genomes at least once in their evolutionary history followed by diploidization and then repeated polyploidization (Adams and Wendel, 2005; Chen, 2007; Birchler and Veitia, 2010; Salmon and Ainouche, 2010; Yang *et al.*, 2011). A substantial fraction of speciation events in plants involve the reunion of divergent genes and genomes through sexual hybridization (Rieseberg and Willis, 2007), and the derived hybrids can show considerable changes in gene expression compared with their parents (Adams, 2007).

Polyploidy is an important speciation mechanism for all eukaryotes and has a profound impact on biodiversity

dynamics and ecosystem functioning (Ainouche and Jenczewski, 2010). The change to a tetraploid state nearly doubles the cell volume and enhances cell surface area by about 1.5 times, often facilitating the development of larger organs in plants, but in animals there is a tendency to preserve the same body size by reducing the overall number of cells, thus indicating a differential effect of polyploidy change across the taxonomic groups (Mable, 2003). As such, larger cells tend to have a smaller surface area to volume ratio, a phenomenon thought to lower the growth rate of polyploid cells (Otto, 2007). Whereas some plants do not experience a change in body size with polyploidization, regulation of cell size and number for ecological adaptation are implicated. The increased cell size in polyploids differentially affects enzyme activity (Galitski *et al.*, 1999), and is often accompanied by changed morphology (Levin, 2002). DNA methylation and histone modifications are said to cooperate to form a 'double lock' on ploidy-associated transcriptional inactivation (Baubec *et al.*, 2010). Nevertheless, the duplicated gene dosage in polyploids offers the opportunity for enhanced metabolic activity/gene expression and enhanced concentration of secondary metabolites in tissues and organs (Lavania, 2005). New phenotypes often arise with polyploid formation that may have value in agriculture. But how the native secondary metabolites in the progenitor diploid influence the growth and development in the polyploid state has not been elucidated, despite its direct bearing on polyploidy breeding. The present study attempts to address this question based on the observations recorded on eight different unique paired sets of diploid versus autotetraploid clones that differ in the qualitative composition of their native secondary metabolites.

RESULTS

To address the key question about the influence of native metabolites on polyploidy-mediated change in body size, we compared growth pattern/biomass productivity of the progenitor diploids *vis-à-vis* their corresponding tetraploids in eight diverse clones of six industrially important species of *Cymbopogon* Sprengel (family: Poaceae) that produce in their vegetative tissues and organs qualitatively different secondary metabolites linked to a common metabolic pathway (monoterpene essential oil) regulated by the terpene synthase (TPSs) gene family. We also attempted to elucidate cytosine methylation on the chromosomes *in situ* to understand possible genome-wide DNA methylation changes with change in ploidy.

Cymbopogon species, their metabolite groups and ploidy-mediated change in biomass

All the *Cymbopogon* species studied have $2n = 20$ except *Cymbopogon khasianus pendulus* that has $2n = 60$ (Lavania, 1988). They are obligatorily endowed with asexual means of reproduction, flower profusely but set seeds only in certain species, e.g. *Cymbopogon flexuosus* and *Cymbopogon martinii* in specific niches. These species could be conveniently clustered into three distinct groups based on the qualitative composition of their essential oil: group I, aldehyde rich; group II, a mix of alcohol and aldehyde, i.e. intermediate type; and group III, alcohol rich, where these components constitute about 70% of the essential oil fraction. Interestingly, one of the species, *C. flexuosus*, exhibits a range of infraspecific variation in its essential oil composition, having genotypic variants that could be clustered into two distinct metabolite groups, i.e. aldehyde-rich or a mix of aldehyde + alcohol. As such, the target species and clones provide model prerequisites to elucidate the influence of native metabolites on polyploidy-mediated change on plant growth.

A quantitative account of the qualitative composition of the major essential oil constituents of all the species examined and their groupings based on essential oil composition is shown in Table 1. The data on biomass yield in the source diploids and their corresponding autotetraploid clones, along with the estimated values of the correlation coefficient (r) between native essential constituents and biomass to elucidate the ploidy-mediated change in biomass yield is given in Table 2.

Histological features, body size, essential oil productivity and cytosine methylation

Metric data and histological features contributing to biomass yield and essential oil productivity are shown in Tables 2 and 3, and their estimated values denoting the key features are graphically depicted in Figure 1. Representative histological/cytological features are shown in Figure 2, immunodetection of 5-methylcytosine (5mC) on the chromosomes *in situ* in Figure 3, and exo-morphological growth features in Figure 4. Observations reveal that autopolyploidy brings about a general increase in cell size/size of essential oil-secreting cells but a decrease in their number, and a significant increase in essential oil concentration. One of the important observations encountered in this study is that polyploidy brings about highly significant change in body size. The latter includes both increase and decrease in biomass unique to the progenitor diploid source depending upon the qualitative composition of its essential oil. There is a strong correlation (details in Table 2) between qualitative composition of the essential oil and ploidy-mediated change in biomass. Species that are rich in aldehyde content in the essential oil show reduced plant growth and those rich in alcohol content in the essential oil show enhanced growth in the autopolyploids.

At an epigenetic level, there is an overall increase in cytosine methylation with autopolyploidy. Although only a representative account from two species is shown here, the trend is the same in all other species as well.

