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(54) Title: METHODS OF OBTAINING HEVEA BRAS ILIENSIS PLANTS

FIG. 1

(57) Abstract: A method for obtaining rubber plants of particular ploidy and genotype useful for seed production, multiplication and crop improvement, the method comprising: (a) providing a population of rubber plants; (b) choosing from the population a subset of individual plants with atypical phenotype; (c) assessing the DNA content of plants in the subset; (d) classifying plants in the subset as haploid, diploid or polyploid according to the results of step (c).

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METHODS OF OBTAINING HEVEA BRAS ILIENSIS PLANTS

Field of invention

The present invention relates to a method of obtaining special forms of rubber plant (*Hevea brasiliensis* Muell. Arg.) useful in plant breeding, or in rubber production, or both: and to plants obtainable thereby. Plants obtainable according to the invention include haploid rubber plants as well as triploids, tetraploids and plants of higher ploidy. In one aspect, the method relates to obtaining doubled haploid rubber plants: in a further aspect, it relates to obtaining haploid rubber plants from which doubled haploid rubber plants can be obtained. The invention further comprises a method of producing uniform F_1 hybrid rubber plants by crossing two different doubled haploids and the F_1 hybrid rubber plants obtainable by the process.

Background of the invention - plant breeding

Although plant breeding programmes worldwide have made considerable progress in developing new cultivars with improved yield, quality, disease resistance and other useful traits, breeding is primarily a stochastic process. It typically involves generating and screening large numbers of individuals to identify rare types containing novel and desirable trait combinations. For this purpose very large numbers of progeny from crosses need to be screened over several seasons in order to select one or a few plants with the desired characteristics.

In a typical plant breeding programme, two plants (parental lines) are crossed: the resultant progeny are screened and one or more plants possessing the desired phenotype (combination of expressed traits) are identified and selected. The selected plant or plants may then be self-pollinated or crossed to yield a second-generation progeny population. This population may undergo another round of individual plant screening to select from it those lines that possess the desired traits originally introduced in the first generation. If, as is often the case, the phenotypic traits are derived from the combined effect of several genes, then the number of progeny plants that must be screened depends on the number of genetic differences between

the two original parental lines. Thus, the greater the number of genetic differences the greater the number of plants that need to be grown and evaluated and the lower the probability of obtaining progeny with all the desired traits. The problem is exacerbated when the desired traits include yield and quality characteristics that can only be determined once the plants have reached maturity.

One possible solution to the problem of screening large numbers of individuals in a progeny that segregates for traits of interest depends on the ability to produce or identify haploid plants derived from the gametic cells of parental individuals. The chromosome complements of these haploids sometimes double spontaneously to produce homozygous doubled haploids (DHs); or they may be induced to double by treatment with certain chemicals, for example colchicine. One way of producing doubled haploids is by the *in vitro* culture of anthers or microspores (androgenesis) or analogously from female gametic cells by culturing flower, ovary and ovule tissues (gynogenesis). A second method of producing haploids and doubled haploids has been via wide-species crosses. The resultant doubled haploid plants, regardless of how they are derived, are instantly and completely homozygous. This means that on selfing the plants breed true, i.e. their progeny are genetically identical to the parent doubled haploid plant, so clones can be generated and multiplied rapidly. Furthermore, when two such doubled haploids are crossed sexually the resultant hybrid is genetically invariant and heterozygous for all loci that differ between the two parents. With a sufficient stock of the two parental doubled haploids, the same F_1 hybrid can be produced repeatedly and reliably and grown commercially.

The ploidy level of somatic cells is defined as the number of genome sets of chromosomes that they contain. A genome set of chromosomes (also known as the base number, x) is most simply described as the number of heterologous chromosomes present in the nuclear genome and equals n, the number present in gametic cells of a diploid organism. For example, humans are a diploid species, having $2n=2x=46$ chromosomes in their somatic cells and $n=x=23$ in their gametes (sperm and egg). When the ploidy level is greater than one, genetic analysis is made more difficult by the effects of dominance. When more than one copy of a gene is present, only one copy, the dominant one, may influence the phenotype: or else both

copies can contribute to the expression of the phenotype (partial or no dominance). With dominance, the other copy of the gene (the recessive allele) is masked: as a consequence its presence cannot be determined by the observed phenotype. Haploid organisms contain the same number of chromosomes (n) in their somatic cells as the normal gametes of the species have. The term haploid sporophyte is generally used to designate sporophytes having the gametic chromosome number.

Haploid sporophytes of higher plants can be distinguished from diploids in many ways. They are usually smaller (partly because of their smaller cell size). In general terms, cell volume in plants is positively correlated with ploidy level. Haploids are also usually sterile. Several methods for the provisional assignment of haploid status to a plant exploit phenotypic characteristics. The most widely used of these methods is the measurement of stomatal guard cell length and chloroplast content in these cells. In some fast-growing annuals such as barley, haploidy is also easily identified because the haploids are sterile. However, none of the phenotypic predictors of haploidy are absolutely reliable. Methods that directly measure genome size are far more reliable. These include direct measurement of the chromosome number using conventional chromosome counting techniques, and measurement of the DNA content using microdensitometry or more especially by flow cytometry (Coba de la Pena and Brown, 2001). It is also possible to exploit the absolute absence of heterozygosity in haploids and doubled haploids to detect such plants using various co-dominantly inherited molecular marker methods (e.g. Chani et al., 2000; Tang et al., 2006).

Haploids may have intrinsic value because of their overall reduction in size compared with diploids. Haploids also have value in allowing the isolation of mutants, which may be masked in a diploid, particularly where the mutant allele is either non-functional or recessive. Haploids also have value in transformation programmes. If haploids are transformed directly, then true-breeding doubled haploid transgenic plants can be produced in one step following chromosome doubling of transgenic haploids. A wide range of techniques for chromosome doubling are known (Kasha, 2005) and these techniques, or modifications of them, may be used in the present invention.

An important use of haploids is based on the fact that marked improvements in the economics of plant breeding can be achieved via doubled haploid production, since selection and other procedural efficiencies can be markedly improved through the provision of elite true-breeding (homozygous) lines (Nei, 1963). With doubled haploid production systems, homozygosity is achieved in one generation. Thus, the breeder can eliminate the numerous cycles of inbreeding that are usually necessary to achieve practical levels of homozygosity by conventional methods. Indeed, absolute homozygosity for all traits is not achievable by conventional breeding methods. Consequently, an efficient doubled haploid technology would enable breeders to reduce the time and the cost of cultivar development relative to conventional breeding practices.

Spontaneous haploids may occur in many species of plants, usually at very low frequencies. For rubber, there are no spontaneous haploid plants reported. Rubber is a clonally propagated crop and as such breeding and planting procedures are geared to selecting uniform high performing plants. Potential off types are therefore eliminated before sowing. Rubber production via seed is subject to a pre-screen in a bounce test. Only normal seed, those that bounce, are selected for sowing. The abnormal, non-bouncing, seed are discarded at the beginning of the process and therefore a major source of atypical seedling phenotypes is lost at the onset, they are never germinated. In addition rapidly germinating seedlings are selected within 20 days of sowing and slow germinating seed (another source of off types) are discarded. The skilled person therefore would have no incentive to try to identify off types in rubber through phenotypic selection because of the high level of prejudice against such selection in normal rubber agriculture.

The small number of haploids so far identified in rubber have been identified by alternative means. In their book "Doubled Haploid Production in Crop Plants", Maluszynski et al. (2003) quoted two recent examples of haploid production in rubber, one from anther culture (Jayasree et al. 1999) and one from unpollinated ovules (Chen et al., 1988, see Clément-Demange et al., 2007). However, there were also earlier publications by Chen et al. (1982) on "Recent advances in anther culture of *Hevea brasiliensis*" and Chen et al. (1987) on

"Production of haploid plantlets in cultures of unpollinated ovules of *H. brasiliensis*". As yet, no double haploids have been reported.

As well as having value in their own right as potential new varieties, homozygous doubled haploid plants also have utility for the generation of F_1 hybrid plants, produced from crosses made between selected homozygous male and female parents. These F_1 plants are also of value as cultivars as they may exhibit so-called hybrid vigour (heterosis), a characteristic often associated with dramatic increases in yield compared with either parent. Furthermore, the production of F_1 hybrids allows the breeder to produce large quantities of uniform seed of a single genotype from homozygous parental lines. F_1 hybrids have many advantages over a genetically heterogeneous mix of genotypes because one can select single elite genotypes that possess various desirable characteristics, for example high yield. One may also achieve higher yields by selecting genotypes adapted to specific environments and to optimise agronomic and management practices. In rubber, as in many other crops, hitherto the only way of producing a single genotype in commercial quantities has been by asexual cloning, using suckers, cuttings or grafts. Thus F_1 hybrid production from doubled haploid parents is a rapid method of achieving distinctive, uniform and stable (DUS) crop varieties demanded by many national regulatory authorities.

