

## Targeted mutation breeding as a tool for tobacco crop improvement

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*Nicotiana tabacum* is a model widely used in functional genomics with transgenesis; however, genetically modified organisms are not accepted by consumers in Europe. Targeted mutagenesis as a non transgenic approach was assessed on a demonstration gene involved in alkaloid metabolism. A population of 4.000 EMS-mutagenized M2 families was created. Single Strand Conformation Polymorphism (SSCP) was used to target mutations. Eleven putative mutants were identified by screening 1344 M2 families. Mutations identified in DNA pools were validated by sequencing. Individual plants carrying missense or truncation mutations were studied for their phenotype. Homozygous plants for one truncation were identified, and the expected phenotype was observed in the field. These plants have been used as genitors to introduce this mutation into elite lines. Backcrosses are being performed to recover the elite line background in combination with SSCP analysis to track the mutation. The amphidiploid nature of tobacco avoids problems related to fertility. Efficiency of this method to create novel genetic variation and to develop cultivars has been demonstrated for the first time in tobacco.

## Introduction

In the last decades, there has been a growing concern about the possible detrimental effect on human health of specific compounds found in tobacco smoke. Among these are the secondary alkaloids derivatives which are supposed to be implicated in the increased risks for various pathologies. Efforts are mostly focused on nicotine and on its conversion product nornicotine, which is implicated in the increased risk for tumors (Hecht 2003).

A new approach to develop non GMO tobacco is needed to adjust to rapidly evolving markets, and to create new varieties of tobacco useful in developing a new generation of products.

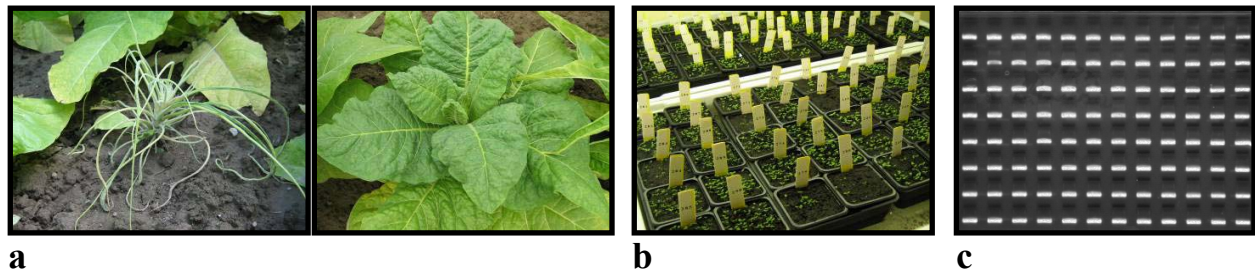
Mutation breeding has been used for decades to modify existing traits or to create new valuable traits within the cultivated varieties. Allele improvement can originate from wild germplasm and related species, or genetic variability can be created by physical or chemical mutagens such as X-ray, fast neutron or ethylmethane sulfonate (EMS). In the past, mutation breeding has produced a set of improved commercial varieties in a wide range of species, including tobacco (see FAO database at <http://www-infocris.iaea.org/MVD/>). Moreover, mutants are not considered as Genetically Modified Organism.

We developed a new strategy relying on mutagenesis coupled with a sensitive detection method allowing rapid, systematic and automated identification of mutations in targeted sequences.

## Results

A mutagenized collection of seeds was developed in Bergerac (in France) during the year 2004. The cultivar BB16NN was chosen for mutagenesis. Two batches of seeds (6000 each) were mutagenized with two different concentrations of a chemical mutagen, ethyl methyl sulfonate (EMS): 0.8% (L1 population) and 0.6% (L2 population). In addition, seeds used for L1 population were pre-germinated in order to increase permeability and penetration of EMS in seed tissues. Seeds were grown in greenhouse. Plantlets were transferred to the field for self pollinating to fix mutations in germinal cells. Some phenotypes deviating from wild-type plants could be observed in the field (**fig.1a**). M2 seeds from each M1 plant were harvested and stocked to develop the seeds collection, or sown to develop the DNA libraries (**fig.1b**). DNA from pools of eight M2 individuals from a single M1 plant was extracted, and DNA quality was assessed on these pooling plates by amplifying a control gene (**fig.1c**).