Table 1 Diploid/autotetraploid paired sets of the *Cymbopogon* species, and their groupings based on major essential oil constituents

No.	Name of the Species (clone)	Concentration (%) of major essential oil constituents in the source diploids (2×) and corresponding autotetraploids (4×)						Essential oil group (major metabolite constituents)
		2×			4×			
		Aldehyde	Alcohol	Alcohol group	Aldehyde	Alcohol	Alcohol group	
1	*C. flexuosus* (Krishna)	Citral-a = 41.57 Citral-b = 29.19 Total = 70.76	Geraniol = 5.99	Geraniol = 5.99 Geranyl acetate = 7.24 Total = 13.23	Citral-a = 42.05 Citral-b = 24.81 Total = 66.86	Geraniol = 10.72	Geraniol = 10.72 Geranyl acetate = 13.14 Total = 23.86	Group I: aldehyde type (rich in citral)
2	*C. flexuosus* (Pragati)	Citral-a = 44.36 Citral-b = 30.22 Total = 74.58	Geraniol = 5.52	Geraniol = 5.52 Geranyl acetate = 6.17 Total = 11.69	Citral-a = 40.44 Citral-b = 28.51 Total = 68.95	Geraniol = 7.47	Geraniol = 7.47 Geranyl acetate = 9.29 Total = 16.76	
3	*C. khasianus* × *pendulus* (CKP-25)	Citral-a = 43.10 Citral-b = 32.16 Total = 75.26	Geraniol = 1.19	Geraniol = 1.19 Geranyl acetate = 4.52 Total = 5.71	Citral-a = 42.33 Citral-b = 29.85 Total = 72.18	Geraniol = 1.30	Geraniol = 1.30 Geranyl acetate = 3.47 Total = 4.77	
4	*C. winterianus* (Bio-13)	Citral-a = 0.67 Citral-b = 0.48 Citronellal = 27.74 Total = 28.89	Citronellol = 6.05 Geraniol = 15.22 Elemol = 16.46 Total = 37.73	Citronellol = 6.05 Citronenyl actetate = 5.56 Geraniol = 15.22 Geranyl actetate = 11.09 Elemol = 16.46 Total = 54.38	Citral-a = 0.91 Citral-b = 0.66 Citronellal = 33.41 Total = 34.98	Citronellol = 5.92 Geraniol = 18.52 Elemol = 10.01 Total = 34.45	Citronellol = 5.92 Citronenyl actetate = 5.10 Geraniol = 18.52 Geranyl actetate = 11.85 Elemol = 10.01 Total = 51.40	Group II: intermediate type
5	*C. flexuosus* (geraniol type)	Citral-a = 19.87 Citral-b = 15.32 Total = 35.19	Geraniol = 37.64	Geraniol = 37.64 Geranyl acetate = 3.65 Total = 41.29	Citral-a = 17.25 Citral-b = 12.47 Total = 29.72	Geraniol = 42.88	Geraniol = 42.88 Geranyl acetate = 6.41 Total = 49.29	
6	*C. jwarunkusha* × *confertiflorus* (Jamarosa)	Citral-a = 1.32 Citral-b = 0.92 Total = 2.24	Geraniol = 52.26	Geraniol = 52.26 Geranyl acetate = 21.84 Total = 74.10	Citral-a = 0.70 Citral-b = 0.43 Total = 1.13	Geraniol = 52.55	Geraniol = 52.55 Geranyl acetate = 26.18 Total = 78.73	Group III: alcohol type (rich in geraniol)
7	*C. nardus* var. *confertiflorus* (CN-5)	Citral-a = 0.50 Citral-b = 0.26 Total = 0.76	Geraniol = 60.93	Geraniol = 60.93 Geranyl acetate = 20.04 Total = 80.97	Citral-a = 0.47 Citral-b = 0.25 Total = 0.72	Geraniol = 58.55	Geraniol = 58.55 Geranyl acetate = 26.79 Total = 85.34	
8	*C. martinii* (Tripta-2)	Total = 0.00	Geraniol = 75.69	Geraniol = 75.69 Geranyl acetate = 18.67 Total = 94.36	Total = 0.00	Geraniol = 76.40	Geraniol = 76.40 Geranyl acetate = 18.02 Total = 94.42	

Table 2 Plant biomass in the diploid/tetraploid paired sets, and pattern of change in biomass yield in the autotetraploid *vis-à-vis* secondary metabolite constituents in *Cymbopogon* species