Haploid (H) plants (and doubled haploids - DH plants) express all their genetic information or, in other words, their genotype is completely displayed by their phenotype. Resistance to pest and diseases or unfavourable external factors (drought, salinity, heavy metal toxicity, temperature, light etc.) can thus be directly recognized and selected. Haploid plants allow the detection of mutants that are unable to pass through the embryonic phases of development. They also allow: 1) screening for both recessive and dominant mutants in the first generation after mutagenic treatment, 2) immediate fixation of mutant genotypes via doubled haploidy, 3) increased selection efficiency, and 4) applying *in vitro* selection methods at the haploid or doubled haploid level. For similar reasons haploid plant tissues make ideal vehicles for genetic transformation, to give genetically modified haploids that on doubling give homozygous diploids containing the introduced gene or genes.

The agricultural applications for haploids exploit their capacity for the rapid generation of homozygous genotypes after chromosome doubling, with advantages including:

•Reduced time for variety development;

5 •Homozygous recombinant lines can be developed in one generation instead of after numerous backcross generations;

•Selection for recessive traits in recombinant lines is more efficient because recessive alleles are not masked by the effects of dominant alleles; and

•Introduction of "alien" genes is speeded up by allowing homozygotes to be developed
10 readily.

The crossing of two homozygous elite lines (such as can be produced by doubling haploids) can generate genetically uniform, highly heterozygous hybrid varieties, as is exemplified by the highly successful hybrid maize varieties first produced in the USA during the 1930s.
15 There have been many efforts to reproduce the yield increase gained in hybrid maize varieties in other crops. Other examples include: F_1 hybrid varieties of sunflower, sugar beet and carrot which are now widely grown, and hybrid lines of oilseed rape (canola) and rice which are becoming increasingly available; more than half the rice grown in China is hybrid, with yields at least 20% higher than the non-hybrid equivalent. To date, there has been no
20 corresponding progress in rubber. The lack of progress with hybrid rubber cultivar production is principally because the traditional cropping system is based on grafted clonal material. F_1 hybrid production has not been an option as no homozygous, true breeding lines (doubled haploids or other inbred lines) have been available in rubber.

25 Background of the invention – ploidy

Ploidy has played a major role in the evolution of plants, including crops, and rubber is no exception. Rubber (*Hevea brasiliensis*) is thought to have become established as a tetraploid (4x=36) during the course of its evolution (Perry, 1943; Clément-Demange et al., 2007). This

is supported by chromosome numbers in phylogenetic studies of the Euphorbiaceae in which rubber is a family member (Perry, 1943), the occurrence of tetravalents at meiosis (Clément-Demange et al., 2007) and the production of viable cells carrying 9 chromosomes in anther culture (Chen et al. 1982). However, rubber cultivated on a large scale is regarded as being
5 diploid (2n=2x=36) (Clément-Demange et al., 2007).

Several important crops are polyploid, for example the Cavandish banana is triploid, potato is tetraploid and bread wheat and oat are hexaploids. Ploidy levels of crops can be either natural or induced. Increasing the ploidy of a crop can have several advantages. By simply increasing
10 the copy number of the genome (from diploid to triploid, tetraploid or higher ploidy) the nucleus of the cell is enlarged proportionally. As a consequence cell size increases, which in turn increases tissue and organ size. The later often being harvestable products. Induced polyploidy to increase crop yields began in the 1930s and was successful in producing new varieties in vegetable crops such as Swedes (rutabaga). Odd ploidy levels such as triploids are
15 meiotically unbalanced and often result in sterile, seedless fruits, as in banana. Sterility of fruits can lead to greater vegetative growth, as resources that would normally be used in seed development are diverted to vegetative tissues. In China, triploids in rubber have been reported to have increased latex yields by about 20% compared to a standard (FAO, 1995) and are therefore of commercial interest.

20

Summary of invention

In accordance with a first aspect of the invention, a method is provided for obtaining rubber plants of particular ploidy and genotype useful for seed production, multiplication and crop improvement, the method comprising:

25 (a) providing a population of rubber plants;

(b) choosing from the population a subset of individual plants with atypical phenotype;

(c) assessing the ploidy level of plants in the subset;

(d) classifying plants in the subset as haploid, diploid or polyploid according to the results of step (c)

The invention may also comprise the additional steps of:

(e) assessing the homozygosity of diploid plants in the subset;

5 (f) discarding from the subset those diploid plants found to be heterozygous;

(g) classifying the remaining diploid plants as doubled haploids.

Preferably the step (c) assessing the ploidy level of atypical plants will apply flow cytometry to assess ploidy levels in cells extracted from root, shoot, leaf or other plant tissue.

10 Preferably, the step (e) of assessing the homozygosity of chosen plants uses molecular, biochemical or phenotypic markers. Phenotypic markers that correlate with homozygosity may be for example, height, pigmentation, leaf size and shape, stem thickness, grassy leaves, leaflet internode length and number and rate of leaf production. It is convenient to assess homozygosity using multiple molecular markers. A preferred technique is to use multiple 15 molecular markers, for example between 2 and 40, which may be microsatellite markers (also known as simple sequence repeats, SSRs): or Sequenced Characterised Polymorphic Regions (SCARs) markers or Single Nucleotide Polymorphism (SNP) markers. It is convenient to employ co-dominant molecular markers, particularly microsatellite markers, although many other marker systems could also be applied, for example, protein profiling, isozymes, High 20 Resolution Melt analysis or pyrosequencing.

It is preferred to carry out stage (b) of the method upon germinating seed or seedlings.

The order in which the various possible stages of the method are carried out is not necessarily 25 fixed. For example, step (e) (assessing homozygosity) may be carried out before step (c) (assessing ploidy levels). In this case, all plants in the set will be screened for homozygosity, not just the diploids: however, since the vast majority of plants in the set are diploid, this makes little difference in practice.

Preferably, the atypical phenotype is an atypical morphology or growth pattern that can be detected in the seed, or during germination of seeds or seedling stages. More preferably, the atypical morphology is one or more of: radicle growth; radicle:plumule length ratio; radicle:plumule angle; colour of radical, plumule or leaf; seed shape or size during germination; radicle width:length ratio; plant height, stem morphology, petiole morphology, venation, distance between leaf whorls and the number of leaves per whorl. The atypical phenotype of a germinated seed may also be the germination of two or more embryos from a single seed.

Definitions

Following are definitions of words used in the specification and claims:

"plant" means rubber seed and any growing rubber plants at any stage of development, for example germinated seeds, seedlings, nursery and mature plants, including plants *in vitro* culture.

"haploid" means any plant cell containing a single set of chromosomes (x=18), or any tissue or plant comprising such cells.

"diploid" means any plant cell containing two sets of chromosomes (2x=36), or any tissue or plant comprising such cells

"triploid" means any plant cell containing three sets of chromosomes (3x=54), or any tissue or plant comprising such cells.

"tetraploid" means any plant cell containing four sets of chromosomes (4x=78), or any tissue or plant comprising such cells.

"polyploid" means any plant cell containing multiple sets of chromosomes, or any tissue or plant comprising such cells.

"mixaploid" means a plant containing cells of two or more different ploidies;

"heterozygous" characterises any cell containing two or more sets of chromosomes that are not all identical sets; or any tissue or plant composed of such cells. Material which is not heterozygous is either homozygous or haploid (containing only one set of chromosomes).

"plantlet" means any small plant which is not fully grown.

One convenient application of the invention is to select candidate haploid seedlings from germinated seed after the plumule and radical have developed and once the first whorl of leaves has developed. Germination usually commences about 10 days from sowing. Cohorts of germinating rubber seedlings typically exhibit a fairly synchronous developmental pathway and reasonably homogenous phenotype (see Figure 1, seedling beds). Abnormal germinated seed may deviate from the characteristic phenotype in one of many ways (Figure 2 and Figure 3 show examples of altered seedling and leaf morphology for haploid, diploid, triploid and tetraploid plants) which may include diverse atypical features of morphology or growth pattern. The atypical morphology or growth pattern may be reduced plant, organ and tissue growth and size, or germination of two embryos from a single seed (multiple seedlings). The atypical morphology or growth pattern may be one or more of atypical: radicle growth; radicle: plumule length ratio; radicle:plumule angle; colour of radicle, plumule or leaf; seed shape or size during germination; altered radicle width:length ratio; altered plant height; stem or petiole morphology; venation; distance between leaf whorls; number of leaves per whorl.

