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Polymorphisms in intron 1 of carrot *AOX2b* – a useful tool to develop a functional marker?

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Abstract

Alternative oxidase (AOX) has been proposed as a promising functional marker candidate for multiple plant stress behaviour. The present paper describes natural polymorphism in *AOX2b* of *Daucus carota* L. (*DcAOX2b*). Exon-primed intron crossing-PCR (EPIC-PCR) revealed length variation (intron length polymorphisms, ILPs) in intron 1. Six fragment patterns were identified in 40 genotypes. However, no more than two fragments were found per genotype, suggesting the presence of two alleles. The ILPs were able to discriminate between single plant genotypes in cv. Rotin and to distinguish individual wild carrot plants. The repetitive pattern of intron 1 length variation allows the grouping of genotypes for functional analysis in future studies. Sequence analysis in intron 1 of polymorphic but also of obviously identical PCR-fragments revealed underlying high levels of sequence polymorphisms between alleles and genotypes. Variation was due to repetitive insertion/deletion (InDel) events and single-nucleotide polymorphisms (SNPs). The results suggest that high *AOX2b* gene diversity in *D. carota* may be a source of functional markers for agronomic traits related to environmental stress responses.

Keywords: *Daucus carota*; exon-primed intron crossing-PCR; insertion/deletion; intron length polymorphism; miRNA; single-nucleotide polymorphism

Introduction

Until the 1970s, eukaryotic introns were considered to be genetically inert sequences with no function in the genome and not subject to evolutionary selection. They were known as dubbed selfish or junk DNA (Orgel and Crick, 1980). However, recent evidences show that plant introns play an active role in the control of gene expression (Gianì *et al*., 2003; Fiume *et al*., 2004) and evolution. Insertions/deletions (InDels) and single-nucleotide polymorphisms (SNPs) are the two main types of DNA polymorphism (Nasu *et al*., 2002) found in intron sequences (Wang *et al*., 2005). These intronic polymorphisms are becoming important genetic markers for genetic research and breeding in major crop species (Bi *et al*., 2006; Braglia *et al*., 2010).

The recent identification of intron variation in carrot *AOX2a* (Cardoso *et al*., 2009) suggested the investigation of intron variation in other carrot *AOX* genes (Campos *et al*., 2009; Costa *et al*., 2009a). Alternative oxidase (AOX) is an inner mitochondrial membrane protein involved in stress acclimation and adaptation of diverse nature (e.g. McDonald and Vanlerberghe, 2006; Plaxton and Podestá, 2006). We describe here the intron variation in carrot *AOX2b* (*DcAOX2b*) among genotypes, with a view to potential applications in carrot breeding (Arnholdt-Schmitt *et al*., 2006; Arnholdt-Schmitt, 2009; Polidoros *et al*., 2009).

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Table 1. Oligonucleotides applied in the present work

Aim	Olinucleotides sequence	Enzyme/kit	T_a (°C)	T_e (min)
ILPs in intron 1	Fw: 5′-TGGGGACAAGGATGATGAG-3′ Rev: 5′-CCCTTAGGTTTATGGTGTTTATTC-3′	Ready-to-go PCR beads (GE Healthcare, Little Chalfont, England)	55	1
ILPs in intron 2	Fw: 5′-TGATCTGAATAAACACCATAAACC-3′ Rev: 5′-GAAGAAAGCATTGAAGAAAACTCC-3′	Ready-to-go PCR beads (GE Healthcare)	55	1
ILPs in intron 3	Fw: 5′-TTTAGCCGTGCAGGGAGTTTTCTT-3′ Rev: 5′-GCATCCCTCAGTTCCTTTCCTTCA-3′	Ready-to-go PCR beads (GE Healthcare)	60	1
Sequence polymorphisms	Fw: 5′-TGCATGCGTCCTTCCTTATTTTTC-3′ Rev: 5′-GCTCTGCTGTGATTTTCTGGAC-3′	Phusion™ high-fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland)	55	2

Fw, forward; Rev, reverse; T_a, annealing temperature; T_e, extension time at 72°C for 35 cycles.