		Concentration (%) of major essential oil constituents in the source diploids (2×) and corresponding autotetraploids (4×)				Fresh herb biomass (kg $10m^{-2}$)			
		2×		4×		2×	4×		
No.	Name of the species (clone)	Aldehydes	Alcohols	Aldehydes	Alcohols			% change in biomass yield in 4× over 2×	Essential oil group (major metabolite constituents)
1	*C. flexuosus* (Krishna)	70.76	5.99	66.86	10.72	20.00 ± 0.25	06.00 ± 0.11[a]	−70.00	Group I aldehyde type (rich in citral)
2	*C. flexuosus* (Pragati)	74.58	5.52	68.95	7.47	24.00 ± 0.20	08.00 ± 0.31[a]	−66.70	
3	*C. khasianus* × *pendulus* (CKP-25)	75.26	1.19	72.18	1.30	25.40 ± 0.65	18.25 ± 0.52[a]	−28.34	
4	*C. winterianus* (Bio-13)	28.89	37.73	34.98	34.45	21.30 ± 0.3	23.30 ± 0.2[a]	09.68	Group II intermediate type
5	*C. flexuosus* (geraniol type)	35.19	37.64	29.72	42.88	31.20 ± 0.35	35.20 ± 0.22[a]	12.11	
6	*C. jwarunkusha* × *confertiflorus* (Jamarosa)	2.24	52.26	1.13	52.55	45.78 ± 0.23	53.96 ± 0.41[a]	17.86	Group III alcohol type (rich in geraniol)
7	*C. nardus* var. *confertiflorus* (CN-5)	0.76	60.93	0.72	58.55	28.80 ± 0.25	38.40 ± 0.11[a]	33.33	
8	*C. martinii* (Tripta-2)	0.00	75.69	0.00	76.40	24.73 ± 0.62	28.13 ± 0.25[a]	13.74	
Coefficient of variation (CV)		93.8	80.9	92.9	76.1	29.6	61.0		All groups
CV for three metabolite groups		3.3	62.5	3.9	73.7	12.1	61.1		Group I
		13.9	00.20	11.5	15.4	26.7	28.8		Group II
		113.9	18.8	92.8	19.8	33.7	32.4		Group III

Correlation coefficient (*r*) between:
(i) Aldehyde concentration in the diploid/autotetraploid and change in biomass in the autotetraploid = −0.895*, **; and −0.882*, **, respectively.
(ii) Alcohol concentration in the diploid/autotetraploid and change in biomass in the autotetraploid = 0.854*, **; and 0.829*, respectively.
Level of significance: *$P < 0.05$ (table value at six degrees of freedom = 0.707); **$P < 0.01$(table value at six degress of freedom = 0.831).
[a]Values significantly different with respect to diploid by Student's *t*-test at $P = 0.01$.

Table 3 Leaf epidermal features, essential oil secretory channels and essential oil concentration in the progenitor diploid and corresponding autotetraploid in the diverse species and clones of *Cymbopogon*

No.	Name of the species (clone)	Ploidy status	Lumen size of essential oil-containing cell (μm^2) ± SE	Frequency of essential oil channels/cm^2 of leaf VS ± SE	Essential oil concentration in fresh herb (%)[a]	Area (cm^2) covered by essential oil-producing cells/cm^2 of leaf VS	Increase in essential oil channel area under cover in 4× over 2× (%)	Increase in essential oil concentration in 4× over 2× (%)
1	*C. flexuosus* (Krishna)	2×	411.5 ± 0.02	4419 ± 199	0.65 ± 0.02	0.018	5.55	7.69
		4×	555.1 ± 0.03	3350 ± 170	0.70 ± 0.03	0.019		
2	*C. flexuosus* (Pragati)	2×	367.1 ± 0.02	5817 ± 77	0.42 ± 0.08	0.021	19.05	19.05
		4×	735.0 ± 0.06	3356 ± 145	0.50 ± 0.06	0.025		
3	*C. khasianus* × *pendulus* (CKP-25)	2×	434.3 ± 0.02	3454 ± 53	0.76 ± 0.03	0.019	10.53	10.53
		4×	880.0 ± 0.03	3409 ± 72	0.84 ± 0.02	0.021		
4	*C. winterianus* (Bio-13)	2×	373.6 ± 0.02	4816 ± 102	0.82 ± 0.12	0.018	16.66	13.41
		4×	798.0 ± 0.01	3642 ± 116	0.93 ± 0.1	0.021		
5	*C. flexuosus* (geraniol type)	2×	231.2 ± 0.02	5598 ± 69	0.40 ± 0.05	0.013	15.38	17.50
		4×	461.3 ± 0.06	3252 ± 55	0.47 ± 0.06	0.015		
6	*C. jwarunkusha* × *confertiflorus* (Jamarosa)	2×	341.5 ± 0.09	4971 ± 48	0.46 ± 0.07	0.017	29.41	17.39
		4×	556.1 ± 0.03	3955 ± 56	0.54 ± 0.11	0.022		
7	*C. nardus* var. *confertiflorus* (CN-5)	2×	305.5 ± 0.04	4586 ± 46	0.51 ± 0.01	0.015	33.33	15.68
		4×	673.0 ± 0.06	3418 ± 62	0.59 ± 0.01	0.020		
8	*C. martinii* (Tripta-2)	2×	355.5 ± 0.05	5971 ± 16	0.67 ± 0.03	0.021	19.05	19.40
		4×	719.0 ± 0.02	3593 ± 119	0.80 ± 0.01	0.025		

[a]Values significantly different in tetraploid (4×) with respect to source diploid (2×) by Student's *t*-test at $P = 0.01$.