Another way of carrying out the invention is to select among a population of nursery or field planted plants. In this method, the atypical morphology or growth pattern used as the basis of selection is preferably one or more of: slower vegetative growth, reduced ratio of leaflet width to length, altered inter-whorl distance, angle of leaf to plant axis, leaf colour, branching pattern and precocious flowering.

A preferred process is one in which the atypical phenotype by which plants are selected is chosen from atypical phenotypes shown from previous tests to correlate with haploid, or doubled haploid, mixaploid, triploid, tetraploid or higher ploidy characters. This process progressively improves accuracy of the phenotypic screen as increasing numbers of off-types are observed, as traits are correlated with ploidy type and uninformative traits are discarded from consideration.

Preferably, the step of further assessing the homozygosity of the chosen plants uses multiple molecular markers. More preferably, this step is performed on a pooled sample of markers. A

chosen plant is identified as highly homozygous if it is homozygous for each molecular marker used.

Preferably, the population of plants comprises at least 500 particularly 750 - 2,500 individual plants. By "providing germinated seeds or seedlings", we refer to any process whereby seeds sprout and seedlings begin to grow. In the case of rubber, this includes any germination technique used by commercial and plant breeding seed production units. Seed tested may include seeds selected using the bounce test (Setyamidjaja, 1993) as well as those that don't bounce and would normally be rejected by commercial breeders and growers. Seeds are normally placed in a sand bed for germination.

Flow cytometry is used for assessing the genome content of plant or animal cells, and can be used to distinguish between haploid, diploid, triploid, tetraploids and polyploids as well as mixaploid material. By "flow cytometry" is meant methods for counting, examining, quantifying and sorting analyte suspended in a stream of fluid. It permits simultaneous analysis of two or more characteristics of single cells flowing through an optical or electronic detection apparatus. In this invention, flow cytometry may be applied to individuals exhibiting an abnormal phenotype. In the first instance flow cytometry will be used to divide the population into haploids, diploids, other higher ploidies and mixaploids (for example individuals having both n and 2n cells). Mixaploids may represent plants that are undergoing chromosome doubling, i.e. they are developing spontaneously into double haploids. This can be monitored by repeating the ploidy analysis at a later stage. Mixaploid plants may also indicate the presence of doubled haploids in a progeny, the progeny can then be screened intensively using molecular markers to select the doubled haploids.

In accordance with a further aspect of the invention, there is provided a method for producing a homozygous doubled haploid rubber plant, the method comprising isolating a haploid plant and obtaining a doubled haploid plant therefrom. The method may include:

(a) selecting a haploid plant using a method according to the first aspect of the invention;

(b) obtaining a doubled haploid plant by doubling the chromosome number by application of an external agent to the haploid plant or clones derived from it; or by application of an external agent to a cell or cells isolated from the haploid or clones derived from it *in vivo* or *in vitro*, followed by regeneration of a plant using tissue culture, methods of regenerating
5 rubber plants via tissue culture are known, from, for example, Clement-Demange *et al* (2007) Plant Breeding reviews, Vol 29, Ch 4, pages 177-283. Plants may also be obtained by application of an external agent to vegetative and generative meristems of haploid plants or clones derived from them; or by application of an external agent to floral tissues of the haploid or clones derived from it; or by selfing the haploid plant by exploiting the occasional
10 spontaneously doubled chromosome number in male and female reproductive cells; or through any other spontaneous doubling event.

In accordance with a further aspect of the invention, there is provided a method for producing a diploid F_1 hybrid rubber plant which comprises crossing two distinct doubled haploid
15 rubber plants obtainable by the method of the invention; and F_1 hybrids so produced.

We are unaware of any prior publication aiming to select doubled haploids, absolutely homozygous plants among sexual offspring. In rubber, no doubled haploid lines and no F_1 hybrids have hitherto been produced. Instead the rubber industry for the last thirty years has
20 been based on the production of grafted clones.

Origin of materials used

The rubber germplasm (*Hevea brasiliensis* Muell. Arg.) used in the following experiments was obtained in Indonesia where the initial stages of the procedure (seed collections and
25 selection of material of atypical phenotype) were carried out. The historic origin of the rubber tree is understood to be the Amazon rainforest, where it still grows wild: it is believed that the species was introduced from Brazil via Kew Gardens (England) to South-East Asia in the last half of the nineteenth century, since when it has been widely cultivated throughout that region.

30

Examples

Specific examples of the invention are now described with reference to the accompanying figures.

5 Brief Description of Figures

In the figures:

Figure 1 is a photograph of a seedling bed of rubber in which off-types may be observed;

Figure 2 is a photograph of selected abnormal seedlings after germination: A: triploid; B: haploid; C: tetraploid; D: diploid;

10 Figure 3 is a photograph of leaves of some abnormal seedlings: A: triploid; B: haploid; C: tetraploid; D: diploid;

Figures 4A - 4F show flow cytometry output histograms: of diploid (Figure 4A-C) and haploid (Figure 4D-F) samples, respectively;

Figure 5 is a photograph of a rubber inflorescence carrying male and female flowers;

15 Figure 6 is a photograph of a rubber inflorescence carrying male flowers at anthesis (pollen shedding);

Figure 7 is a photograph of the removal of a staminal column containing dehiscing anthers from a male flower;

Figure 8 is a photograph of insertion of the excised stamina column into a female flower, thus
20 making contact with the stigma and effecting pollination;

Figure 9 is a photograph of artificially pollinated female flowers, covered with cotton wool to prevent uncontrolled pollinations;

Figure 10 is a photograph of the labelling of crosses;

Figure 11 is a photograph of crossed flowers bagged for protection.

Detailed Description of the Preferred Embodiments

1) Seedling screening

5 A total of 44,742 rubber seed were collected and sown directly into outside sand beds from the period September 2007 – January 2008, i.e. during the seed fall season of North Sumatra. In commercial usage, the "bounce test" would typically be applied to such seed, to remove seed less likely to germinate, but the test was not used on these seeds. Seed were planted so that ⅔ of each seed was embedded in the sand, at a density of approximately 1,200 seeds per 10 square metre. The exposed parts were covered with sacking until seedling emergence had commenced. The seedbeds were shaded (Fig. 1). Seeds were watered daily. Germination began 7-10 days from sowing and continued for two to three months.

2) Morphological screen

15 Of the 44,742 seed, 36,604 germinated: this high percentage of ungerminated seed (18%) is quite typical of seed sown without a pre-sowing 'bounce test' .Seedlings were examined for off-types of which 916 were detected. Examples of off-type seedlings and leaves are shown in Figures 2 and 3. Off-type seedlings were removed from the seedbed and potted into individual pots.

20

3) Assessment of nuclear genome content by flow cytometry

Sample preparation

The cell nuclei were prepared from fresh plant material (leaves or roots), by chopping the 25 tissue (about 0.5 cm square leaf tissue or about 1 cm length of root of about 20-25 mg) with a clean, sharp razor blade in 0.5 ml buffer, or more depending in amount of tissue chopped, in a

plastic Petri dish. The DNA buffer is based on Arumuganathan and Earle (1991) and consisted of:

5 mM Hepes

10 mM Magnesium sulphate heptahydrate

5 50 mM Potassium chloride

0.2% Triton X-100

2% DTT (Dithiothreitol)

2 mg/litre DAPI

at pH 8

10

DAPI, a fluorescent dye that selectively binds to form a complex with double stranded DNA and gives a product that fluoresces at 465 nm wavelength, was introduced to the solution. DAPI has specific DNA-binding properties, with preference for adenine-thymine (AT)-rich sequences. After chopping, the buffer (ca. 2 ml), containing cell constituents and larger tissue
15 remnant, was passed through a filter of 40 μm mesh. This method produces thousands of nuclei from a leaf piece of 0.5 cm square.

A solution containing stained nuclei was passed through the flow cytometer. A standard normal diploid rubber sample was used as a reference.