Embryo lethality of EMS-treated seeds reached 36% and 20% in L1 and L2 populations, respectively. Fertility of the M1 plants was also strongly affected since only 34.5% (L1) and 73.3% (L2) of the selfed field-grown M1 plants yielded seeds. Final ratios of harvested M1 plants to sown M1 seeds were 22% (L1) and 58.6% (L2).

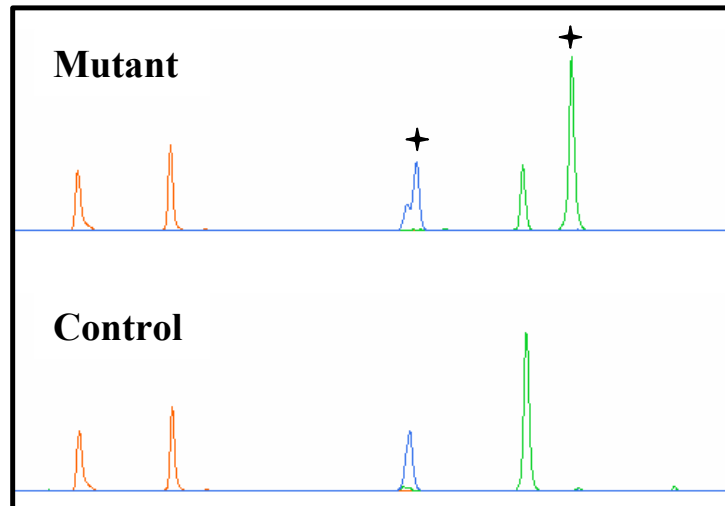


**Figure 1:** After EMS mutagenesis on seeds, some abnormal phenotypes could be observed in the field in the M1 generation (a). Fertile plants were self pollinated, and M2 seeds were harvested. M2 seeds from a single M1 plant were sown to extract DNA on young plantlets (b). Total DNA quality was checked by amplifying the nitrate reductase gene, which produces a 100 bp fragment on an agarose gel (c).

The mutation detection strategy is based on high throughput conformation-sensitive capillary electrophoresis (CSCE) analysis (Davies *et al.*, 2006). Single-stranded conformation polymorphism (SSCP) analysis detects electrophoretic mobility differences that can result from small changes in nucleotide sequence, such as point mutations. The targeted gene is amplified by PCR with dye labeled primers. The PCR product is denatured, and electrophoretic separation of the single-stranded DNA is performed on a nondenaturing sieving medium on an ABI3100Avant capillary electrophoresis (Applied Biosystems). A single base change can cause a conformational change in the DNA molecule. These conformational changes result in detectable mobility differences, characterizing a mutant in the analysed DNA pool.

To evaluate the potential of targeted mutation breeding in tobacco, we targeted a gene controlling normicotine content. Nicotine to normicotine conversion is catalyzed by the nicotine N-demethylase *NtabCYP82E4* gene (Siminszky *et al.*, 2005). Normicotine is highly undesirable because the tobacco nitrosamine N-nitrosornicotine (NNN), which is formed from normicotine, has been implicated in the increased risks for certain diseases. Primers were designed to specifically amplify exons in the *NtabCYP82E4* genes. Among the available mutant collection, we screened 1,311 M2 families. **Figure 2** shows the CSCE detection of

*NtabCYP82E4* mutated allele in one pooled M2 family i.e. in one pool of 8 M2 plants coming from the same M1 plant. Additional peaks corresponding to the mutated allele labelled with two different dyes can be clearly and reproducibly identified on the chromatogram.

