**Supplemental Data**

**Development of a nornicotine-reduced flue-cured tobacco line via EMS mutagenesis of nicotine N-demethylase genes**

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Supplementary Table S1 List of primers and restriction enzymes.

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| --- | --- | --- | --- | --- | --- | --- | --- |
| **Analysis** | **Gene/Marker** | **Forward primer**  | **Forward primer sequence** | **Reverse primer**  | **Reverse primer sequence** | **Enzyme** | **Products (bp)** |
| Sequencing | *CYP82E4* | Tilling-E4-F-2 | 5’-TTTTCTATTTCGGTACGAC-3’ | Tilling-E4-R-2 | 5’-TAACTTTACAGTTGCTCCA-3’ |   |   |
| *CYP82E5* | Tilling-E5-F | 5’-GGTAATTTTGTATTTATTATATTATGCG-3’ | Tilling-E5-R | 5’-TCATCCTTAGTATTTAGATAATCTAATT-3’ |   |   |
| *CYP82E10* | Tilling-E10-F | 5’-GTCAAATACCACCTCTTAATAGTAA-3’ | Tilling-E10-R | 5’-AAAAGTCCCTATTGGTAGGAAGTGC-3’ |   |   |
| dCAPS | E4\_*Xho*I | E4dCAPS\_F1 | 5’-TTCCAATCGTCCAGCTTTTCTTTACGGCGAT-3’ | E4dCAPS\_R1 | 5’-AGTTAAATTTATCGTACTCGAATTTCCATCAACTC-3’ | *Xho*I | E4:196+36; e4:232 |
| E5\_ *Bal*I | E5dCAPS\_F1 | 5’-CCTTACACTTCTCTTCTACTTCCTATGGCCC-3’ | E5dCAPS\_R1 | 5’-GAATGACTAATTTTCTATTTTTTGGC-3’ | *Bal*I | E5:355+25; e5:380 |
| E10\_ *Bam*HI | E10dCAPS\_F1 | 5’-GTGAAATTCAGACGAGCATTAAGAA-3’ | E10dCAPS\_R1 | 5’-TTTGAACAATGGAATTGGAAAAGGATC-3’ | *Bam*HI | E10:219+26; e10:245 |
| qRT-PCR | *CYP82E4* | CYP82E4q\_F\_1 | 5’-CATTGCTACTGATATTGACTTT-3’ | CYP82E4q\_R\_1 | 5’-GCGCTATTATCAGTTCCAC-3’ |   |   |
| *CYP82E5* | CYP82E5q\_F\_1 | 5’-CTTCGCTGATGATATTGACTAC-3’ | CYP82E5q\_R\_1 | 5’-CCGTAATTGTCACTTCTAC-3’ |   |   |
| *CYP82E10* | CYP82E10q\_F\_1 | 5’-TTTCGCTGCTGATATTGACTTT-3’ | CYP82E10q\_R\_1 | 5’-GCGTAATTACCACTTCTAT-3’ |   |   |
| *NtPMT1a* | PMT1aqF1 | 5’-AAATGGCACTTCTGAACACCTC-3’ | PMT1aqR1 | 5’-CCCATTCTGGTGGCCGTTCC-3’ |   |   |
| *NtQPT2* | QPT2-F | 5’-TACAAGAGTGGAGTCATTAGAG-3’ | QPT2-R | 5’-GCAAGTGCAATTCCTGCTATG-3’ |   |   |
| *NtMPO* | qNtMPOf1 | 5’-CCTACTAAGCTTCCTCCAAG-3’ | qNtMPOr1 | 5’-TCGAGCAGCAGCATGTACTT-3’ |   |   |
| *NtA622* | qNtA622f1 | 5’-GGATGATAGAGGCAGAAGGA-3’ | qNtA622r1 | 5’-TGACAACTTTGTCTCTAGGAG-3’ |   |   |
| *NtActin* | NtACTINq\_F\_1 | 5’-CTGAGGTCCTTTTCCAACCA-3’ | NtACTINq\_R\_1 | 5’-TACCCGGGAACATGGTAGAG-3’ |   |   |