20

The fluorescence of the stained nuclei, passing through the focus of a light beam from a high-pressure mercury lamp was measured by a photomultiplier and converted into voltage pulses. These voltage pulses were processed electronically to give integral and peak signals for processing by a computer. By running the samples with the appropriate filter settings for
25 excitation and emission, quantitative DNA histograms were produced.

Material

Flow cytometer: CyFlow ML (Partec GmbH, Otto Hahnstrasse 32, D-4400 Münster, Germany) with a high-pressure mercury lamp, OSRAM HBO 100 long life. Objective: 40 x N.A. 0.8air (Partec)

5 Filter combination with DAPI:

Heat protection filter KG-1

Excitation filters: UG-1 and BG-38

Dichroic mirrors: TK 420 abd TK 560

Emission filter: GG 435

10 Software: Flomax version 2.4 d (Partec)

Results

Off-types were analysed for ploidy level using flow cytometry. Figures 4A and 4B show histograms obtained from flow cytometry for diploid and haploid samples, respectively. Note
15 that the comparative DNA content is indicated by the x-axis, the haploid (about 100) having half the DNA of the diploid (about 200). From the 916 off-types selected and analysed over a period from 28 November 2007 to 2 July 2008, one haploid, two triploids and one tetraploid were detected (see Table 1 below).

Table 1

No	Batch No	Clone	Result	
			Ploidy	Date
1	R1070002	Unknown	2x	02 Jul 07
2	R1070007	Unknown	2x	02 Jul 07
...	...	...	...	...
90	R3070015	RRIC100	2x	25 Jan 08
91	R3070209	RRIC100	2x	25 Jan 08
92	R3070016	RRIC100	4x	25 Jan 08
93	R3070210	RRIC100	2x	25 Jan 08
94	R1070004	Unknown	2x	25 Jan 08
...	...	...	...	...
156	R3070030	RRIC100	2x	14 Feb 08
157	R3070031	RRIC100	2x	14 Feb 08
158	R3070032	RRIC100	3x	14 Feb 08
159	R3070033	GT1	2x	14 Feb 08
160	R3070034	RRIC100	2x	14 Feb 08
...	...	...	...	...
400	R2070167	PB260	2x	20 Mar 08
401	R2070168	PB260	2x	20 Mar 08
402	R1080001	PB260	x	20 Mar 08
403	R1080002	PB260	2x	20 Mar 08
404	R1080003	PB260	2x	20 Mar 08
...	...	...	...	...
699	R3070206	RRIC100	2x	24 May 08
700	R3070207	RRIC100	2x	24 May 08
701	R1070054	PB260	3x	24 May 08
702	R2070177	PB260	2x	24 May 08
703	R2070178	PB260	2x	24 May 08
...	...	...	...	...
915	R1080414	PB260	2x	02 Jul 08
916	R1080415	PB260	2x	02 Jul 08

Most negative results (plants found to be diploid) are omitted from the Table above. Positive results (Nos 92, 158, 402 and 701) are highlighted.

5 4) Genome characterization

Genome characterisation is used in two ways: firstly to identify homozygous diploids among off-types confirmed by flow cytometry to be diploid and secondly, if so desired, as a prescreen for homozygous off-types before flow cytometry.

10 *Marker strategy.*

The protocol applied to perform a molecular pre-screen of seedlings showing abnormal phenotypes to discard heterozygotes comprises the following stages.

1. *DNA extraction*

Around 50 mg of the fresh leaves (approximately 2 leaves) was removed from the seedling and used to extract DNA using the Qiagen 96 DNeasy extraction kit according to the manufacturer's instructions as described below, although other systems for DNA extraction could also be used.

A. PREPARATION

1.For new kits, add 100% ethanol to AP3/E buffer and AW buffer
2.Set water bath to 65°C
3.Pre-heat AE and AP1 buffer to 65°C
4.If AP1 buffer has a cloudy appearance, heat to 65°C and shake until the solution becomes clear

B. PROTOCOL

1.Add 50 mg plant material into each tube in two collection microtube racks. Retain the clear cover.
2.Add one tungsten carbide bead into each microtube.
3.Prepare the lysis solution: (400 μl AP1+ 1 μl RNAse + 1 μl Reagent DX)/reaction plus 15% of each component.
4.Disrupt the sample using MM 300, 30 Hz for 1.5 minutes.
5.Pulse centrifuge to 3000 rpm.
6.Remove and discard caps, add 130 μl AP2 buffer into each collection microtube.
7.Close the microtubes with new caps. Place a clear cover (from step 1) over the 96 well plate. Shake the plate vigorously for 15 sec. Pulse centrifuge to 3000 rpm.
8.Incubate the racks for 10 minutes at -20°C.
9.Remove and discard the caps. Transfer 400 μl of each supernatant to new plate of collection microtubes (provided). Do not transfer pellet and floating particles. Hold the strips and use the lowest pipette speed. Recover the tungsten beads.

10. Add 1.5 volume (typically 600 μl) of AP3/E buffer.
11. Close the microtubes with new caps and mix vigorously.
12. Pulse centrifuge (3,000 rpm) to collect solution).
13. Place 96 well plates on top of S-Blocks provided.
5 14. Transfer 1ml of sample into each well of the 96 well plate.
15. Seal with Airpore Tape sheet and centrifuge for 4 min at 6,000 rpm.
16. Add 800 μl of Buffer AW to each sample.
17. Centrifuge for 15 min at 6,000 rpm.
18. Add 100 μl of buffer AE to each sample and seal with new AirPore sheets.
10 19. Incubate for 1 min at room temperature (15-25°C).
20. Centrifuge for 2 min at 6,000 rpm.

2. Amplification of microsatellite markers by PCR

15 Primers

The following microsatellite markers have been used:

SSR Name	Forward Primer	Reverse Primer
M 197	ACAAGAGATGCGAGAAGAAATACCC	GTTTTATTTTCATTCAGCTGTTATG
M 622	TAAGAAACACCAGTCATCAAAAATT	GTACAGTATGGCGATTTGAAACCTG
M 616	ATGCATATGGAAGTGAGAACAAAAA	GATTGCTCTGTCTTCTCTTGCGATT
M 613	CTGCATAGGATGTGAACAAGTAGGC	GATTTTTGACCTATCTGCCGTTTCT
M 574	TGTGTCCTCTACTTGTCTTCATTTG	ATAAAGGAGAAAGAAAAGTAGAGGC
M 508	GCTAACCCTCTCTTCATTGATA	CTGTCTGAGAAAAGGCGAATCT
M 340	ATCTGCATAATAGTTGTAACCACA	CCAGAACTTCACTATGATGAGAGCT
M 127	GGACCAAAGGAATGTCAAAGC	GTTTAATGGCCTTACCTCACG
M 124	TCATTTCAAGTTCACCGTGCTTATT	GAGACATAAGGCAAATACATGCGCT

Markers were selected from Lespinasse et al. (2000) from which additional markers may be chosen. Examples of these are set out below.

Microsatellite	Sequence	
	Forward	Reverse
M382	TTTCCAATAAGATTGATTCC	
M379	GGTTATCAAAGAGAAGATGCCAAGA	GCAATATCTGATTCCAGCATTTGGA
M291	TTTGGCATTGTTGATGTTGA	TCCTGAAACAGCATATTTGG
M249	TTTGCAGTGTATGCGTTTGGAAGTTC	ATAGCACTGAAAAAGAAAACTGCAG
M72	CGAGCACCAAAATCCCACCA	TGGAAAAACCAGAGGTGAGGA
DNA gHbCIR692	GGATGGTTTAACTGCTTGAAGAGAG	GACATCTTAGCGAGCAAAACAGACA
DNA gHbCIR425	AGTCTAGCAATGTATGGTTG TTTCC	CAAACCAAGTGATAAAGAGGAAGAT
DNA gHbCIR412/493	CATTAGTTGGCTGCTCTTTCATTTC	GTGGTAGATGGAACATAAGATAAGT
DNA gHbCIR273	GCATCACATTTTATTGGCATCATTT	ATAGGTGATGGTTAGGATAGTGACA
DNA gHbCIR256	TTTTCCTGATAAGGGCTGCTGGTTT	ACTATTGAATCAGGAAGCGGCAGCT

Reaction Mixtures

In all cases, 10 ml of PCR reaction mixture contained the following reagents; 1.0 ml of 10x PCR buffer (Bioline), 0.3 ml $MgCl_2$ (10 mM), 0.4 ml dNTPs (10 mM of each), 0.2 ml of each
5 primer pair (10 mM), 1-5 ng of DNA (extracted as above) and 1 U of *Taq* DNA polymerase (5 U ml-1 Bioline).

