

Multiplex single-base extension typing to identify nuclear genes required for RNA editing in plant organelles

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ABSTRACT

We developed a multiplex single-base extension single-nucleotide polymorphism-typing procedure for screening large numbers of plants for mutations in mitochondrial RNA editing. The high sensitivity of the approach detects changes in the RNA editing status generated in total cellular cDNA from pooled RNA preparations of up to 50 green plants. The method has been employed to tag several nuclear encoded genes required for RNA editing at specific sites in mitochondria of *Arabidopsis thaliana*. This approach will allow large-scale screening for mutations in genes encoding *trans*-factors for many types of RNA editing as well as for other RNA modifications.

INTRODUCTION

RNA editing in mitochondria of flowering plants changes about 450 specific cytosines to uridines mostly in mRNAs (1,2). To date, neither the enzyme(s) involved in the biochemical reaction nor any of the factors involved in recognition of the specific editing sites have been identified. In recent years several *cis*-determinants of RNA editing sites have been characterized by *in organello* and *in vitro* analyses of RNA editing in mitochondrial RNA molecules, but have not yet led to any of the *trans*-factors (2–5).

The biochemical purification of these presumed *trans*-factors, site-specific or more general, may be difficult if not impossible primarily due to extremely low concentrations of these factors. As an alternative and/or complementing approach, screening for mutants may provide more rapid access to these factors and their genes. Indeed, in plastids, nuclear mutants detected by various secondary phenotypic defects incidentally led to the identification of three genes required for editing at specific sites (6–9). Such indirect phenotypic screens are not feasible for mitochondria, since most mitochondrial genes code for subunits of the

respiratory chain and most disturbances in any of these genes will be very severe if not lethal. We therefore set out to develop a method to directly detect RNA editing defects at individual sites in populations of randomly mutagenized plants.

METHODS

Arabidopsis thaliana growth conditions and preparation of nucleic acids

A. thaliana seeds for the wild-type (wt) Col and C24 ecotypes were kind gifts of J. Forner and S. Binder (Universität Ulm). An EMS-mutant library of *A. thaliana* ecotype Col was obtained commercially (Lehle seeds). Growth of the *A. thaliana* plants and preparation of DNA or RNA from leaves were as described (2,10). Leaves of similar sizes were collected and pooled to ensure that each plantlet analyzed was represented approximately equally in the RNA preparations. Portions of the first supernatants of the RNA extraction assays from three of the small pools of eight plants were combined to further purify total RNAs from 'large pools' of 24 plants (Figure 2). The RT-PCR products from 18 reactions for 16 genes from each 'large pool' were analyzed for single-nucleotide polymorphisms (SNPs) in six assays. For RNA extraction all samples were extensively treated with RNase-free DNase from the RNAspin mini kit (GE Healthcare, Freiburg, Germany) to remove the unedited genomic DNA sequence.

Analysis of RNA editing sites

Gene-specific cDNA fragments were generated by RT-PCR amplification by established protocols (10) in independent reactions for each fragment. Simultaneous amplification of several fragments in one reaction proved to be too variable to yield comparable amounts of products. To avoid any potential residual contamination of the generated cDNA with genomic sequences, the RT-PCR primers were designed to anneal only to mRNA

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and cDNA by placing the 3' terminal nucleotides of the primers at editing sites to allow efficient binding only at edited mRNAs. Additional primer-internal editing sites were included where possible. Editing site locations are given in the RNA editing database (11).

Amplified fragments were designed to cover several editing sites to allow parallel screening with the SNP primers. Comparable amounts of the targeted RT-PCR fragments were pooled for the separate single-nucleotide primer extension analyses.

Multiplex SNP analysis of the cDNAs

For the multiplex SNP assays, specific oligonucleotides (Biomers, Ulm, Germany) were designed with regard to staggered lengths of 3–5 nucleotides and to similar annealing/melting temperatures in the separately mixed batches of 10–11 such primers (Table 1). Single-nucleotide primer extension reactions used the commercial kits (ABI SNAPshot™) with concentrations of the individual compounds reduced to one-twelfth of the recommended amounts for optimal enhancement of the unedited C-signal. To maintain the necessary magnesium concentration in the routinely performed 5 µl half-volume reactions, 2.1 µl of a buffer containing 80 mM Tris-HCl (pH 9.0) and 2 mM MgCl₂ were added to the 0.4 µl reaction mix. The extensions were run with 0.6 pmol of each primer. The single-nucleotide primer extension cycle numbers were increased to 60 to further enhance the unedited mutant C-signal after depletion of the edited wt T-corresponding nucleotide. Samples were analyzed on an ABI 3100 machine.

