Intronic polymorphisms in *Daucus carota AOX2b* generate putative genotype specific miRNAs

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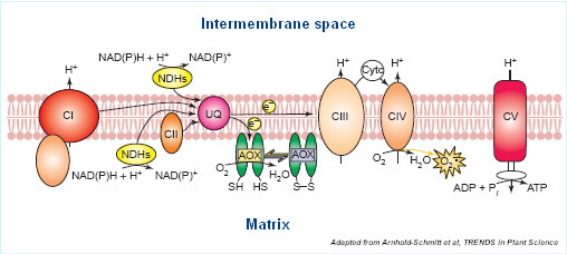
ABSTRACT



Plant mitochondria play an important role in diverse metabolic pathways and are involved in pathogen stress responses and in the process of programmed cell death. Under diverse biotic as well as abiotic stress conditions, plant mitochondrial can control the reactive oxygen species (ROS) generation by means of energy-dissipating systems (Clifton et al. 2005. *Plant Mol Biol* 58:193-212).

In higher plants, AOX is codified by a small multigene family with at least five genes (Clifton et al. 2006. Biochim Biophys Acta 1757:730-741) belonging to two subfamilies: AOX1-type and AOX2-type genes. Results achieved in our laboratory has demonstrated that the *D. carota AOX* multigene family is characterized by two *AOX1* (*DcAOX1a and DcAOX1b*) *and two AOX2* (*DcAOX2a* and *DcAOX2b*) genes (Costa et al. 2009. Plant Physiol Biochem 47:753-759).

The involvement of introns in the regulation of gene expression can be due to intronic sites for important regulatory elements, such as miRNAs. This knowledge together with the recent identification of a putative pre-miRNA in a polymorphic region of the *DcAOX2a* (Cardoso et al. 2009. Physiol Plant 137:592-608) made it reasonable to investigate the effect of the variability in intron sequences on the predictability of putative miRNAs also in *DcAOX2b* in view of the development of functional marker candidates for carrot plant breeding. A study on several individual plant genotypes of *D. carota* cv. Rotin confirmed a frequent occurrence of intron length polymorphisms in *DcAOX2b* that result in three different sizes of intron 1 (Cardoso et al. 2011. Plant Genet Res: Char Util 9:177-180). Here we will present the result of an *in silico* analysis performed at the intron 1 sequences of different *D. carota* genotypes in order to identify putative pre-miRNA sites.

MATERIALS AND METHODS

Cloning of *DcAOX2b*

Complete *DcAOX2b* genomic sequences were amplified in 14 *Daucus carota* L. cv. Rotin genotypes. Specific primers were designed in the 5'UTR (Fw: 5'-TGCATGCGTCCTTCCTTATTTTTC-3') and 3'UTR (Rev: 5'-GCTCTGCTGTGATTTTCTGGAC-3') of *DcAOX2b* (acc. EU286576). Amplicons were generated by using *Phusion™ High-Fidelity DNA Polymerase* (Finnzymes Oy) and cloned in a pGEM®-T Easy System I vector (Promega).
Two plant genotypes representing each band pattern were selected for sequence analysis. From each those genotypes three bacterial clones were sequenced.

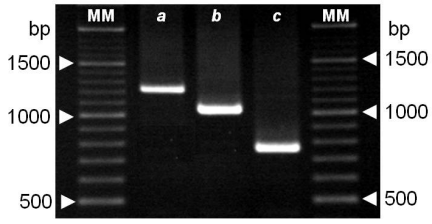
Intron 1 sequence analysis

For sequence analysis EditSeq and Clustal W algorithm of Megalign (Lasergene, GATC Biotech, Konstanz) were applied. Putative miRNA precursors (pre-miRNAs) were searched by using the software miR-*abela* (http://www.mirz.unibas.ch/cgi/pred_miRNA_genes.cgi). For validation of potential pre-miRNAs the software MiPred was applied (Jiang et al., 2008. Clin Cancer Res 14:419-427). Prediction of the secondary structure of pre-miRNA was run on the web-based software MFOLD 3.1 (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) (Zuker, 2003. Nucleic Acids Res 31:3406-3415).

RESULTS AND DISCUSSION

Previous analysis of *DcAOX2b* performed by Exon Primed Intron Crossing (EPIC)-PCR allowed to the identification of differences at the intron 1 size.

Sequence analysis of the three intron 1 sequences revealed that the intron 1 size can be 1019, 822 or 557 bp (named fragment *a*, *b* and *c*). Those differences are due by Insertions/deletions (InDels) located along the sequence.



Sequences of fragment *a* and *b* are more similar between each other whereas fragment *c* shows a higher degree of divergence.

17 InDels with positions conserved in all analyzed sequences were identified:
- 9 within 1 - 10 bp (2 bp was the most frequent);
- 5 within 10 - 50 bp;
- 2 within 50 - 100 bp (61 and 97 bp);
- 1 higher than 100 bp (246/248 bp).

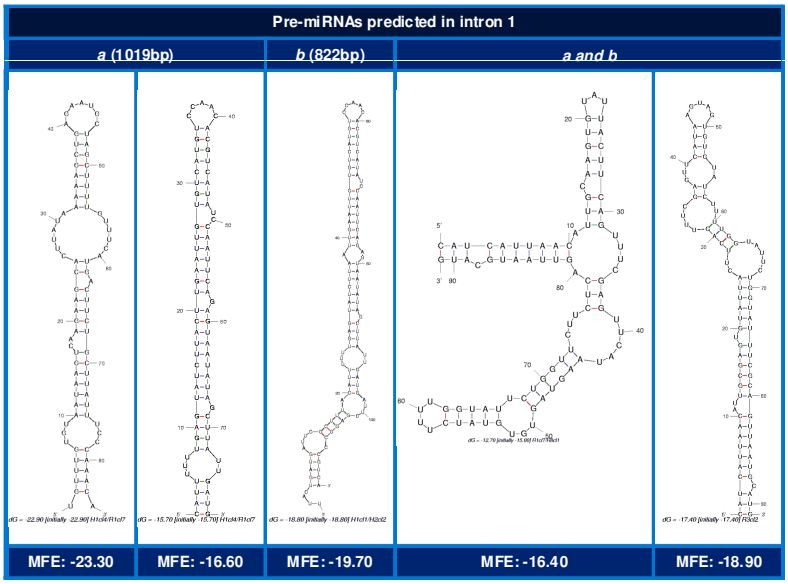
15 putative single-nucleotide polymorphisms (SNPs) were identified between intron fragments *a* and *b*.

The comparison of the three intron fragments allowed to identify 74 putative SNPs, 3 exclusively of fragment *a* (427: T/C, 611: A/C and 852: T/C).

Five putative pre-miRNAs were predicted: 2 exclusively of fragment *a* (intron of 1019 bp), [minimal free energy (MFE)= -16.60 and -23.30], and 1 of the fragment *b* (intron of 822 bp) (MFE= -19.70).

An additional pre-miRNA was found in both larger introns (MFE= -16.40 or -18.90, depending on the existence of single nucleotide polymorphisms (SNPs).

No pre-miRNA was predicted in the smallest version of intron 1 (fragment c corresponding to intron size of 557bp).



CONCLUSIONS

► Polymorphisms at intron 1 sequence influence the pre-miRNA prediction.
► Functional analysis should also consider introns to establish the link between the DNA polymorphisms and the phenotype.
► Further work will be made in order to confirm the existence of the predicted miRNAs and its function at the plant.

ACKNOWLEDGEMENTS

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