Materials and methods

In a search for intron length polymorphisms (ILPs) in the three introns of *DcAOX2b* (see gene structure in Campos *et al.*, 2009), exon-primed intron crossing-PCR (EPIC-PCR) was performed using 10 ng DNA of 40 *Daucus carota* L. genotypes (14 of cv. Rotin and 26 of wild carrot). Specific primers were designed in the exons flanking each intron (Table 1). To avoid the occurrence of heteroduplex fragments, electrophoresis was performed in denaturing conditions in 3-(*N*-morpholino) propanesulphonic acid buffer (BDH, Haasrode, Belgium) supplied with 2% formaldehyde (Sigma-Aldrich, Steinheim, Germany).

For analysis of intron variation in the *DcAOX2b* sequence, the gene was isolated from six genotypes of cv. Rotin using a forward primer located in the 5′-untranslated region (UTR) and a reverse in the 3′UTR (Table 1). For sequence analysis, EditSeq and the ClustalW algorithm of Megalign (Lasergene 7; GATC Biotech AG, Konstanz, Germany) were applied. Putative miRNA precursors (pre-miRNAs) located in intron sequences were searched and validated as described in Cardoso *et al.* (2009).

Results and discussion

EPIC-PCR fragments from intron 2 and intron 3 show lengths consistently around 430 or 400 bp in all carrot genotypes analyzed. Sequence analysis yielded a size for intron 2 of 91 bp and for intron 3 of 85 bp. All intron 2 sequences were identical. In intron 3 sequences, four putative SNPs were identified.

ILP was found in intron 1 in the genome of individual plants and between plant genotypes. Six polymorphic patterns were identified: three single-band patterns (homozygous) named fragments a, b and c, and three double-band patterns (heterozygous) named ab, ac and bc (Fig. 1). In cv. Rotin and in wild carrot, the PCR-fragment pattern related to the single 1 kb (b) band was found more frequently than other patterns (Fig. 1). No more than two fragments were observed in the same individual plant, consistent with biallelic nature in a diploid species. Breviario *et al.* (2007), using ILPs as molecular markers, described a link between the number of amplified bands and ploidy level of the taxon. ILPs were also reported in the other member of the *AOX2*-subfamily in *D. carota* (Cardoso *et al.*, 2009) and in the *AOX1*-subfamily of other plant species (Naydenov *et al.*, 2005; Costa *et al.*, 2009b; Ferreira *et al.*, 2009).

The length of inton 1 of *DcAOX2b* was reported as 822 bp (Cardoso *et al.*, 2009). The present extended analysis reveals that intron 1 has at least three length variants of 1019 (a), 822 (b) and 557 bp (c).

The existence of SNPs and InDels was confirmed by sequencing. Both types of polymorphisms are known

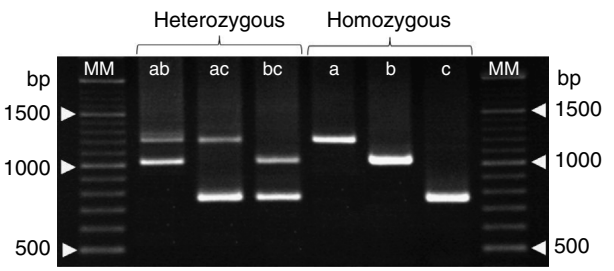

Fig. 1. Agarose gel showing the six-band patterns identified by EPIC-PCR for intron 1 of *DcAOX2b*. From the homozygote genotypes, 40% demonstrated fragment b (seven genotypes of cv. Rotin and nine of wild genotypes), fragment a was detected once in Rotin and also in wild genotypes, fragment c was not detected in cv. Rotin, but was present in four genotypes of wild carrot. From the heterozygous genotypes, the combination of fragments bc was more frequent than other combinations (22%: two genotypes in cv. Rotin and seven in the wild genotypes), the combination ab amplified in three genotypes of cv. Rotin and wild carrots and ac appeared once in cv. Rotin and twice in wild carrot plants. MM: molecular marker (O'Range Ruler 100bp + 500bp Ladder; Fermentas, Ontario, Canada).

as two of the major driving forces in genome evolution (e.g. Gregory, 2004). Twelve putative SNPs were identified between the intron 1 sequences of fragment a and b (the most similar fragments). When fragment c was included in the alignment, 64 additional putative SNPs were identified. Four of them were specific to fragment a (427:T/C, 611:A/C, 852:T/C and 1061:T/C).