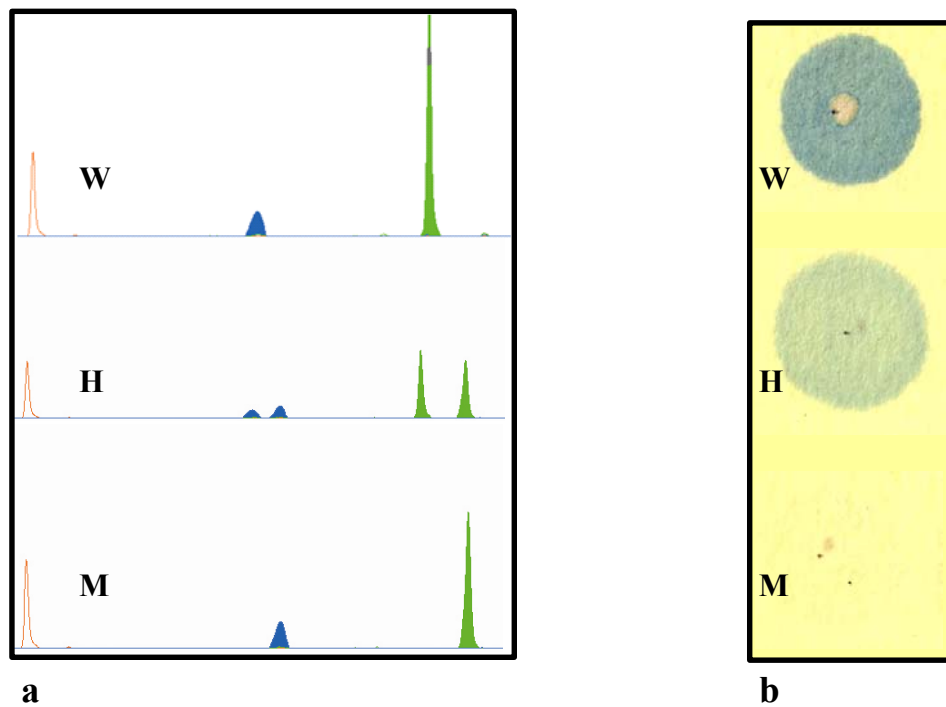


**Figure 2:** CSCE profiles of *NtabCYP82E4* amplified fragment analyzed on the ABI3100Avant capillary electrophoresis. The LIZ500 size standard is labeled with orange dye. Amplified fragments are labeled with blue and green dyes. The mutant allele produces two additional peaks (indicated with stars) compared to the control BB16NN.

DNA fragments from mutant were cloned and sequenced to identify the mutation, and its consequences on the protein. Mutations can range from nonsense (apparition of a stop codon, resulting in a truncated protein), missense mutation (amino acid change) and silent mutation (no amino acid change). Of 10 alleles isolated by screening 0,532 kb of *NtabCYP82E4* DNA, 1 was silent, 5 were missense and 4 were truncation mutations. At least 0.64 % to 1.07 % of the screened M2 mutant families contained plants with mutated alleles of *NtabCYP82E4* gene.

Seeds of M2 families carrying a mutation into the *NtabCYP82E4* gene were sown in greenhouse and plants were individually analyzed by CSCE. Heterozygous and mutant plants could be easily distinguished from wild type plants (**fig.3a**). Nornicotine synthesis in leaf was induced by a bicarbonate treatment according to Shi et al. (2003). Nornicotine presence was assessed by a rapid colorimetric test, adapted from Stephens & Weybrew (1959) which highlight nornicotine presence with blue spot. Correlation between genotype and phenotype

was studied in individual plants. In a family carrying a nonsense mutation, we could observe that no nornicotine was detected in homozygous plants for the mutation (**fig3b**).

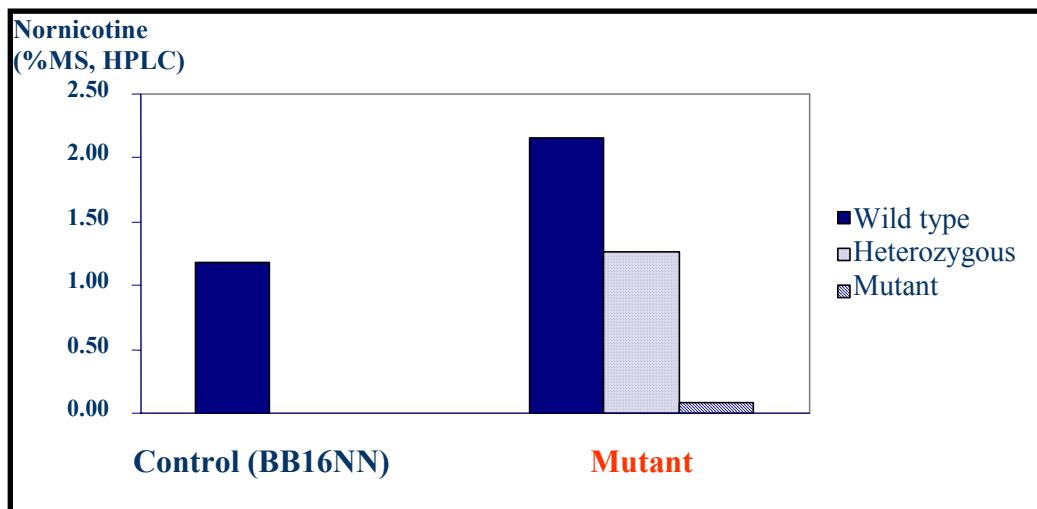


**Figure 3: (a)** CSCE profiles of NtabCYP82E4 amplified fragment in three M2 individuals of the family L2-37, carrying a nonsense mutation. The LIZ500 size standard is labeled with orange dye. Amplified fragments are labeled with blue and green dyes. W=wild type; H=heterozygous; M=mutant for the NtabCYP82E4 gene. **(b)** Results of the nornicotine colorimetric test show that near null nornicotine content is found in homozygous plants carrying the truncated protein.