PCR conditions

The following conditions were used for the Polymerase Chain Reaction for all microsatellite
10 markers: an initial 94°C denaturing step for 2 min followed by 34 cycles of: 94°C for 30 sec, 52°C for 30 sec and 72°C for 45 sec, with a final extension step of 72°C for 7 min.

3. *Separation of PCR products by agarose gel electrophoresis or other method*

Agarose gel electrophoresis and ethidium bromide staining are used to fractionate and visualise products generated by microsatellite PCR.

(1) Reagents

5 TBE running buffer : 0.089 M Tris base, 0.089 M boric acid,(pH 8.3) and 2 mM Na2EDTA

Loading buffer: 0.23% (w/v) bromophenol blue 60 mM EDTA 40% (w/v) sucrose

Ethidium bromide stain: 1% (w/v) ethidium bromide

Ladder 100 bp (Gibco Life Science BRL)

10 (2) Gel preparation and loading

2 % (w/v) agarose (prepared in 1 x TBE buffer and subjected to heating in a microwave (700W) for 2 x 1 min at full power to create a gel solution. The gel solution is cooled to approximately 55°C prior to the addition of ethidium bromide (3.5 μl per 100 ml gel). The ends of a suitable gel tray rig (midi-gel tray for 100 ml gels, maxi-gel tray for 250 ml gels)
15 are sealed with masking tape and an appropriate number and type of combs placed in position. Combs with 16 x 20 μl wells are most often employed. The gel solution is carefully poured into the prepared tray and allowed to cool for at least 20 min. Combs and tape are then removed and the gel tray submerged into a tank 10 containing 1 x TBE buffer.

20 Generally, 5 μl of sample are mixed with 2 μl of bromophenol blue buffer prior to loading. The loading buffer serves two functions: first, it increases the specific gravity of the sample thereby preventing diffusion of DNA from the top of the well into the surrounding buffer, and second, it indicates the progress of product as they migrate through the gel by electrophoresis (the blue dye migrates at approximately the same position as DNA fragments 200 bp in
25 length). To estimate the size of the amplicons, 4μl of 100 bp Gibco's ladder (Gibco Life Science BRL) are loaded together with the analysed samples.

Electrophoresis of mid-gels (100 ml) is performed at 75 Volts in 1X TBE buffer for approximately 2 h. Following electrophoresis, gels are removed from the rig and post-stained in 5 mg/l aqueous ethidium bromide solution for 30 min, destained in distilled water for 20 min and then viewed under Ultra Violet Illumination using a UVP Bio-Doc-system. Images
5 of the gels are captured by the UVP Bio-Doc system as Polaroid format and used for scoring.

4. Scoring of results to discard individuals with one or more heterozygous loci

PCR products generated by each microsatellite-genotype combination are evaluated for the presence of one or two distinct bands after fractionation by agarose gel electrophoresis
10 (stages 1-3 above). Any genotype that yields two products (two bands) for any of the microsatellite loci is deemed to be heterozygous and so discarded as a possible doubled haploid plant.

To asses the levels of homozygosity, DNA from 130 abnormal seedlings has been screened
15 using microsatellite (SSR) markers: M124 and M574.PCR products produced using these primers were subjected to capillary electrophoresis, and outputs analysed using gene mapper software. From this initial screen 69 abnormal seedlings were identified as homozygous for the two markers.

20 Generating doubled haploids from haploids

Haploid cells will sometimes undergo "spontaneous doubling" whereby failure of complete mitosis gives rise to a doubling of the chromosomes. If this occurs early in development, the seed, plantlet and plant derived is a doubled haploid. If no such doubling occurs then a haploid is obtained and in most circumstances such haploid plants are intrinsically infertile, in
25 that the process of meiosis is unable to generate gametes capable of fertilisation. In order to produce a fertile plant from which sexual progeny can be produced it is necessary either to double the chromosome number of a haploid by application of an external agent, or to rely on the process by which a haploid cell can spontaneously double; this is often a rare event though in some species, e.g. barley, spontaneous chromosome doubling is of the order of 60-

90% and precludes the necessity of artificial chromosome doubling. Induced chromosome doubling of haploids is usually adopted, and usually involves the application of a chemical agent capable of inhibiting mitosis and thereby inducing the formation of a diploid cell. There are several chemicals known to induce such a chromosome doubling process and of these
5 colchicine is the best known, and most commonly utilised. Other similar agents include microtubule inhibitors such as the herbicides trifluralin, and oryzalin. Such chemicals can either be applied to a whole plant and fertile seeds may be produced on that plant, or they can be applied *in vitro* to isolated cells from which an intact plant can be regenerated using conventional tissue culture techniques. For a full description of available chemical and other
10 methods and their means of application see Kasha (2005). An alternative to the external application of chemicals is the exploitation of spontaneous doubling. For example, in a haploid, the nucleus of an individual cell may occasionally fail to divide normally at mitosis and thus form a diploid cell that ultimately gives rise either to a diploid sector(s) that may encompass most or all of the main shoot axis or (if it occurs in the first embryonic division) a
15 doubled haploid plant. In either case, the selfed seed secured from such individuals will be completely homozygous and genetically identical to the parent. This process can occur during the formation of reproductive cells and in this case it is possible that fertile gametes (pollen or egg cells) may be produced. When both male and female gametes form on the same plant then successful fusion of gametes can take place and an embryo will develop. Such an
20 embryo will be a homozygous diploid, and will breed true in all future selfed generations; all its selfed progeny will be genetically identical. In some cases seedlings are found to be mixaploid (carrying cells of different ploidy level) and may develop into one ploidy class. Thus a seedling carrying n and 2n cells may develop to be completely homozygous diploid. As such they provide another route of obtaining doubled haploids that can be detected by
25 monitoring their ploidy development using flow cytometry.

Examples of methods for chromosome doubling of haploids

The examples given below use colchicine as the doubling agent, but other chemicals that induce chromosome doubling may also be used. Various plants, plantlets and plant parts *in*
30 *situ* or *in vitro* are treated with colchicine. The stock colchicine solution is prepared in a fume hood to avoid inhalation of the colchicine powder. The stock solution contains 1 g colchicine

in 1 litre of water to which 20 ml DMSO (Dimethyl sulphoxide), 1 ml of a 10 ppm solution of GA3 (Gibberelin A3) and 10 drops of Tween 20 (surfactant) may also be added. The stock solution is diluted to give working solutions of colchicine between 0.2 and 12 ppm. The concentration used is inversely related to the length of exposure. Colchicine is toxic and is handled in a designated area using protective clothing. Typically, treatments involve colchicine concentrations in the range of 2.5 – 12 ppm for 2 – 10 hours.

1.Seedlings: Seedlings detected as being haploid are washed clean with water and the bare rooted plants placed into a colchicine solution so that their roots and crowns are fully immersed. Treatment is done in the light at ambient temperatures for 2-10 hours. After treatment seedlings are rinsed in water for 15 minutes, potted into soiled-filled pots and placed into nurseries or greenhouses where they receive shading and misting. Chromosome doubling is monitored by flow cytometry analysis of leaves or roots emerging after treatment. Once doubled haploidy is confirmed, established plants are removed to harden off.

2.In vitro micropropagation of rubber is well established (Mendanha et al. 1998) and can be exploited to clonally propagate haploid plants. The clonal material can then be used to obtain doubled haploids from each original haploid genotype. Rooted micropropagated plantlets can be treated with colchicine using the *in vivo* method described for seedlings above. Alternatively, treatment with colchicine may be carried out *in vitro*. *In vitro* methods involve either 1) placing a clonal haploid plantlet into a sterile culture medium containing colchicine for a suitable period, or 2) treating a shoot meristem in a solution of colchicine and growing on the treated meristem *in vitro*.

3. Floral organs: Floral buds developing on haploid plants can be treated with colchicine to induce doubling and restore fertility to male and female gametic cells. Self pollination of such flowers will yield doubled haploids.

Producing F_1 hybrids

The F_1 hybrid is produced by crossing 2 different homozygous doubled haploid rubber plants. The inflorescence of rubber contains separate male and female flowers (see Fig. 5). Crossing is done artificially by following the following procedure:

5 1.Female flowers are selected from an inflorescence a day before pollination takes place (Fig. 5)

2.Male flower selection is conducted on the day of pollination. Male flowers are selected that exhibit ongoing anthesis (Fig. 6). Cross pollination is done by excising the staminal column from a dehiscing male flower (Fig. 7) and plugging it into the female flower,
10 making contact with the stigma (Fig. 8).

