

# Genetic diversity in intron1 of carrot *AOX2b* – a useful tool to develop a functional marker?

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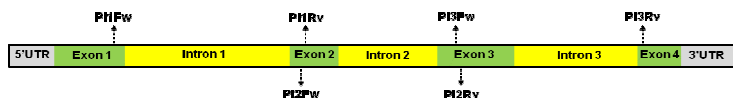
## ABSTRACT

Alternative respiration is enhanced after various developmental or environmental stimuli (Purvis and Shewfelt, 1993. *Physiol Plant* 88:712-718). Alternative oxidase (AOX) genes are recently suggested to serve as a source for functional markers for efficient cell reprogramming under stress (Arnholdt-Schmitt et al., 2006. *Trends Plant Sci* 11:281-287). The development of functional markers directed to assist selection of genotypes with defined traits for plant breeding requires identification of gene-specific polymorphisms and a linkage to important agronomic traits. Recent knowledge on the regulatory functionality of intronic regions and sequence variability identified in intron 3 of the *AOX2a* carrot gene from diverse carrot genotypes and breeding lines (Cardoso et al., 2009. *Physiol Plant*, 137:592-608) made it reasonable to investigate intron variability also in other carrot AOX genes. The complete cDNA sequence of the *DcAOX2b* is recently available (Campos et al., 2009. *Physiol Plant*, 137:578-591). This allowed us to investigate the diversity in this gene among genotypes in view of potential future applications in carrot plant breeding. In the presented study we focus on sequence variations at genomic DNA level.

## MATERIALS AND METHODS

### 1. Intron analysis by exon-primed-intron-crossing-polymerase-chain-reaction (EPIC-PCR)

Intron regions of *DcAOX2b* were amplified by EPIC-PCR in 40 *Daucus carota* L. genotypes (14 genotypes of cv. 'Rotin' and 26 of wild carrot plants). Specific primers were designed in the exons boundaries for each of the three introns (see below the scheme of the structure of the *AOX2b* gene in carrot, acc. EU286576, showing the primers position).



### 2. Intron sequence analysis

Amplicons of interest generated by using *Phusion™ High-Fidelity DNA Polymerase* (Finnzymes Oy) were cloned in a pGEM®-T Easy System I vector (Promega). Two genotypes representing each band pattern were selected for sequence analysis. From each those genotypes three bacterial clones were sequenced. 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](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).

### 3. Gene mapping

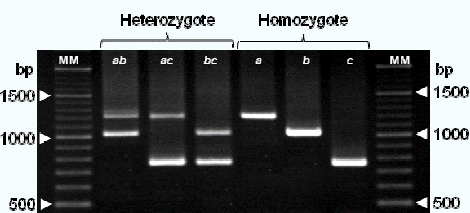
A linkage map of carrot was developed using a F<sub>2</sub> population DM19 (Nothnagel and Straka, 2003. *Plant Breeding* 122:339-342; Nothnagel et al., 2005. *Plant Breeding* 124:481-486). A total of 161 F<sub>2</sub> plants was used to investigate the inheritance of *DcAOX2b* and its localization in the carrot genome. An integrated linkage map was designed using the JoinMap 3.0 programme with a conservative LOD score of 6.0 (Ooijen van and Voorrips, 2001. *Plant Research International B.V.*, Wageningen). Map distances (cM) between ordered *loci* were calculated using the recombination fraction and Kosambi mapping function.

## RESULTS AND DISCUSSION

### Band pattern analysis by EPIC-PCR:

- Intron length polymorphism (ILPs) was found by EPIC-PCR in intron 1 in individual plants (two bands) and between plant genotypes.

- Six different band patterns were identified:  
- 3 single band patterns (homozygous): named **a**, **b** and **c**  
- 3 double band patterns (heterozygous): **ab**, **ac** and **bc**



- In cv. Rotin and in carrot wild plants the PCR-fragment pattern related to the single band of 1 kb (**b**) was found more frequently than the other patterns:

Band Pattern Frequency							
Genotype	n	ab	ac	bc	a	b	c
Rotin	14	3	1	2	1	7	0
Wild	26	3	2	7	1	9	4

- EPIC-PCRs from intron 2 and 3 show stable fragment lengths of around 430 and 400 bp, respectively, in all plants.

### Sequence analysis

- Sequences of fragment **a** and **b** are more similar between each other whereas fragment **c** shows a higher degree of divergence (see table on right).

		Percent Identity			
		a	b	c	a
Divergence	a	69.3	51.4	a	
	b	1.7	66.9	b	
	c	12.4	13.8	c	
		a	b	c	



- The comparison of the three intron 1 sequences revealed 17 Insertion/Deletions (InDels) with positions conserved in all analyzed sequences:

- 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**.

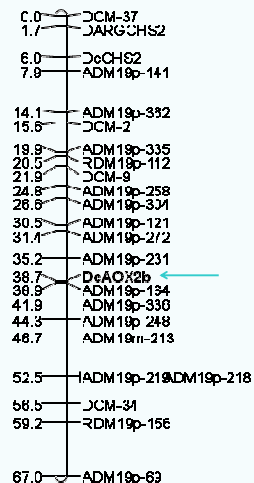
- The comparison of the three intron fragments allowed to identify 74 putative SNPs, 3 exclusively of fragment **a** (427: T/C, 611: A/C and 852: T/C).

- 5 putative pre-miRNAs were predicted: 2 exclusively of the fragment **a** (see secondary structure on the left of the pre-miRNA with the lower minimal free energy) and 1 of the fragment **b**.

- No pre-miRNA was predicted for fragment **c**.

### Gene mapping

The codominant marker could be mapped on **linkage group 4** together with four SSR markers, two gene specific markers for the chalcone synthase gene, 15 AFLP and 2 RAPD markers (see figure below).



## CONCLUSIONS

- ▶ ILPs identified in intron 1 of *DcAOX2b* are an useful tool to search for gene variability in carrot plants.
- ▶ ILPs enable mapping of *DcAOX2b* in different carrot populations.
- ▶ SNPs and InDel events can be important sources for functional marker development to assist carrot breeding.
- ▶ Functional analysis should also consider introns to establish the link between the DNA polymorphisms and the phenotype.

## ACKNOWLEDGEMENTS

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