Three InDels were identified by alignment of fragments a and b. Integrating the 557 bp intron sequence into the alignment allowed the identification of nine new InDels. In total, 12 InDels were identified, three larger than 50 bp, six with a size between 5 and 50 bp and three shorter than 5 bp. This observation is not in full agreement with Wang *et al.* (2005), who reported that the most frequent InDels (72.6%) had a size of <5 bp, followed (23.5%) by 5–50 bp and very few (3.9%) longer than 50 bp.

The involvement of introns in the regulation of gene expression can be due to the coding of regulatory elements such as miRNAs, recently associated with plant stress responses (Chiou *et al.*, 2006) and development (Wang *et al.*, 2004). In *DcAOX2b* intron 1 sequences, five pre-miRNAs were predicted: two exclusively in fragment a, one in fragment b and two common to both. No pre-miRNA could be predicted for the short fragment c.

The data obtained for the orthologous gene *AOX2b* in *Vigna unguiculata* point to its function in modulating abiotic stress response (Costa *et al.*, 2007). McCabe *et al.* (1998) reported an enhancement of *AOX2b* expression during the ageing of soybean cotyledons concomitant to a reduction of *AOX2a* transcripts and a low level of *AOX1* expression.

The polymorphisms identified by us in *DcAOX2b* are of special interest due to their location in the first intron. Introns that are most proximate to the 5′ end of a gene were observed to exert a more pronounced effect on gene expression (Rose *et al.*, 2008). In future experiments, genotypes with homozygous and heterozygous polymorphic intron 1 sequences will be explored for an association to diverse abiotic stress phenotypes and differential expression of miRNA.

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References

Arnholdt-Schmitt (2009) Alternative oxidase (AOX) and stress tolerance – approaching a scientific hypothesis. *Physiologia Plantarum* 137: 314–315.

Arnholdt-Schmitt B, Costa JH and Fernandes de Melo D (2006) AOX – a functional marker for efficient cell reprogramming under stress? *Trends in Plant Science* 11: 281–287.

Bi McMullenMD IV, Sanchez-Villeda H, Schroedor S, Gardiner J, Polacco M, Soderlund C, Wing R, Fang Z and Coe EH (2006) Single-nucleotide polymorphisms and insertion–deletions for genetic markers and anchoring the maize fingerprint contig physical map. *Genomics, Molecular Genetics and Biotechnology* 46: 12–21.

Braglia L, Manca A, Mastromauro F and Breviario D (2010) cTBP: a successful intron length polymorphisms (ILP)-based genotyping method targeted to well defined experimental needs. *Diversity* 2: 572–582.

Breviario D, Baird W, Sangoi S, Hilu K, Blumetti P and Gianì S (2007) High polymorphism and resolution in targeted fingerprinting with combined β-tubulin introns. *Molecular Breeding* 20: 249–259.

Campos MD, Cardoso HG, Linke B, Costa JH, Fernandes de Melo D, Justo L, Frederico AM and Arnholdt-Schmitt B (2009) Differential expression and co-regulation of carrot AOX genes (*Daucus carota*). *Physiologia Plantarum* 137: 578–591.

Cardoso HG, Campos MD, Costa AR, Campos MC, Nothnagel T and Arnholdt-Schmitt B (2009) Carrot alternative oxidase gene *AOX2a* demonstrates allelic and genotypic polymorphisms in intron 3. *Physiologia Plantarum* 137: 592–608.