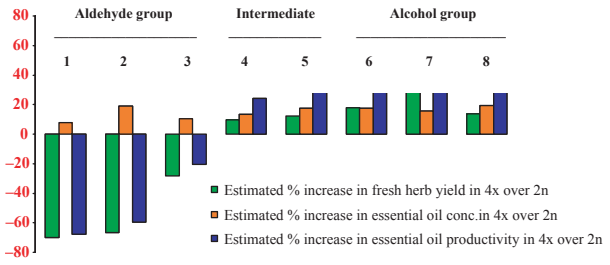

Figure 1. Polyploidy-mediated differential change in biomass yield and productive potential in different *Cymbopogon* species.
Polyploidy-mediated differential change in biomass yield and productive potential exhibited by different species/clones of *Cymbopogon* arranged in order 1–8 as in Table 1. Note the apparent link between qualitative composition of the secondary metabolite in the progenitor diploid and relative biomass in the corresponding autotetraploid, whereby aldehyde-rich clones show a reduction but alcohol-rich species show an increase in biomass when changed to autotetraploid state (see Table 2).

DISCUSSION

We used carefully selected experimental material, i.e. unique diploid/autotetraploid pairs of diverse species of *Cymbopogon* that produce in their vegetative tissues and organs qualitatively different secondary metabolites linked to a common metabolic pathway (monoterpene essential oil) regulated by the TPSs gene family. To ensure genetic fidelity and to exclude all other variation only 'clone' material was used as the diploid progenitor source to develop the corresponding autotetraploid clone. Most species in the genus *Cymbopogon* are diploid, $2n = 20$ (Lavania, 1988), and appear to have undergone a sort of homoploid speciation (Riddle *et al.*, 2007) enabling cryptic species diversification for variation in growth habit and qualitative differentiation of essential oil components, supporting an obligatory vegetative mode of reproduction (Sharma and

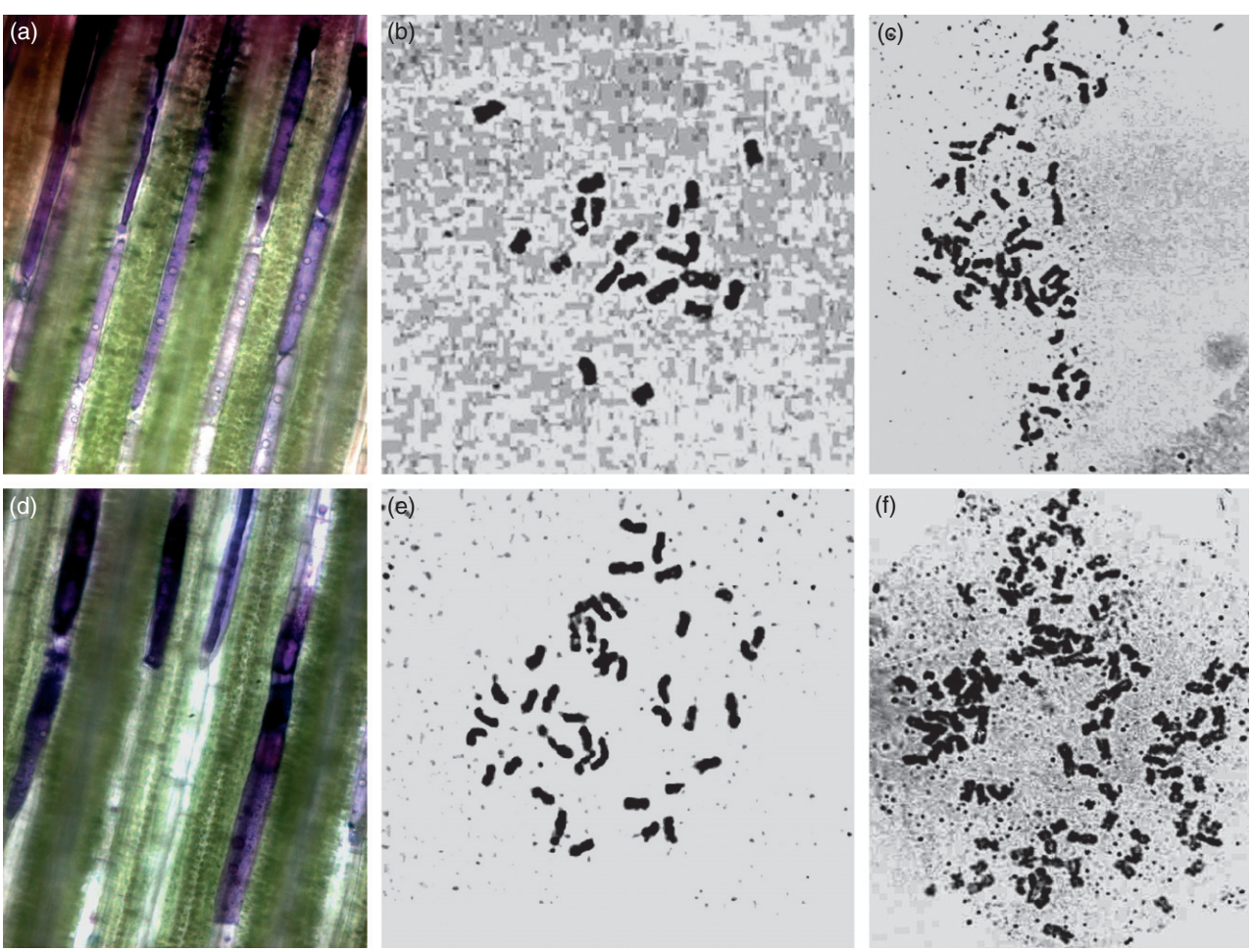

Figure 2. Essential oil secretory cells in the leaf epidermal layers and somatic chromosomes.
Representation of essential oil secretory cells in the leaf epidermal layers stained magenta with Schiff's reagent, and somatic chromosomes in the diploid (upper row) and tetraploid (lower row) respectively in (a, d) *Cymbopogon flexuosus*, (b, e) *C. nardus* and (c, f) *C. khasianus* × *pendulus*.

