CLAIMS

1. A method for identifying a mutant plant of a predefined species carrying one or more mutation(s) in nucleotide(s) of interest [NOI(s)], in a predetermined target sequence, said method comprising the steps of:
    a) Providing Np regenerative parts of a parent plant of said species, wherein Np is an integer of at least 5,000, wherein Np is in the range of $0.7 * ONp$ to $1.3 * ONp$,
    wherein ONp $= \frac{\frac{OLS \times 100}{Hr}}{GECN}$ and wherein Hr is the expected average harvest rate of said plant species after mutagenesis in %; and
    OLS is optimal library size, wherein
    $OLS = \frac{\log\left(1-\frac{PS}{100}\right)}{\log\left(1-\left(1-\left(1-(Mf \times n)\right)\right)\right)}$
    wherein PS is the probability of success in %; and
    Mf is the mutation frequency; and
    n is the number of mutations screened for; and
    Mf is the rate of random mutagenesis of step b);
    b) subjecting said regenerative parts to a step of mutagenesis, preferably of random mutagenesis, leading to a low rate of mutagenesis, thereby generating a pool of regenerative parts of generation M0 representing a plurality of genotypes;
    c) growing said regenerative parts of generation M0 into mature plants of generation M1 and obtaining regenerative parts of generation M1 from said mature plants;
    d) optionally repeating the previous step X times to obtain plants of generation M(1+X) comprising regenerative parts of generation M(1+X);
    e) dividing regenerative parts of a plurality of generation M1 or M(1+X) plants into sub-pools, each sub-pool comprising regenerative parts representing a plurality of genotypes, wherein all regenerative parts from one given mature plant are placed in the same sub-pool , and wherein this step further comprises:
        i. determining the optimal size of the sub-pool, wherein the maximum sub-pool size (SPm) SPm = Y/GECN
        ii. dividing said regenerative parts into sub-pools, wherein each sub-pool comprises regenerative parts from a number of mature plants, said number being in the range of 0.5SPm to 1.1*SPm, wherein all regenerative parts from one given mature plant are placed in the same sub-pool;

wherein the detection sensitivity of sensitive detection means is 1 in Y, and wherein the plants have a genetically effective cell number of GECN;

f) preparing DNA samples, such as gDNA samples or cDNA samples obtained from mRNA samples, from each sub-pool, wherein the step of preparing DNA samples comprises

- dividing each sub-pool into fractions;
- preparing DNA samples, such as gDNA samples or cDNA samples obtained from mRNA samples, of an entire fraction of each sub-pool;

g) identifying sub-pool(s) comprising DNA comprising the mutation(s) using sensitive detection means;

h) identifying regenerative parts within said sub-pool comprising said mutation, thereby identifying said mutant plant,

wherein a low rate of mutagenesis in diploid plants is that an average at least 99.8, preferably at least 99.9% of all genes are free of non-synonymous mutations and in plants of higher ploidy that an average of at least 98.0%, preferably at least 99.0% of all genes are free of non-synonymous mutations in a regenerative part of generation M1, preferably wherein the regenerative part is a vegetative tissue, a seed, a grain, a germline cell in a seed, a grain or an embryo of said plant, pollen or an embryo, preferably the regenerative part is a seed.

2. The method according to claim 1, wherein said regenerative part is a seed.

3. The method according to any one of the preceding claims, wherein the rate of mutagenesis is at the most 1 mutation every 1000 genes, such as at the most 1 mutation every 5000 genes, such as at the most 1 mutation every 10000 genes, such as at the most 1 mutation every 20000 genes, such as at the most 1 mutation every 30000 genes.

4. The method according to any one of the preceding claims, wherein said mutagenesis leads to at the most 25 non-synonymous mutations in coding regions, such as at the most 20 non-synonymous mutations in coding regions, such as at the most 15 non-synonymous mutations in coding regions, such as at the most 10 non-synonymous mutations in coding regions, such as at the most 5 non-synonymous mutations in coding regions, such as 1 non silent-mutation in a coding region in plants of generation M1.