Chiou TJ, Aung K, Lin SI, Wu CC, Chiang SF and Su CL (2006) Regulation of phosphate homeostasis by MicroRNA in *Arabidopsis*. *Plant Cell* 18: 412–421.

Costa JH, Jolivet Y, Hasenfratz-Sauder M-P, Orellano EG, Lima MGS, Dizengremel P and Fernandes de Melo D (2007) Alternative oxidase regulation in roots of *Vigna unguiculata* cultivars differing in drought/salt tolerance. *Journal of Plant Physiology* 164: 718–727.

Costa JH, Cardoso HC, Campos MD, Zavattieri A, Frederico AM, Fernandes de Melo D and Arnholdt-Schmitt B (2009*a*) *D. carota* L. – an old model for cell reprogramming gains new importance through a novel expansion pattern of AOX genes. *Plant Physiology and Biochemistry* 47: 753–759.

Costa JH, Fernandes de Melo D, Gouveia Z, Cardoso HG, Peixe A and Arnholdt-Schmitt B (2009*b*) The alternative oxidase family of Vitis vinifera reveals an attractive model for genomic design. *Physiologia Plantarum* 137: 553–565.

Ferreira A, Cardoso HG, Macedo ES, Breviario D and Arnholdt-Schmitt B (2009) Intron polymorphism pattern in *AOX1b* of wild St John's wort (*Hypericum perforatum*) allows discrimination between individual plants. *Physiologia Plantarum* 137: 520–531.

Fiume E, Christou P, Gianì S and Breviario D (2004) Introns are key elements of rice tubulin expression. *Planta* 218: 693–703.

Gianì S, Morello L, Bardini M and Breviario D (2003) Tubulin intron sequences: multi-functional tools. *Cell Biology International* 27: 203–205.

Gregory TR (2004) Insertion–deletion bases and the evolution of genome size. *Gene* 423: 15–34.

McCabe TC, Finnegan PM, Millar H, Day DA and Whelan J (1998) Differential expression of alternative oxidase genes in soybean cotyledons during postgerminative development. *Plant Physiology* 118: 675–682.

McDonald AE and Vanlerberghe GC (2006) The organization and control of plant mitochondrial metabolism. In: Plaxton WC and McManus MT (eds) Control of Primary Metabolism in Plants. *Annual Plant Reviews* 22: 290–324.

Nasu S, Suzuki J, Ohta R, Hasegawa K, Yui R, Kitazawa N, Monna L and Minobe Y (2002) Search for and analysis of single nucleotide polymorphisms (SNPs) in rice (*Oryza sativa, Oryza rufipogon*) and establishment of SNP markers. *DNA Research* 9: 163–171.

Naydenov N, Takumi S, Sugie A, Ogihara Y, Atanassov A and Nakamura C (2005) Structural diversity of the wheat nuclear gene *WAOX1a* encoding mitochondrial alternative oxidase, a single unique enzyme in the cyanide-resistant alternative pathway. *Biotechnology and Biotechnological Equipment* 19: 48–56.

Orgel LE and Crick FH (1980) Selfish DNA: the ultimate parasite. *Nature* 284: 604–607.

Plaxton WC and Podestá FE (2006) The functional organisation and control of plant respiration. *Critical Reviews in Plant Sciences* 25: 159–198.

Polidoros AN, Mylona PV and Arnholdt-Schmitt B (2009) *Aox* gene structure, transcript variation and expression in plants. *Physiologia Plantarum* 137: 342–353.

Rose AB, Elfersi T, Parra G and Korf I (2008) Promoter-proximal introns in *Arabidopsis thaliana* are enriched in dispersed signals that elevate gene expression. *Plant Cell* 20: 543–551.

Wang XJ, Reyes JL, Chua NH and Gaasterland T (2004) Prediction and identification of *Arabidopsis thaliana* microRNAs and their mRNA targets. *Genome Biology* 5: R65–R6515.

Wang X, Zhao X, Zhu J and Wu W (2005) Genome-wide investigation of intron length polymorphisms and their potential as molecular markers in rice (*Oryza sativa* L.). *DNA Research* 12: 417–427.