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The alternative oxidase family of *Vitis vinifera* reveals an attractive model to study the importance of genomic design

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'Genomic design' refers to the structural organization of gene sequences. Recently, the role of intron sequences for gene regulation is being better understood. Further, introns possess high rates of polymorphism that are considered as the major source for speciation. In molecular breeding, the length of gene-specific introns is recognized as a tool to discriminate genotypes with diverse traits of agronomic interest. 'Economy selection' and 'time-economy selection' have been proposed as models for explaining why highly expressed genes typically contain small introns. However, in contrast to these theories, plant-specific selection reveals that highly expressed genes contain introns that are large. In the presented research, 'wet' *Aox* gene identification from grapevine is advanced by a bioinformatics approach to study the species-specific organization of *Aox* gene structures in relation to available expressed sequence tag (EST) data. Two *Aox1* and one *Aox2* gene sequences have been identified in *Vitis vinifera* using grapevine cultivars from Portugal and Germany. Searching the complete genome sequence data of two grapevine cultivars confirmed that *V. vinifera* alternative oxidase (Aox) is encoded by a small multigene family composed of *Aox1a, Aox1b* and *Aox2*. An analysis of EST distribution revealed high expression of the *VvAox2* gene. A relationship between the atypical long primary transcript of *VvAox2* (in comparison to other plant *Aox* genes) and its expression level is suggested. *V. vinifera* Aox genes contain four exons interrupted by three introns except for *Aox1a* which contains an additional intron in the 3′-UTR. The lengths of primary Aox transcripts were estimated for each gene in two *V. vinifera* varieties: PN40024 and Pinot Noir. In both varieties, *Aox1a* and *Aox1b* contained small introns that corresponded to primary transcript lengths ranging from 1501 to 1810 bp. The *Aox2* of PN40024 (12 329 bp) was longer than that from Pinot Noir (7279 bp) because of selection against a transposable-element insertion that is 5028 bp in size. An EST database basic local alignment search tool (BLAST) search of GenBank revealed the following ESTs percentages for each gene: *Aox1a* (26.2%), *Aox1b* (11.9%) and *Aox2* (61.9%). *Aox1a* was expressed in fruits and roots, *Aox1b* expression was confined to flowers and *Aox2* was ubiquitously expressed. These data for *V. vinifera* show that atypically long *Aox* intron lengths are related to high levels of gene expression. Furthermore, it is shown for the first time that two grapevine cultivars can be distinguished by *Aox* intron length polymorphism.

Abbreviations – Aox, alternative oxidase; BLAST, basic local alignment search tool; BLAT: blast-like alignment tool; EST, expressed sequence tag; *TvvAox2*, *Aox2* containing a Ty1/*copia*-retroelement; UTR, untranslated region.

Introduction

A few years ago some efforts were initiated to understand genome evolution through structure models (Castillo-Davis et al. 2002, Huang and Niu 2008, Seoighe et al. 2005, Stenoien 2007). Several hypotheses were proposed to explain the compact organization of highly expressed genes. Among these models, the predominant one proposed that natural selection favored short introns in highly expressed genes in order to minimize the cost of transcription and other molecular processes, such as splicing (Castillo-Davis et al. 2002). However, not all studied organisms show a correlation between gene expression and short introns. It is known that highly expressed genes of various yeasts and unicellular organisms have longer introns than genes that are expressed at low levels (Vinogradof 2001). It has been hypothesized that selection for gene configuration might have been less important in plant evolution because of the fact that selection on genome organization may have acted differently in plant and animal phyla (Ren et al. 2006). It has been reported both in monocots (*Oryza sativa* L.) and dicots [*Arabdopsis thaliana* (L.) Heyenh] that highly expressed genes contain more and longer introns and larger primary transcripts than genes expressed at low levels in spite of the statement that transcription of a single gene requires several minutes and thousands of ATP molecules (Ren et al. 2006). In this case, neither transcriptional efficiency, regional mutational bias, or genomic design favoring open chromatin seems necessary, or appropriate, to explain the relationship between gene structure and gene expression in *Arabidopsis* and rice (Ren et al. 2006). Considering the limited number of studies that have evaluated evolutionary mechanisms related to genome organization in multicellular organisms, it is advantageous to further investigate plant genome structure organization. In this context, using a bioinformatics approach, we evaluated the gene expression pattern of the *Vitis vinifera* alternative oxidase (Aox) family because it relates to intron length differences and in comparison to other plant Aox families.