5. The method according to any one of the preceding claims, wherein the mutagenesis is an irradiation-induced mutagenesis or a chemical mutagenesis performed with a mutagenizing chemical such as a mutagenizing chemical selected from the group consisting of sodium azide (NaN_3), ethyleneimine, hydroxylamine (NH_2OH), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), diazomethane, methylnitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS) and alkylating agents such as sulfonates, such as ethylmethane sulfonate (EMS), diethyl sulfonate (DES) or N-ethyl-N-nitrosourea (ENU), or such as sulphur mustards, such as ethyl-2-chloroethyl sulphide, or such as nitrogen mustards such as 2-chloroethyl-dimethyl amine, or such as epoxides, such as ethylene oxide, preferably the step of random mutagenesis is performed with sodium azide, preferably at a concentration of 1 mM or less for 2 hours or less, such as 0.9 mM or less, such as 0.8 mM or less, such as 0.7 mM or less, such as 0.6 mM or less, such as 0.5 mM or less, such as 0.4 mM or less, such as 0.3 mM or less, or the step of random mutagenesis is performed with EMS, preferably at a concentration of 4% or less for 2 hours or less, such as 3% or less, such as 2% or less, such as 1.5% or less, such as 1% or less, such as 0.75% or less, such as 0.5% or less, such as 0.4% or less, such as 0.3% or less, such as 0.2% or less, such as 0.1% or less, such as 0.1% for 24 hours or less.

6. The method according to any one of the preceding claims, wherein said mutant plant has the same phenotype as the parent plant, except from the phenotypic trait(s) caused by the mutation(s) in the target sequence.

7. The method according to any one of the preceding claims, wherein mutations of interest occur at a frequency corresponding to 1 mutation per 1.5 million plants.¨

8. The method according to any one of the preceding claims, wherein N_p is in the range of 0.8*ONp to 1.2*ONp.

9. The method according to any one of the preceding claims, wherein the probability of success is at least 85%, preferably at least 90%, more preferably at least 95%, such as at least 98%.

10. The method according to any one of the preceding claims, wherein at least 5,000 regenerative parts, such as at least 10,000 regenerative parts, such as at least 15,000 regenerative parts, such as at least 20,000 regenerative parts, such as at least 25,000 regenerative parts, such as at least 30,000 regenerative parts, such as at least 40,000 regenerative parts, such as at least 50,000 regenerative parts, such as at least 60,000 regenerative parts, such as at least 70,000 regenerative parts, such as at least 80,000 regenerative parts, such as at least 90,000 regenerative parts, such as at least 100,000 regenerative parts, such as at least 200,000 regenerative parts, such as at least 300,000 regenerative parts, such as at least 400,000 regenerative parts, such as at least 500,000 regenerative parts, such as at least 600,000 regenerative parts, such as at least 700,000 regenerative parts, such as at least 800,000 regenerative parts, such as at least 900,000 regenerative parts, such as at least 10^6 regenerative parts, such as at least 10^6 mature plants, such as 2.10^6 mature plants, such as at least 3.10^6 mature plants, such as at least 4.10^6 mature plants, such as at least 5.10^6 mature plants, such as at least 6.10^6 mature plants, such as at least 7.10^6 mature plants, such as at least 8.10^6 mature plants, such as at least 9.10^6 mature plants, such as at least 10^7 mature plants are obtained in step c).

11. The method according to any one of the preceding claims, wherein the plant is a crop plant or a flowering plant, such as a plant belonging to the Brassicaceae family, the Fabaceae family, the Poaceae family, the Amaranthaceae family, the Asteraceae family, the Solanaceae family, the Rubiaceae family or the Orchidaceae family, preferably wherein the plant is barley.

12. The method according to any one of the preceding claims, wherein the plant species has an average number of regenerative parts per plant of RP_p, and wherein step f) comprises

i. randomly dividing each sub-pool into fractions, wherein a first fraction comprises in the range of $\frac{\mathrm{GECN}*100}{\mathrm{RPp}}$% to $\frac{\mathrm{GECN}*500}{\mathrm{RPp}}$% of the regenerative part of the sub-pool; and wherein said first fraction comprises at the most 50% of said regenerative part;

ii. preparing DNA samples of the entire first fraction of each sub-pool.