3.Pollinated female flowers are then covered with cotton wool to protect from uncontrolled cross pollination, chiefly by insects (Fig. 9). The flower is then labelled at the base of the inflorescence (Fig. 10). Data are recorded for: cross number or code,
15 female and male parent, pollination and harvest dates, pollinator's identity. The inflorescence is then bagged (Fig. 11): the bag ensures that mature seeds that fall out of ripe fruits are collected.

4.The F_1 seed are then sown. They may be cultivated and tapped for the production of latex, in conventional manner.

20

The following references are incorporated by reference:

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CLAIMS

1. A method for obtaining rubber plants of particular ploidy and genotype useful for seed production, multiplication and crop improvement, the method comprising:

5 (a) providing a population of rubber plants;

(b) choosing from the population a subset of individual plants with atypical phenotype;

(c) assessing the DNA content of plants in the subset;

(d) classifying plants in the subset as haploid, diploid or polyploid according to the

10 results of step (c).

2. A method according to claim 1 which further comprises:

(e) assessing the homozygosity of diploid plants in the subset;

(f) discarding from the subset those diploid plants found to be heterozygous;

15 (g) classifying the remaining diploid plants as doubled haploids.

3. The method according to claim 2 wherein the homozygosity screen step (e) uses molecular or biochemical markers.

20 4. The method according to claim 2 or 3, wherein the homozygosity screening step (e) uses multiple co-dominant molecular markers, for example microsatellite markers or Sequenced Characterised Polymorphic Regions (SCARs) markers or Single Nucleotide Polymorphism (SNP) markers.

25 5. The method according to any of claims 1 to 4, wherein the atypical phenotype is an atypical morphology or growth pattern.

6. The method according to claim 5 wherein the atypical morphology or growth pattern is one or more of atypical: radicle growth; radicle:plumule length ratio;

30 radicle:plumule angle; colour of radicle, plumule or leaf; seed shape or size during germination; radicle width:length ratio; plant height; stem or petiole morphology; venation; distance between leaf whorls; number of leaves per whorl.

7 The method according to claim 5 in which the population of plants comprises nursery or field planted plants wherein the atypical morphology or growth pattern is one or more of slower vegetative growth, reduced ratio of leaflet width to length, altered inter-whorl distance, angle of leaf to plant axis, leaf colour, branching pattern and precocious
5 flowering.

8. The method according to claim 5 wherein the atypical phenotype is germination of two or more embryos from a single seed.

10 9. The method according to any of claims 1 to 6 in which the atypical phenotype by which plants are selected is chosen from atypical phenotypes shown from previous tests to correlate with haploid, doubled haploid or polyploid character.

10. The method according to claim 3 wherein the assessment of homozygosity uses
15 between 2 and 40 microsatellite markers.

11 The method according to any of claims 1 to 10 in which the plants are germinated seeds or seedlings.

20 12. The method according to any of claims 2 and 10 wherein the step of assessing the homozygosity of the chosen plants using multiple molecular markers uses pooled samples.

13. The method of any of claims 2, 3, 10 and 11 wherein a diploid is classified as
25 heterozygous unless it shows only one allele per locus for each molecular marker used.

14. The method according to any of the preceding claims wherein the population comprises at least 500 plants.

30 15. The method of claim 14 wherein the population comprises between 750 and 5,000 plants.

16 A method according to any of claims 1 to 15 in which one or more plants classified as haploids, doubled haploids or polyploids are subsequently used in breeding, multiplication or seed production.

5 17. Progeny plants from somatic or reproductive cells of a haploid, doubled haploid or polyploid rubber plant obtained by a method according to any of claims 1 to 15.

18. Clones, pollen or ovules of a haploid, doubled haploid or polyploid rubber plant obtained by a method according to any of claims 1 to 15 or of a plant according to
10 claim 17.

19. A method for producing a homozygous doubled haploid rubber plant, the method comprising:

(a) isolating a haploid plant using a method according to any of claims 1 to 15;

15 (b) obtaining a doubled haploid plant therefrom.

20. A method according to claim 19 in which the doubled haploid is obtained through spontaneous chromosome doubling; or by doubling the chromosome number by application of an external stimulus to the haploid plant *in vivo* or *in vitro*; or by
20 application of an external stimulus to a cell or cells isolated from the haploid plant, followed by regeneration of a plant using tissue culture; or by selfing, cloning or pollinating the haploid plant.

21. A method according to claim 16 which comprises crossing two distinct doubled
25 haploids obtainable by the methods of claim 2 or claim 19 to give an F_1 hybrid.