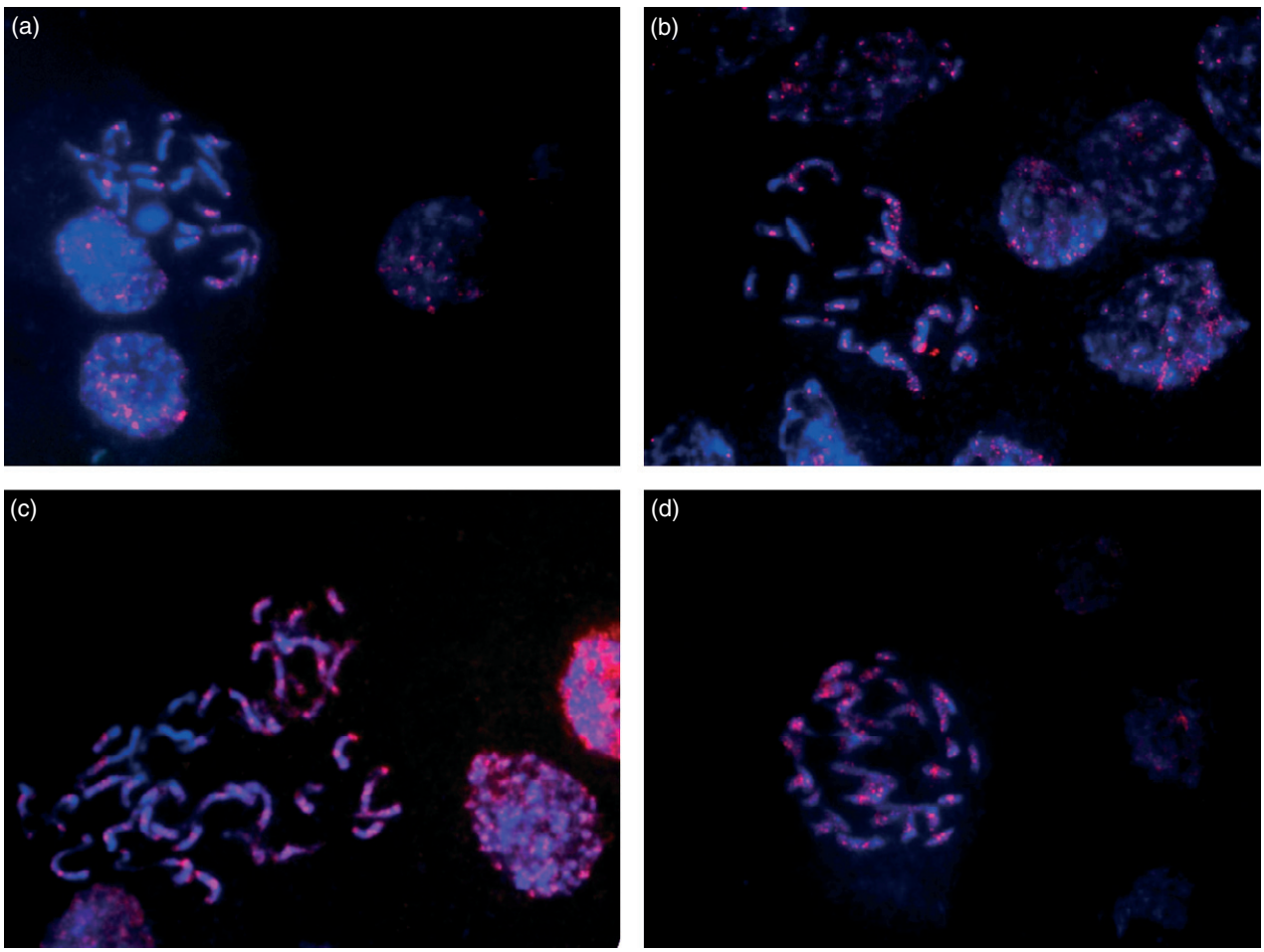

Figure 3. 5-Methylcytosine (5mC) immunodetection.
Immunodetection of 5mC *in situ* on somatic chromosomes of a diploid and corresponding autotetraploid of *Cymbopogon jwarunkusha* × *confertiflorus* (a, c), and *C. winterianus* (c, d). Note the relative abundance of methylation in the tetraploid.

Ram, 2000). Such available diversification in native metabolites in a single homoploid species complex, and also within a single species such as *C. flexuosus*, offers the ideal opportunity to understand the significance of attendant secondary metabolites in response to polyploidy change.