The Aox is regulated at different levels in plant metabolism by the amount of Aox protein, the redox state of sulfhydryl groups of the Aox dimer, and the reversible stimulation of Aox activity by α-keto acids and pH-dependence (Lima-Júnior et al. 2000, Millenaar and Lambers 2003). Plant Aox is typically encoded by a small gene family of three to five members. All genes are encoded in the nucleus and imported to mitochondria (Considine et al. 2001, Tanudji et al. 1999, Thirkettle-Watts et al. 2003). The multigene family is divided in the two subfamilies *Aox1* and *Aox2*, which contain variable gene numbers depending on the species. *Aox1* is found in monocot and eudicot plants and shows higher protein sequence similarity between species than to *Aox2* genes of the same species. The *Aox1* subfamily appears to be present as several gene copies in a large number of plants while the *Aox2* subfamily appears less expanded (Considine et al. 2002). *Aox2* is only present in eudicots and, so far, multiple copies are found in few plants such as in soybean (Whelan et al. 1996), cowpea (Costa et al. 2004) and carrot (Costa et al. 2009). *Aox1* expression is related to stress responses while *Aox2* expression is defined as being more constitutive. However, more recently, it has been shown that *Aox2* is not just a 'housekeeping' gene but also appears to play a role in plant stress responses (Clifton et al. 2005, Costa et al. 2007).

We report the identification of two *Aox1* and one *Aox2* gene sequences in *V. vinifera* in several cultivars from Portugal and Germany. These data were further supported by recovering Aox genes in the complete genome sequences of two recently published grapevine cultivars (Jaillon et al. 2007, Velasco et al. 2007) that demonstrate that *V. vinifera* possesses a small multigene family composed of *Aox1a, Aox1b* and *Aox2*. The *V. vinifera Aox* family revealed some peculiarities not yet found in other Aox genes, namely, exceptionally long introns, the presence of an additional intron in the 3′-UTR of *VvAox1a* and a retrotransposon element integrated in the ubiquitously expressed *VvAox2*. We propose that *V. vinifera* can be used as a model organism in *Aox* research to study the importance of genome design for the control of gene expression.

Materials and methods

Plant material

The Portuguese *V. vinifera* cultivars 'Touriga Nacional,' 'Gouveio,' 'Trincadeira,' 'Antão Vaz' and 'Aragonês,' and the German cultivar 'Regent' were used in this study. Cuttings from each cultivar were provided from plants growing under field conditions in southern Portugal (Montemor-o-Novo). After being submerged for 50 min in a 1% Benomyl solution, the cuttings were forced to sprout in growth chambers under controlled conditions of humidity (80%) at 27°C and a 16-h photoperiod. Sprouting occurred after 30–45 days. The developed shoots were used to establish in vitro cultures.

The shoots were surface sterilized in 70% (v/v) ethanol for 2 min, followed by immersion in a filtered calcium hypochlorite solution with 10% (w/v) active chlorine for 20 min, and were rinsed three times with sterile water. The uninodal portions of the stem were aseptically excised and inoculated per Nitsch and Nitsch (1969)

in basal medium supplemented with 2% (w/v) sucrose solidified with 0.2% (w/v) Phytagel (Sigma). The pH was adjusted to 5.75 prior to autoclaving (121°C, 98 kPa for 15 min). Cultures were kept at 25 ± 1°C, with a 16-h photoperiod and 34 μmol $m^{-2}s^{-1}$ of light intensity provided by day-light fluorescent lamps (Philips).

Cloning and sequence analysis of *Aox* genes

Young leaves from in vitro grown plants were used for genomic DNA extraction using the DNeasy Plant Mini Kit (50) (Qiagen cat. no. 69104) according to the manufacturer's protocol. The quantification was made by electrophoresis in a 1% agarose gel using Lambda DNA standards and visualized by ethidium bromide staining using the gel image system Gene Tools (Syngene).

The DNA of the different *V. vinifera* cultivars was used as template for *Aox* gene amplification by PCR. Several combinations of degenerate primers were used, some of which targeted the exon 3 conserved region: P1 with P2 (Saisho et al. 1997), and 42AOXFw (5′-GCDGCDGTBCCDGGVATGGT-3′) with 45AOXRev (5′-TCVCKRTGRTGHGCYTCRTC-3′); and some of which targeted the exon 1 region: 40AOX1 (5′-TGGAARTGGAATWGYTTYAGG-3′) combined with 45AOXRev located at the exon 3 region. For the PCR mix, 0.5 U of a Taq DNA Polymerase was used (Fermentas) with 1 × *Taq* Buffer $(NH_4)_2SO_4$, 0.2 m*M* of the four dNTPs (Fermentas) and 0.2 μM of each primer.