These observations could be confirmed in the field. Mutant and control tobaccos were grown as burley type tobacco. As already observed in greenhouse, plants displayed near-normal phenotype in field conditions, with however slightly retarded growth, reduced height and smaller leaves than BB16NN elite variety (**fig.4a**). Nornicotine was analyzed by HPLC on cured leaf. The homozygous plants from the truncation mutant L2-37 displayed near-null nornicotine contents while heterozygous mutants showed an intermediate phenotype (**fig.4b**). The mean nicotine to nornicotine conversion rate was as low as ~3.1% in homozygous non converter mutant, compared to ~59% in heterozygous mutant and ~77.5% in wild type plants.



**a**



**b**

**Figure 4: (a)** BB16NN control (left) and L2-37 M2 family (right) in the field in Bergerac, 2006. Plants were individually labeled. A normal phenotype was observed on the mutant family, except a slight growth delay compared to the control. **(b)** Leaf material was air-cured until midribs turned dry and alkaloids were analyzed by HPLC on lamina samples ground to powder. A near null amount of nornicotine was found in homozygous plants for the mutation, compared to the control.

We checked for the transfer of the mutation into non mutant tobacco. For that, we crossed plants carrying the mutation with conventional lines, and we checked for the presence of the mutated allele in the segregating progeny. After one backcross, BC1 plants were undistinguishable from the parental lines without negative collateral effects either on visual phenotype as exemplified in **figure 5**, nor on alkaloid content. It is therefore expected that the strategy of backcrossing until BC5 to BC6 commonly used for the introgression of valuable traits in tobacco is well adapted to mutation breeding of commercial elite tobacco lines.



**Figure 5:** A normal phenotype is completely recovered with the first backcross (left) between a mutant from the L2-37 M2 family and a conventional line (right).

## Discussion

We demonstrate here that mutation breeding can be used as an alternative to GM plants for generating nicotine-free tobacco with reduced risks for human health (Gavilano *et al.*, 2006).

Tobacco lines created by mutation breeding have several advantages. Point mutations are stable whereas transgene may be silenced in some of the transgenic plants and after a number of generations (McGinnis *et al.*, 2007). Moreover, low nitrosamine plants obtained through mutation technology are not considered as Genetically Modified (GM) in European Union. As a consequence, there is no need of containment of plants from mutation breeding for field trials and the time between the targeting of a candidate gene and the production of the improved crop can be considerably shortened.

We didn't need to screen the entire collection to obtain interesting mutants. The mutation load may be buffered by the gene duplication due to the amphidiploid nature of cultivated tobacco. This would explain the high mutation frequencies found in the polyploid species tobacco and wheat (Slade *et al.*, 2005). As a consequence, small mutant libraries are sufficient to identify large allelic series comprising both severe and hypomorphic mutations in tobacco.

The efficiency of TILLING technology using CEL1 endonuclease is proved for a wide range of plants (Colbert *et al.*, 2001; Henikoff and Comai 2003), however, the cost of

commercially-available enzyme remains prohibitive for routine use, hence the Conformation Sensitive Capillary Electrophoresis (CSCE) used here appears as a good alternative as it is robust, cost-effective and versatile, and it can be used for all molecular markers applications. Screening ~4000 M2 mutant families with 16-capillary equipment requires ~2 days and produces up to 35 mutated alleles per target sequence, a throughput compatible with the objective of mutation breeding of multiple quality traits in tobacco.

## **Conclusion**

To conclude, we used the suppression of nornicotine as a case study in tobacco, to demonstrate the potential of the targeted mutation breeding method. The study presents evidence that tobacco improvement does not necessarily rely on lengthy breeding programs or on GM plant generation but can make use of comparatively rapid and simple mutant-based strategy for modifying specific traits of interest.

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