Qualitative composition of secondary metabolites in the source diploid has a decisive bearing on the body size response to genome duplication

The experimental results presented here clearly show that the qualitative composition of the secondary metabolites produced in the source diploid has a decisive influence on the performance of the derived autotetraploid. Polyploidy brings about enlarged cell size, including that of the cells secreting essential oil, and is accompanied by an associated increase in concentration of essential oil in all the species (Table 3). Although no significant change in oil quality occurs with ploidy change (Table 1), there is significant change in plant biomass (Table 2). Both increases and decreases in body size specific to species group are encountered with ploidy change. Observations recorded in different species/secondary metabolite groups clearly underpin that the differential response to ploidy change is closely associated with the qualitative composition of monoterpene in the essential oil produced therein (Table 2). This study unequivocally demonstrates that the polyploidy advantage associated with increased cell size (thereby contributing to plant biomass) is greatest in those species having alcohol-rich essential oils (there is a strong positive correlation between alcohol concentration in the source diploid and increase in plant biomass in the derived autotetraploid; $r = 0.854$, $P < 0.01$, table value 0.831), but is drastically reduced in those species with aldehyde-rich oils (there is strong negative correlation between aldehyde concentration in the source diploid and change in plant biomass/reduction in the derived autotetraploid; $r = -0.895$, $P < 0.01$). Some species have intermediate advantages with respect to polyploidy change for growth (see Table 2 and Figures 1 and 4). Huge changes observed in biomass yield – ranging from –70 to 33.33% – with ploidy change in diverse

Figure 4. Diploid versus autotetraploid *Cymbopogon* species.
Exo-morphological field view of diploid (left) versus autotetraploid (right) in *Cymbopogon*: (a). *C. flexuosus* (clone 'Pragati'), (b) *C. nardus*, and (c) *C. martinii*. Note that the tetraploids in (a) (i.e. citral type) evince a reduction in body size, but in (b) and (c) (i.e. geraniol type) the tetraploids are more vigorous.

related species with a coefficient of variation for qualitative composition in their secondary metabolites of 93.8% for aldehydes and 80.9% for alcohols (Table 2), amply suggest that there is a clear-cut metabolite-dependent link to polyploidy-mediated change in body size.

It may be surmised that such a differential response to polyploidy-mediated change in body size is apparently an effect of the length of the metabolic steps involved in the synthesis of a given essential oil fraction. The accumulation and storage of terpenoids may entail substantial costs in terms of energy and nutrients and partitioning of a significant amount of metabolic resources into the production of secondary metabolites. Terpenoids are more expensive to manufacture per gram than most other primary and secondary metabolites due to their extensive chemical reduction. The enzymatic costs of making terpenoids (monoterpenes in the present case) could be high, since terpenoid biosynthetic enzymes are apparently not shared with other metabolic pathways, and plant cells may possess more than one set of enzymes for catalyzing the basic steps of terpenoid formation (Gershenzon, 1994). It may be difficult to estimate overall global metabolic cost differences between group I, II and III polyploids, but the costs may be substantial since their yield constitutes about 5% of the plant's dry mass. In a broad sense the average cost of monoterpenes is estimated to be about 3.37 g glucose g^{-1}, far more expensive than nearly all types of primary metabolites, including carbohydrates (Gershenzon, 1994). The number of enzymatic conversion steps involved in the biosynthesis of a given terpenoid may correspondingly add to the metabolic costs. As such, an inverse correlation exists between terpenoid accumulation and growth since several processes involved in terpenoid accumulation demand a large outlay of a plant's resources (Gershenzon, 1994).

Accordingly, it may be emphasized that progenitor diploids requiring longer metabolic steps in the production of their metabolites experience more stress in obtaining resources for plant growth, and those having shorter metabolite routes utilize their resources more efficiently with regard to plant growth and vigour with increase in ploidy state. This fits well with the scheme of monoterpene biosynthesis in *Cymbopogon*, where condensation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) facilitates the formation of geranyl pyrophosphate – a C_{10} compound which serves as the precursor for further transformation into various alcoholic, aldehydic and other mon-

oterpenes. While geraniol is the main monoterpene constituent in *Cymbopogon martinii* (an alcohol-rich species), in *Cymbopogon winterianus* (having a mix of alcohol and aldehyde fractions as major constituents) the geraniol is first reduced and converted into citronellol (the alcohol compound) which in turn converts into citronellal (the aldehyde compound) (Akhila, 1986). In *Cymbopogon flexuosus* (an aldehyde-rich species) geraniol is converted into citral-a (an aldehyde) which is isomerized to citral-b (also an aldehyde) via a redox mechanism (Akhila, 1985). In *C. martinii* (a geraniol-rich species), a fraction of geraniol may be converted to geranyl acetate (an ester) by acetylation, but during inflorescence development deacetylation of the geranyl acetate is effected (Dubey *et al.*, 2003). As such the conversion from alcohol to aldehyde involves additional metabolic steps, stressing the developmental resources of the plant. Thus the citral-producing species are more constrained with regard to metabolic resources in the tetraploid state, further affecting their growth and development.