PCR with primers P1 and P2 was carried out according to the conditions previously described by Saisho et al. (1997). PCR with the other two primer combinations was carried out with an initial step at 94°C for 5 min, 35 cycles consisting of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 2 min at 72°C for DNA synthesis and a final step at 72°C for 10 min.

The fragments generated by PCR were separately cloned into the pGEM T-Easy vector (Promega, Madison, WI) and used to transform *Escherichia coli* JM109 (Promega) competent cells. Plasmid DNA was extracted from putative recombinant clones (Birnboim and Doly 1979) and analyzed with the restriction enzymes *Eco*RI, *Hpy*F3I, *Alu*I and *Bsp*143I (all from Fermentas). Clones showing different restriction patterns were completely sequenced (Macrogen company: www.macrogen.com) in the directions of sense and antisense strands using the primers T7 and SP6 (Promega). Sequence homology was searched for in the NCBI database (National Center for Biotechnology Information, Bethesda, MD; http://www.ncbi.nlm.nih.gov/) using the BLASTn and BLASTp algorithms (Altschul et al. 1997).

Clustal X (Thompson et al. 1997) was used for sequence alignment and molecular evolution analysis. A phylogenic tree was constructed using the neighbor-joining method, and the reliability of each node was established by bootstrap methods using MEGA4 software (Tamura et al. 2007).

Bioinformatics search of *Aox* genes in genomic or expressed sequence tag databases

In order to identify different members of the *Aox* multigene family for several angiosperms, a basic local alignment search tool (BLAST) search of available non-annotated genomic sequences was carried out in several public databases. Poplar and Sorghum *Aox* genes were retrieved from complete genome databases at the DOE Joint Genome Institute (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html and http://genome.jgi-psf.org/Sorbi1/Sorbi1. home.html, respectively). Lotus *Aox* genes, AP009156 (*Aox1*) and AP007304 (*Aox2a* and *Aox2b*), were obtained from sequences in the high throughput genome sequence (HTGS) database at NCBI. The new Maize *Aox1d* (AC198384) was also retrieved from the HTGS database. *V. vinifera* cultivars PN40024 and Pinot Noir *Aox* genes, AM466432 (*Aox1a*), AM472072 (*Aox1b*) and AM459831 (*Aox2*), were retrieved from the Vitis complete genome (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis) and from sequences in the non-redundant (nr) database at NCBI.

The expressed sequence tag (EST) occurrence of *VvAox1a*, *VvAox1b* and *VvAox2* in different tissues and conditions was obtained using a BLASTn search of each gene nucleotide sequence against the 35 3706 DNA sequences of the *V. vinifera* EST database at NCBI.

Search for the integrated retrotransposon identified within the *VvAox2* intron 2 in other species using PCR

The integrated retrotransposon identified in the *VvAox2* intron 2 (*TvvAox2*) of cultivar PN40024 was searched for in the *V. vinifera* cultivars 'Regent,' 'Touriga Nacional,' 'Gouveio,' 'Trincadeira,' 'Antão Vaz,' and 'Aragonês.' Genomic DNA (10 ng DNA) was used as template in PCR using a specific forward primer *TvvAox2*: 5′-ACCATTACTCGTCCAGACAT-3′ combined with a reverse primer located at the third exon of *VvAox2*: 5′-GAGATCTTAGATGCAGTAGC-3′. For the PCR mix, 0.4 U of Phusion™ high-fidelity DNA polymerase were used (Finnzymes Oy.) with 1× Phusion HF buffer, 0.2 m*M* of the four dNTPs (Fermentas) and 0.2 μ*M* of each primer. PCR was carried out with 35 cycles, each consisting of

10 s at 98°C, 20 s at 52°C and 90 s at 72°C. After the last cycle, a 10 min elongation step at 72°C occurred. The PCR products were analyzed by electrophoresis in a 1.4% (w/v) agarose gel, stained with ethidium bromide and photographed with the gel imaging system Gene Tools (Syngene).

Results

Identification of the Aox multigene family in *V. vinifera* cultivars

Partial DNA sequences of *Aox* genes from the *V. vinifera* cultivars 'Regent,' 'Touriga Nacional,' 'Gouveio,' 'Trincadeira,' 'Antão Vaz' and 'Aragonês' were obtained and indicated the presence of three different genes: *Aox1a*, *Aox1b* and *Aox2*.