RESULTS AND DISCUSSION

Increasing the sensitivity for the expected mutant signal

To directly detect mutations in RNA editing in plant mitochondria (or chloroplasts) the nucleotide identity change from cytosine to uridine needs to be followed. In consequence, a given editing event creates a single-nucleotide polymorphism which can be detected in the cDNA population by various approaches, most of which are sensitive, expensive and time-consuming, or usually less sensitive when less expensive.

A novel, sensitive and affordable approach is required to solve the major problem in the direct identification of mutants of RNA editing, which is to screen large numbers of mutant plants for variations in RNA editing at many editing sites simultaneously and in parallel. Towards this aim, we adapted SNP-genotyping protocols developed for the detection of SNPs in multiplex assays for genotyping (12,13). The single-base extension approach can be used after RT-PCR to detect differences in the editing status at a given site (14), but needs to be multiplexed and enhanced to make it affordable and feasible. As a compromise between the number of SNPs addressed in a single reaction and a reproducible and safely detectable signal, we optimized the assays with the analysis of 10–11 sites in a single reaction by spaced primer lengths with 3–4 nucleotide intervals (Figure 1A and B; Table 1).

To specifically increase the signal of the C-reaction resulting from the contribution of an individual unedited plant in a pool of random mutants, a combination of more amplification cycles and a lower concentration of nucleotides was introduced: While in the cDNA analysis the T-nucleotides of a very limited reaction mix have all been used up in the extension of the C to T processed RNA editing sites in the majority of the editing-wt plants, the C nucleotide targeting the rare specific mutant is still available. Furthermore, cycles will continue to add this nucleotide to the excess primer molecules and will thus increase the fluorescence signal for the unedited site from the mutant individual. Optimal enhancement of the C-signal was achieved when the reaction reagents were scaled down to one-twelfth of the recommended concentrations and when the number of single-nucleotide elongation cycles was increased to 60 rather than the usual 25 (Figure 1C).

Determining the detection limits for mutant individuals

Depending on the density of the chemically or otherwise induced mutations, several thousand individual plants have to be screened. In the typical EMS-mutant populations of the model plant *A. thaliana* the average incidence of mutations should yield a dysfunctional variant of a given gene in about 2000 plants. As a proof of concept and to verify the validity of the approach, we performed a screen in which we tested for 62 editing sites in mRNAs of

Table 1. Oligonucleotide primers designed for the simultaneous investigation of 10 RNA editing sites in one reaction of single-nucleotide primer extensions (Figure 1)

Gene	Editing position	Primer sequence	Orientation	Primer length (nucleotides)
cob	286	AAAATGCTAATGGGGCAAGTATGTTT	F	26
cob	325	AAAAAAAAAAGGACTGCTATAACTCGCAT	R	30
cob	568	AAAAAAAAAACCTACTAAAATAAAGGGGAGTAAAT	R	34
nad5	1275	AAAAAAAAAAAAAGTAATAAGAAGTGAAAAGGACAGA	R	38
nad5	1918	AAAAAAAAAAAAAAAAAAGAAGATAGAGAATCCACATAC	R	41
nad5	1610	AAAAAAAAAAAAAAAAAAAAAGTTGGAATTGATCCGCTACG	R	44
nad5	1550	AAAAAAAAAAAAAAAAAAAAAGAGTTTGCTGTCCAACCATTA	F	47
rps12	146	AAAAAAAAAAAAAAAAAAAAACGTACTTTGGCTATCTTACGT	R	50
rps12	84	AAAAAAAAAAAAAAAAAAAAAAGGCATACTCTGTTTTCTG	R	53
rps12	221	AAAAAAAAAAAAAAAAAAAAAACACCGTAGAATGTTCTCTGC	R	56

The polyA tail is adjusted to provide gaps of 3 or 4 nucleotides between primers. Positions of editing sites are given as nucleotides from the respective AUG codon.