Autopolyploidy may cause gene expression bias at the genome level but has limited effect on the qualitative profile of secondary metabolites

Autopolyploidy is more common in angiosperms than traditionally assumed, and is characterized by genomic redundancy and polysomic inheritance (Soltis *et al.*, 2007; Parisod *et al.*, 2010). Rapid changes in genomic composition and gene expression have been observed in both auto- and allopolyploids. Most results concerning alterations in gene expression after polyploidy formation have been obtained from studies in allopolyploids, in which changes to the nuclear environment are more profound than in autopolyploids. Loss and/or gain of parental restriction sites, gene silencing/activation (Song *et al.*, 1995; Kashkush *et al.*, 2002), gene expression bias at the genome level (Hovav *et al.*, 2008; Flagel and Wendel, 2010), changes in post-transcriptional regulation and translational modification of proteins (Ng *et al.*, 2012), and changes in cytosine methylation appear to be occurring in early generations after polyploidization in allopolyploids (Xu *et al.*, 2009). However, the limited evidence currently available suggests that autopolyploids experience neither strong genome restructuring nor wide reorganization of gene expression during the first generations following genome doubling, but these processes may become more important in the long run (Church and Spaulding, 2009; Parisod *et al.*, 2010). This is consistent with the present observation that in the first instance there is no significant change in the quality of the essential oil with ploidy change (Table 1), notwithstanding that the growth behaviour of the derived autopolyploids is significantly affected (Table 2) and there is enhancement in essential oil concentration *per se* (Table 3).

In autopolyploids, a smaller number of studies have shown that there may be only small changes in gene expression between ploidy levels. No significant differences in gene silencing or novel gene expression could be observed in the diploid, autotetraploid and autohexaploid lineages of *Helianthus decapetalus* (Church and Spaulding, 2009). An increase in gene expression on a per cell basis in proportion to the gene dosage has been demonstrated in comparisons of monoploid, diploid, triploid and tetraploid lines of maize (Guo *et al.*, 1996; Riddle *et al.*, 2007). Using synthetic autopolyploid series 1× to 4× in potato (*Solanum phureja*), it has been shown that cell size and organ thickness are positively correlated with the ploidy level, but only about 10% of the genes exhibit linear expression with the ploidy and could be dramatically changed because of ploidy alteration (Stupar *et al.*, 2007). However, the expression of genes duplicated by polyploidy could be partitioned between the duplicates exhibiting differential expression and silencing between the different organs of the same plant, indicating subfunctionalization of homoeologous genes as a consequence of polyploidization (Adams *et al.*, 2003; Liu and Adams, 2007). Recent studies using newly formed synthetic auto- or allopolyploid plants have shown that polyploidization is associated with genome-wide changes in gene expression, and these changes appear to be controlled primarily by epigenetic mechanisms such as cytosine methylation and heterochromatic histone modifications (Comai *et al.*, 2000; Kashkush *et al.*, 2002; Wang *et al.*, 2004; Xu *et al.*, 2009; Baubec *et al.*, 2010; Yu *et al.*, 2010). The present observation about differential response to growth rate in the analogous autopolyploids underpins the significance of steps in the metabolic pathway in functionalization of duplicated genes.

Ploidy change and cytosine methylation

Widespread changes to gene expression, including effects on the plant phenotype and the response to environmental and genomic stress, have been shown to result from interspecific hybridization and polyploidy in a number of plant species, and attention has now shifted to determining the epigenetic processes that drive these changes (Hegarty *et al.*, 2011). Epigenetic mechanisms such as DNA methylation can be triggered by environmental and genomic stresses and can cause stable heritable phenotypic modifications without changes in the underlying DNA sequence (Verhoeven *et al.*, 2010). The DNA methylation status of newly formed species is found to be consistently affected following genome doubling (Salmon and Ainouche, 2010). The genetically identical offspring of asexual triploid F_1 *Taraxacum officinale* (dandelion) plants showed modest levels of methylation variation, indicating that *de novo* methylation was triggered by the formation of triploids (Verhoeven *et al.*, 2010).

The observations recorded here on physical detection of 5mC *in situ* clearly reveal the incidence of large-scale genome-wide cytosine methylation. While it is difficult to pinpoint specific chromosomal sites that are more highly

methylated, it is amply clear that there is enhanced global methylation with the change to autotetraploidy. This is consistent with the findings of Finn *et al.* (2011), who used a different but related approach to show that the transgenes are expressed at a lower level in autotetraploid (4×) than in diploid (2×) Arabidopsis, but are more methylated in 4× than 2× Arabidopsis. They have suggested that transgenes are transcriptionally repressed in the 4× background, resulting in expression levels that are lower than in the 2× background. Accordingly, it may be inferred that higher cytosine methylation caused by ploidy elevation, as observed here, may lead to the formation of a 'double lock' on the ploidy-associated influence of native secondary metabolites on growth and body size in autopolyploids. A form of 'double lock' cooperation between DNA methylation and histone modification with ploidy-associated transcriptional inactivation was proposed by Baubec *et al.* (2010), and a corollary to this is likely for different situations, as reported in the present study.

In conclusion, the present study provides categorical information that qualitative composition of the secondary metabolites present in the vegetative biomass has a decisive bearing on the growth potential of derived autotetraploids. Only those plants having the lowest secondary load on their vegetative growth are favoured by ploidy elevation. Our study provides insights for genetic enhancement of cryptic species where sexual means of recombination are deficient and the secondary metabolites produced in the biomass constitute the economic product.