22. A doubled haploid rubber plant.

23. An F_1 hybrid rubber plant

30

24. Latex from a plant claimed in claim 22 or 23.

25. A method of obtaining latex comprising tapping an F_1 hybrid rubber plant created from doubled haploid parents.

FIG. 1

FIG. 2

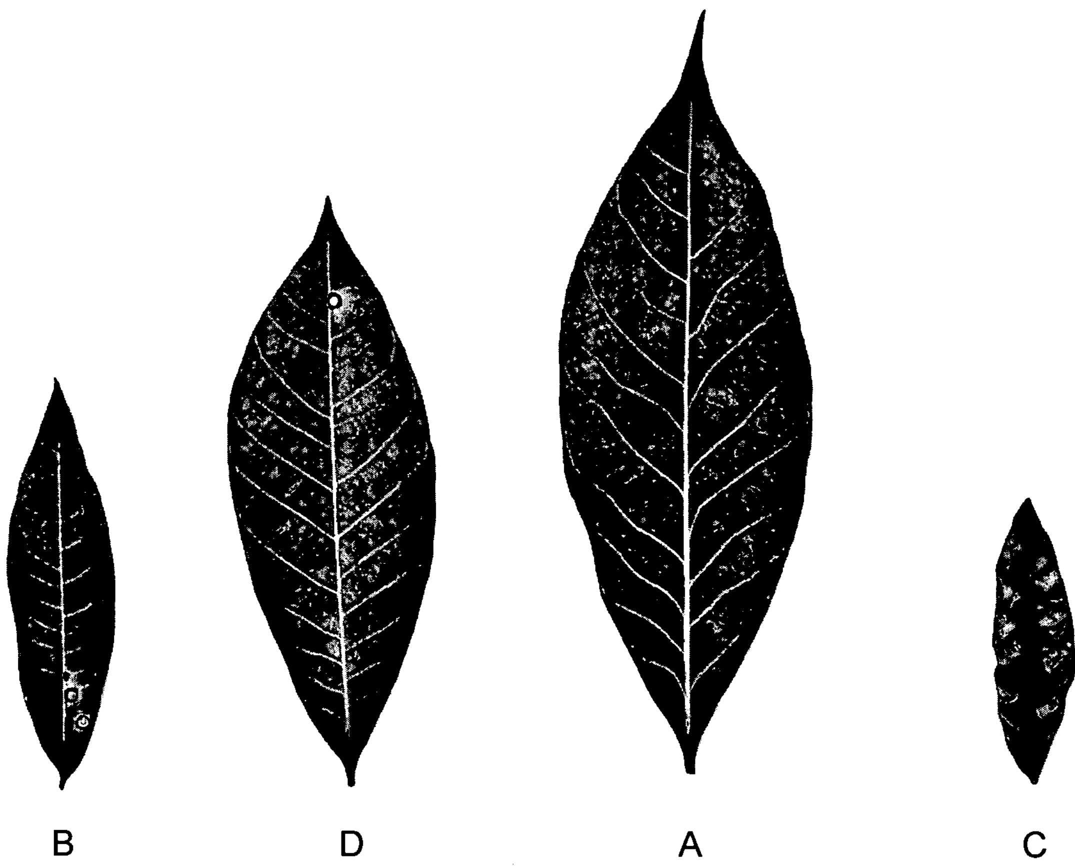

FIG. 3

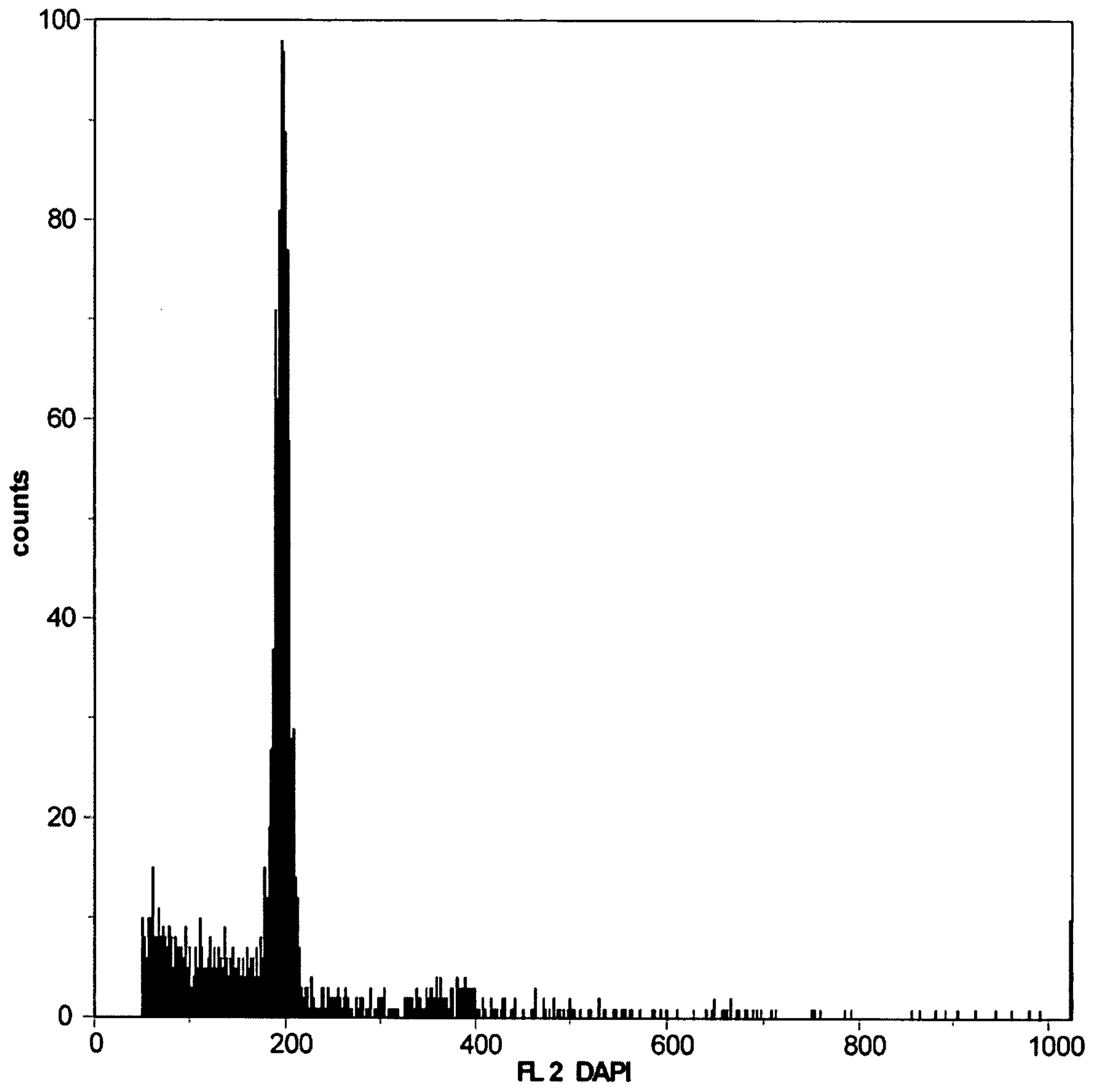

FIG. 4a

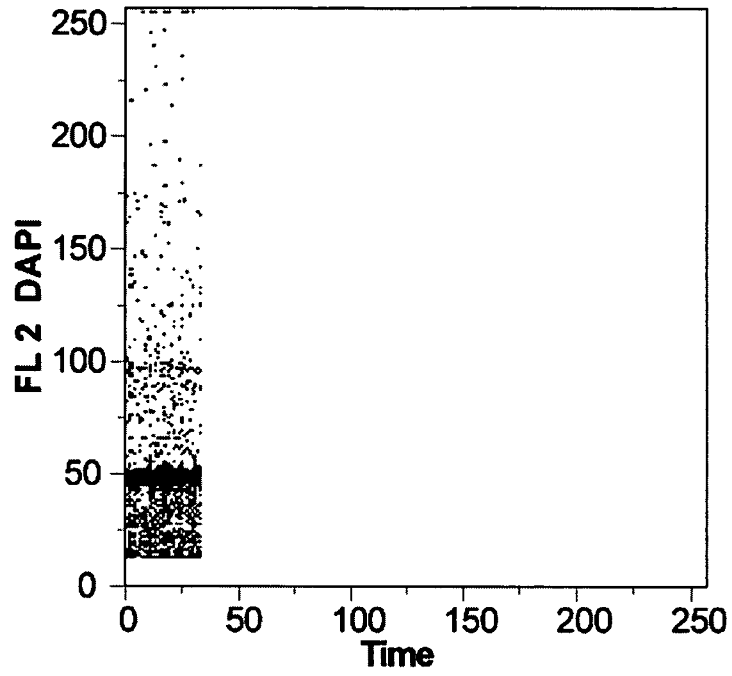

FIG. 4b

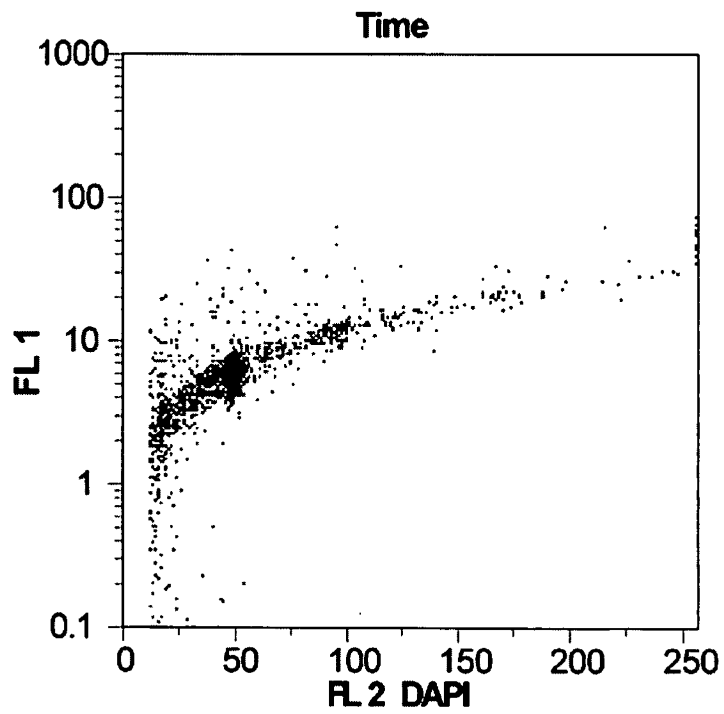

FIG. 4c

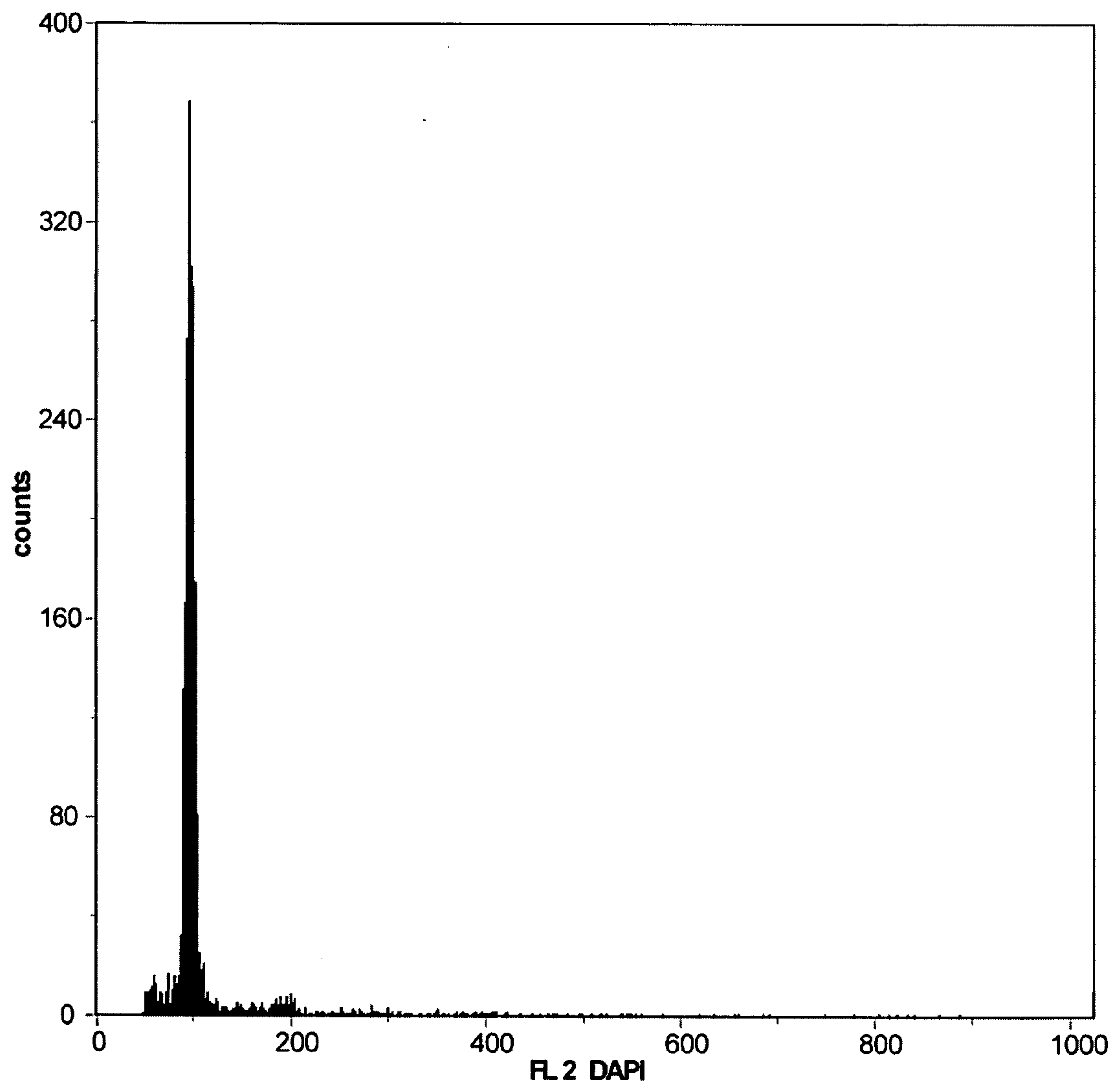

FIG. 4d

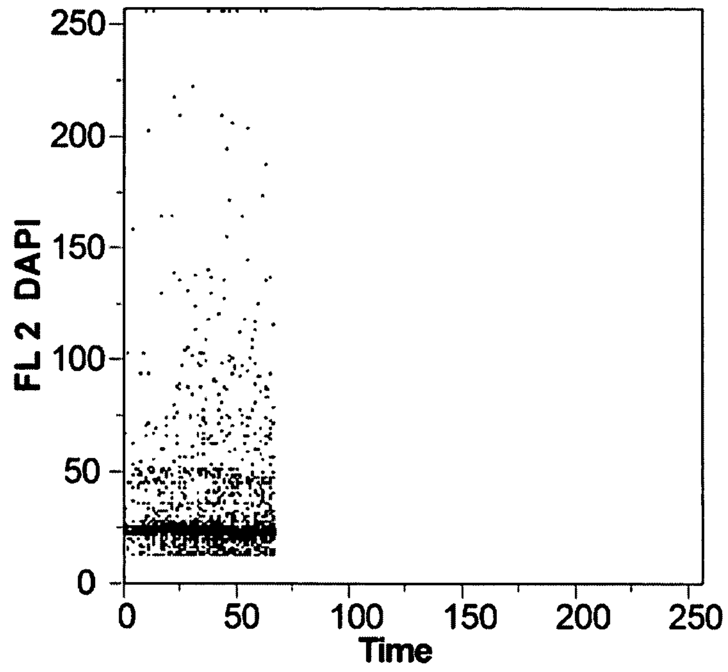

FIG. 4e

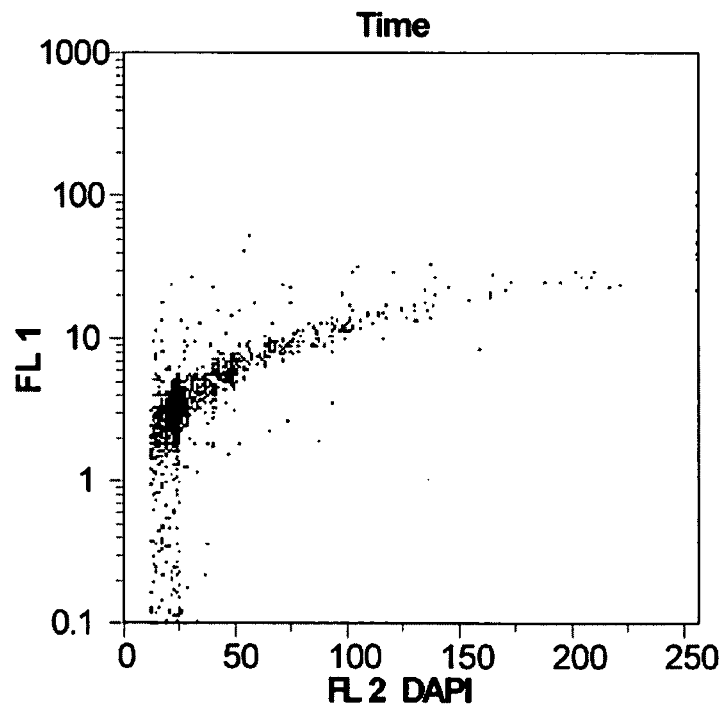

FIG. 4f

FIG. 5

FIG. 6

FIG. 7

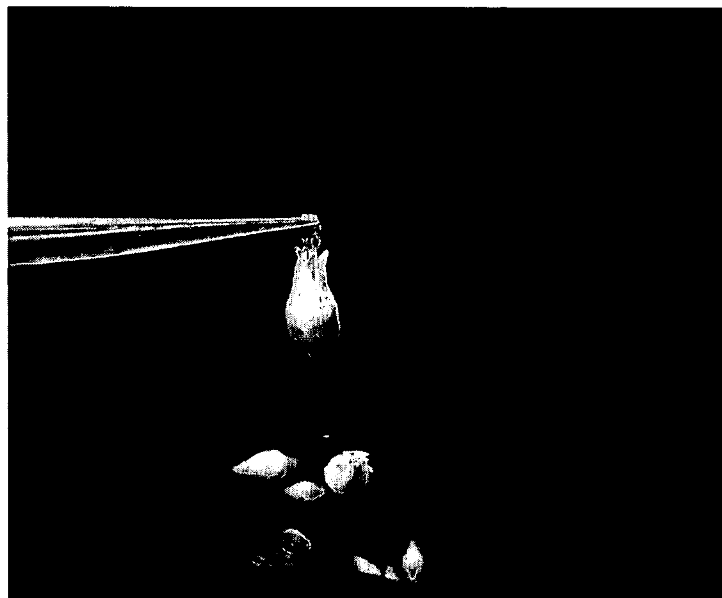

FIG. 8

FIG. 9

FIG. 10

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/003227

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01H5/00 A01H1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	C. ZHENGHUA: "III. 1. Rubber (Hevea brasiliensis) in vitro production of haploids in: Bajaj, Y.P.S., Palmer, C.E. : Biotechnology in Agriculture and Forestry 12; Haploids in crop improvement I" 1990, SPRINGER-VERLAG , XP008106750 page 215 - page 236 page 230, last paragraph page 234, last paragraph - page 235 ----- -/--	17,18, 22,24 1-7,9-25

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 May 2009	12/06/2009

Name and mailing address of the ISA/
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NL - 2280 HV Rijswijk
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Authorized officer
Holtorf, Sönke

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ASSANI, A., ET AL.: "Floriculture, Ornamental and Plant Biotechnology: v. 2: Advances and Topical Issues; Haploid production in trees, ornamental and floricultural plants, pages 360-375" June 2006 (2006-06), GLOBAL SCIENCE BOOKS, LTD , XP008106769 the whole document abstract	17,18,22
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Y	BOUVIER LYDIE ET AL: "Chromosome doubling of pear haploid plants and homozygosity assessment using isozyme and microsatellite markers" EUPHYTICA, KLUWER ACADEMIC PRESS, AMSTERDAM, NL, vol. 123, no. 2, 1 January 2002 (2002-01-01), pages 255-262, XP002446607 ISSN: 0014-2336 page 255; figure 1 page 261, paragraph 2	1-7,9-25
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