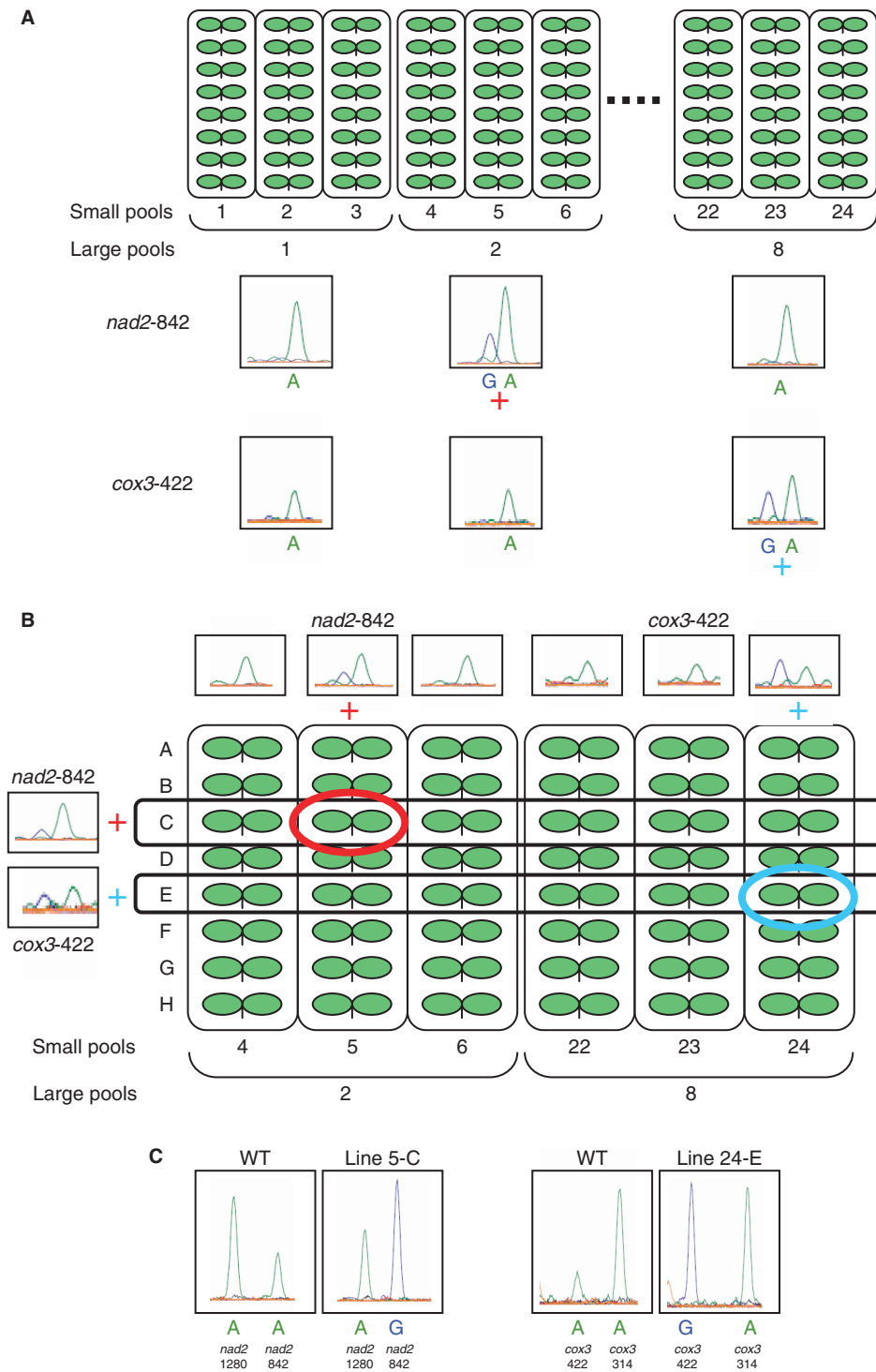


Figure 2. Detection of specific RNA editing mutants in the mutated plant population. (A) Green leaves of similar sizes were pooled from eight plants in the ‘small pools’ and three of the small pools were combined for ‘large pools’ of 24 plants. The RT–PCR products from 18 reactions for 16 genes from each ‘large pool’ were analyzed for SNPs in six assays. Sample traces of the single base extension assays are shown for pools without any mutants for the monitored RNA editing sites in pool 1. In pool 2 a mutant is identified which has lost editing at site *nad2-842* and pool 8 contains a mutation of editing at site *cox3-422*. The gene *nad2* codes for subunit 2 of the NADH-dehydrogenase of the respiratory chain and *cox3* codes for subunit 3 of the cytochrome oxidase of the respiratory chain. (B) The mutated individual plants are identified in the vertical and horizontal pools of eight plants each. (C) The isolated individual plants are compared with wt primer extensions for the editing levels at sites *nad2-842* (line 5-C) and *cox3-422* (line 24-E). No trace of an edited nucleotide is detected, showing that the underlying mutations have incapacitated genes required for editing at these respective sites.