EXPERIMENTAL PROCEDURES

Plant material

Eight diploid clones of six species of *Cymbopogon* Sprengel (family: Poaceae), listed in Table 1, all having $2n = 20$ (except *C. khasianus pendulus* with $2n = 60$) were targeted for the study. They were categorized into different metabolite groups based on the content of major essential oil constituents estimated by standard GLC analysis.

Realization of clonal polyploids

Basal meristem on the exposed axillary buds on fast-growing slips (tillers) was immersed for 7 h at 25°C in 0.1% (v/w) aqueous solution of colchicine in 2% DMSO, followed by thorough washing in running water and planting in soil. Emerging plantlets were screened for leaf stomata size, and those with uniformly and distinctly enlarged stomata, roughly twice the volume of source diploids, were selected to isolate polyploids. As such both diploid and polyploid clones derived from the 'same source tiller' were isolated and multiplied for comparative study. Clonal progenies of autopolyploid clones stabilized for at least 2 years (i.e. five or six passages of clonal propagation) were used for recording the observations.

Immunodetection of 5-methylcytosine

Fast-growing root tips from both diploid and corresponding autotetraploid clones of different *Cymbopogon* species were excised, pre-treated in saturated aqueous solution of *para*-dichlorobenzene for 3 h at 12–14°C, washed thoroughly in running water and then fixed overnight in ethanol:acetic acid (3:1). Fixed roots were washed in 1× enzyme buffer at pH 4.6 (prepared by mixing 40 ml of 10 mM citric acid + 60 ml of 10 mM trisodium citrate) to remove the fixative and then transferred to an enzyme solution (mixture of 3% v/v pectinase + 2% w/v cellulase from *Aspergillus niger*) for 45 min at 37°C to soften the cell wall and facilitate squashing. The softened material was again washed in 1× enzyme buffer, and meristematic cells from the root-tip zone were squeezed out in 45% acetic acid on a clean slide and squashed under a cover glass to realize good metaphase spreads. The cover glass was removed after freezing the slide on dry ice, and the preparations were allowed to air dry and were then stored in moisture-free box for at least 12 h. The slide preparations containing metaphase spreads were denatured in 70% formamide/2× SSC at 70°C for 2 min, and then dehydrated in an ethanol series at −20°C. Air-dried slides were treated with 1% BSA, 1× PBS solution at 37°C for 30 min in a moisture chamber, and then incubated with a mouse monoclonal antibody against 5mC i.e. 5-Methylcytidine (Eurogentec, http://www.eurogentec.com/), diluted 1/500 with 3% BSA, at 4°C for about 18 h. After three washes with 1× PBS for 5 min, the slides were incubated with anti-mouse Alexa Fluor 546 IgG A11030 (Invitrogen, http://www.invitrogen.com/), diluted 1/200 with 3% BSA, at 25°C for 2 h in a dark moisture chamber, and washed in 4× SSC, 4× SSC with 0.1% Tween 20 at 42°C for 3 min, 4× SSC at 42°C for 3 min, and 2× SSC at room temperature (23°C) for 5 min. The slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in an anti-fade solution [1.25% 1,4-diazabicyclo[2.2.2]octane (DABCO) in 90% glycerol] for observation and recording under the microscope.

Growth potential and essential oil productivity

Both progenitor diploids and their corresponding autopolyploid clones were grown in 10-m^2 experimental plots replicated three times, plant to row distance 50 cm × 50 cm, for recording observations on biomass and essential oil yield. Data on herbage yield were taken at 4-monthly harvests, except in *C. martinii* where it was taken on a 6-monthly harvest due to a different harvest stage, in the second year when the crop growth is at the optimum. As such the sample size constituted of nine observations per 10-m^2 plot size with 36 hills per plot for each plant type, except *C. martinii* where only six observations were recorded. To minimize any error in data recording, both the diploid and its corresponding tetraploid clones were harvested simultaneously at the pre-flowering full-grown stage and weighed immediately. Similar care was taken with recording data on essential oil content. Essential oil concentration in the leaves harvested at a similar growth stage was estimated by hydrodistillation in Clevenger's equipment adjusted to 60°C and run for 2 h. The percentage area covered by essential oil secretory channels was estimated from micromorphological analysis of leaf vertical sections and histological localization of essential oil secretory channels, where aldehyde-producing cells were localized by staining in Schiff's reagent (Lewinsohn *et al.*, 1998) and with fluorescent localization of similar lignified sites for geraniol-producing cells.

Statistical analysis

Coefficient of variation (CV), being a good unitless measurement for comparing the extent of variation between different characters with different scales, was used to delineate the incidence of qualitative differences for secondary metabolite content amongst the related species. The correlation coefficient (r) that facilitates the measure of association between variables was applied to deduce the relation-

ship between the secondary metabolite composition and growth pattern/biomass yield. Student's *t*-test was applied to test the significance of differences in biomass yield and essential oil concentration between the diploid and autotetraploid pairs. Statistical analyses (mean, standard deviation, standard error, CV, correlation coefficient and *t*-value) were done using the statistical software available in the Division of Genetics and Plant Breeding of the Central Institute of Medicinal and Aromatic Plants, Lucknow, India, that is based on the standard methods described in Panse and Sukhatme (1967).

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