13. The method according to any one of the preceding claims, wherein step f) is performed by dividing each sub-pool into at least 2 fractions, such as at least 3 fractions, such as at least 4 fractions, and preparing DNA samples from an entire fraction of each sub-pool, and/or

wherein step f) comprises obtaining a fraction comprising in the range of 10 to 50% of regenerative parts of each sub-pool and preparing DNA samples from the entire fractions of each sub-pool, and/or

wherein step g) for each sub-pool comprises the steps of:

- performing one or more PCR amplifications, comprising the DNA sample from said fraction of the sub-pool, one or more set(s) of primers each set flanking the target sequence and PCR reagents, thereby amplifying the target sequence(s);
- detecting PCR amplification product(s) comprising the target sequence(s) comprising the one or more mutation(s) in the NOI(s).

14. The method according to any one of the preceding claims, wherein 10^6 regenerative parts of generation M0 with different genotypes are obtained in step b), such as at least 2.10^6 regenerative parts, such as at least 3.10^6 regenerative parts, such as at least 4.10^6 regenerative parts, such as at least 5.10^6 regenerative parts, such as at least 6.10^6 regenerative parts, such as at least 7.10^6 regenerative parts, such as at least 8.10^6 regenerative parts, such as at least 9.10^6 regenerative parts such as at least 10^7 regenerative parts or more.

15. The method according to any one of the preceding claims, wherein the method comprises a step of reproduction of the organisms, or regenerative parts thereof, within the pool, and wherein said step of reproducing may be performed simultaneously with, or subsequent to, step e) of dividing the organisms into sub-pools and/or wherein the regenerative parts of step e) are of generation M2.

16. The method according to any one of the preceding claims, wherein step h) comprises the following steps:

- Providing a sub-pool comprising a plurality of regenerative parts of a plant, wherein said sub-pool comprises regenerative parts comprising one or several mutation(s) of the NOI(s);
- Dividing the regenerative parts of said sub-pool into secondary sub-pools,;
- Obtaining a sample from each regenerative part of the secondary sub-pools – in a manner leaving the regenerative parts sufficiently intact to develop into a plant – and combining all samples from all regenerative parts of each secondary sub-pool;
- Preparing a DNA sample from said combined samples;
- Identifying secondary sub-pool(s) comprising DNA comprising the mutation(s).

17. The method according to claim 16, further comprising the steps of:

- Providing the identified secondary sub-pool comprising regenerative parts comprising the mutation in the NOI, wherein the regenerative parts are seeds;
- Cultivating all seeds within said secondary sub-pool to allow germination, and optionally develop plants from each seed;
- Obtaining a sample from each germinated seed;
- Testing said sample for the presence of said mutation in the NOI, thereby identifying a plant carrying the mutation in the NOI;
- Optionally growing said plant to maturity.

18. The method according to claim 17, wherein the method further comprises the following steps performed after step f):

- Obtaining a fraction of each DNA sample from each sub-pool;
- Combining a plurality of fractions into super-pools, thereby obtaining DNA super-pools comprising DNA samples from a plurality of sub-pools, wherein DNA from each sub-pool is only present in one super-pool;
- Performing a plurality of PCR amplifications, each comprising a DNA sample super-pool, wherein each PCR amplification comprises a plurality of compartmentalised PCR amplifications, each comprising part of said DNA sample, one or more set(s) of primers each set flanking a target sequence and PCR reagents, thereby amplifying the target sequence(s);
- Detecting PCR amplification product(s) comprising one or more target sequence(s) comprising the mutation(s) in the NOI(s), thereby identifying super-pool(s) comprising said mutation,

preferably wherein PCR amplifications are performed only on samples of DNA from sub-pool(s) comprising one of the mutation(s).