16 mitochondrial genes. The 62 editing sites are located in 16 different mRNAs from which the investigated regions were amplified to cDNA fragments in 18 separate RT-PCR reactions. These were analyzed in six multiplexed SNP panels. If indeed single nuclear genes are involved in addressing each of the 450 editing sites, this screen should search for any of 62 nuclear genes. Ideally, one nuclear mutant defective or severely disturbed in editing at one of the 62 sites should thus be identified in about 30 plants.

However, several caveats will lower the probabilities unpredictably and have to be accommodated. Many if not most of the editing events in plant mitochondria are required for proper mitochondrial function and non-editing may be lethal. Under this condition a homozygous knockout mutant for a given site will not be viable. If one nuclear gene is involved in editing at several sites, one or the other site may be essential. A nuclear mutation may affect the gene function only partially and an effect of only slightly reduced RNA editing may be difficult to detect and to follow through a genetic screen. To allow for these uncertainties, the number of mutant plants investigated has to be increased for a reasonable chance of success. To minimize the probability of lethal phenotypes, we preferentially selected editing sites which are either silent or absent in other plant species with the rationale that *A. thaliana* plants lacking editing at any of these sites should more likely be viable.

To optimize the screening procedure, we experimentally determined the number of plants, which can be pooled and still allow detection of the absence of an editing event or of a reduction by at least 50% in one of the plants. For a pilot assay, leaves from *A. thaliana* ecotypes C24 and Columbia (Col) were mixed and the extracted RNAs were surveyed for the RNA editing site *rps4-956*, which is edited to only 50% in C24 (15). At a ratio of 1:9 of C24:Col plants, the signal difference is still clearly detectable (not shown). In subsequent optimization assays, a mutant plant deficient for RNA editing at site *cob-325* was pooled with increasing numbers of wt Col plants to explore more rigorously the limits of detection (Figure 1C). At a dilution of 1 mutant plant in 100 plants the mutant signal is barely detectable, reproducible results with a safety margin are obtained with a ratio of 1:50 plants. To also accommodate detection of a plant with 50% diminished editing at a given site, about 25 plants can thus be routinely pooled in a single reaction.

Proof of concept: identification of nuclear mutants for RNA editing

With these parameters we screened 384 individual plants of a population of chemically mutagenized *A. thaliana* plants from ecotype Columbia (Figure 2). Candidate traces of mutants disturbed in RNA editing are detected for several sites (Figure 2A and B). These plant individuals were identified and propagated by selfing. Under standard growth conditions no phenotypic difference to wt Col is obvious in most of these plant lines. Analysis of the RNA editing status in these mutant lines shows no detectable editing at the affected sites (Figure 2C). The mitochondrial

cDNA sequences are identical between the mutant and the wt Col plants for more than 100 nucleotides around the respective affected editing sites, suggesting that no mitochondrial mutation is responsible for the loss of RNA editing (data not shown).

To corroborate this conclusion, inheritance of individual RNA editing phenotypes was investigated in crosses with wt plants. Transmission of the RNA editing phenotype clearly indicates a Mendelian mode of inheritance of a recessive trait (data not shown). This result confirms that mutations in nuclear genes have been identified, which block RNA editing at specific sites in the mitochondrial mRNA population. These nuclear genes are now amenable to identification by genomic mapping.

CONCLUSIONS

In summary, we have established a procedure to screen large mutant populations of plants for defects in RNA editing at specific sites in mitochondria. This method represents an alternative to a recently developed method based on high resolution melting (HRM) of amplicons (16). The strengths of the HRM approach are the identification of new RNA editing sites and the screening for affected site(s) in an isolated mutant line or individual, but does not allow to screen large mutant populations for a mutant affected at a given editing site. Our multiplexed base extension approach can also be used to search e.g. T-DNA insertion lines of candidate genes potentially involved in RNA editing such as any or all of the more than 400 PPR-genes encoded in plant genomes (17,18). The procedure can equally be employed for the identification of nuclear mutants in RNA editing in chloroplasts. Screening for mutants of the U to C editing events in plastids and mitochondria of non-flowering plants as well as for mutants of RNA editing in other systems and organisms and the subsequent search for the mutant gene will then mostly depend on the availability of gene identification procedures. Where these are available, screening will be feasible for any of the various nucleotide differences caused by enzymatic or chemical reactions such as tRNA modifications, rRNA methylation and even intron splicing.

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