The *Aox* sequences from the different cultivars are available in the GenBank database (NCBI): 'Regent' *Aox1a* (EU165202) and *Aox1b* (EU165203); 'Touriga Nacional' *Aox1a* (EU165201) and *Aox2* (EU165200); 'Gouveio' *Aox1b* (EU165199); 'Trincadeira' *Aox1a* (EU165197) and *Aox1b* (EU165198); 'Antão Vaz' *Aox1a* (EU165195) and *Aox1b* (EU165196); 'Aragonês' *Aox1a* (EU165193), *Aox1b* (EU165194) and *Aox2* (EU165192).

A search in the complete genome sequence data of the *V. vinifera* cultivars Pinot Noir and PN40024 confirmed this pattern of AOX gene distribution. A phylogenetic tree based on the deduced amino acid sequences of the three identified genes and various Aox proteins revealed that in *V. vinifera* Aox is encoded by a small multigene family composed of *Aox1a*, *Aox1b* and *Aox2* (Fig. 1).

Intron/exon structure of the full-length *Aox* sequences in cv. Pinot Noir and cv. PN40024

Fig. 2 shows alignments that highlight the intron/exon structures of the *Aox1* (Fig. 2A) and *Aox2* (Fig. 2B) genes of Pinot Noir and PN40024. All genes contained four exons and three introns in the coding region, however, the *VvAox1a* had an additional intron in the 3′-UTR. Analysis of the *Aox* chromosomal position in PN40024 in the Grape Genome Browser (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis) shows that *Aox1a* and *Aox1b* are located at the end of chromosome 2 on anti-parallel strands and are separated by 1857 bp (Fig. 2A), whereas *Aox2* is located on chromosome 12. Both *Aox1a* and *Aox1b* of cv. PN40024 have high sequence similarity, ranging from 96 to 100%, with the orthologous genes of cv. Pinot Noir, including within the promoter and intron regions (Fig. 2A). Similar identities are observed between orthologous *Aox2* genes, except for a 5028 bp insertion in intron 2 in cv. PN40024 (Fig. 2B). Comparison among the paralogous *Aox* genes showed particularly long introns for *Aox2* compared with *Aox1*.

Analysis of the 5028 bp insert in intron 2 of the *VvAox2*

A BLASTn search against the nr *V. vinifera* database using this insert revealed many similar sequences in this species and indicates that it is a highly repeated genomic element. Structure analysis of the repeated element showed three open reading frames (ORFs) flanked by a 5′-PBS (primer binding site) and 3′-PPT (polypurine tract) which are attached to 5′ and 3′ long terminal repeat (LTR) regions of 163 and 162 bp, respectively (Fig. 3). The ORF1 containing 2112 bp encodes for putative gag protein (GAG), protease (PR) and integrase (IN) proteins, the ORF2 of 894 bp encodes for a putative reverse transcriptase (RT) while ORF3 which is 324 bp in length encodes for a putative RNase H (RH) exhibiting the typical order in Ty1/*copia*-retroelements (Pelsy and Merdinoglu 2002).

The sequences of the different domains from the integrated retrotransposon identified in intron 2 of *VvAox2* (*TvvAox2*) were compared with those reported for Ty1/*copia*-retrotransposons found in other species. This revealed identity with characteristically conserved amino acids (Fig. 3).

A blast-like alignment tool (BLAT) search at the Grape Genome Browser (PN40024 cultivar genome) using the identified *TvvAox2* revealed several similar sequences (97 to 98.4% identity) located in different chromosomes. Insertion of this retroelement was identified in the introns of several other genes (Table 1).

Search for the retrotransposon insertion in intron 2 of *VvAox2* in six *V. vinifera* cultivars

Insertion of the Ty1/*copia*-retrotransposon in intron 2 of the *VvAox2* was explored in the *V. vinifera* cultivars 'Regent,' 'Touriga Nacional,' 'Gouveio,' 'Trincadeira,' 'Antão Vaz' and 'Aragonês' by PCR using a specific forward primer at the *TvvAox2* with a reverse primer at exon 3 of *VvAox2*. No band was amplified, indicating that this insertion might not be present in these cultivars (data not shown).

Primary transcript length of different plant Aox multigene family members

The estimated primary transcript length for the members of the *Aox* multigene family in angiosperms was investigated by taking into account the publication data and conducting a bioinformatics search for available

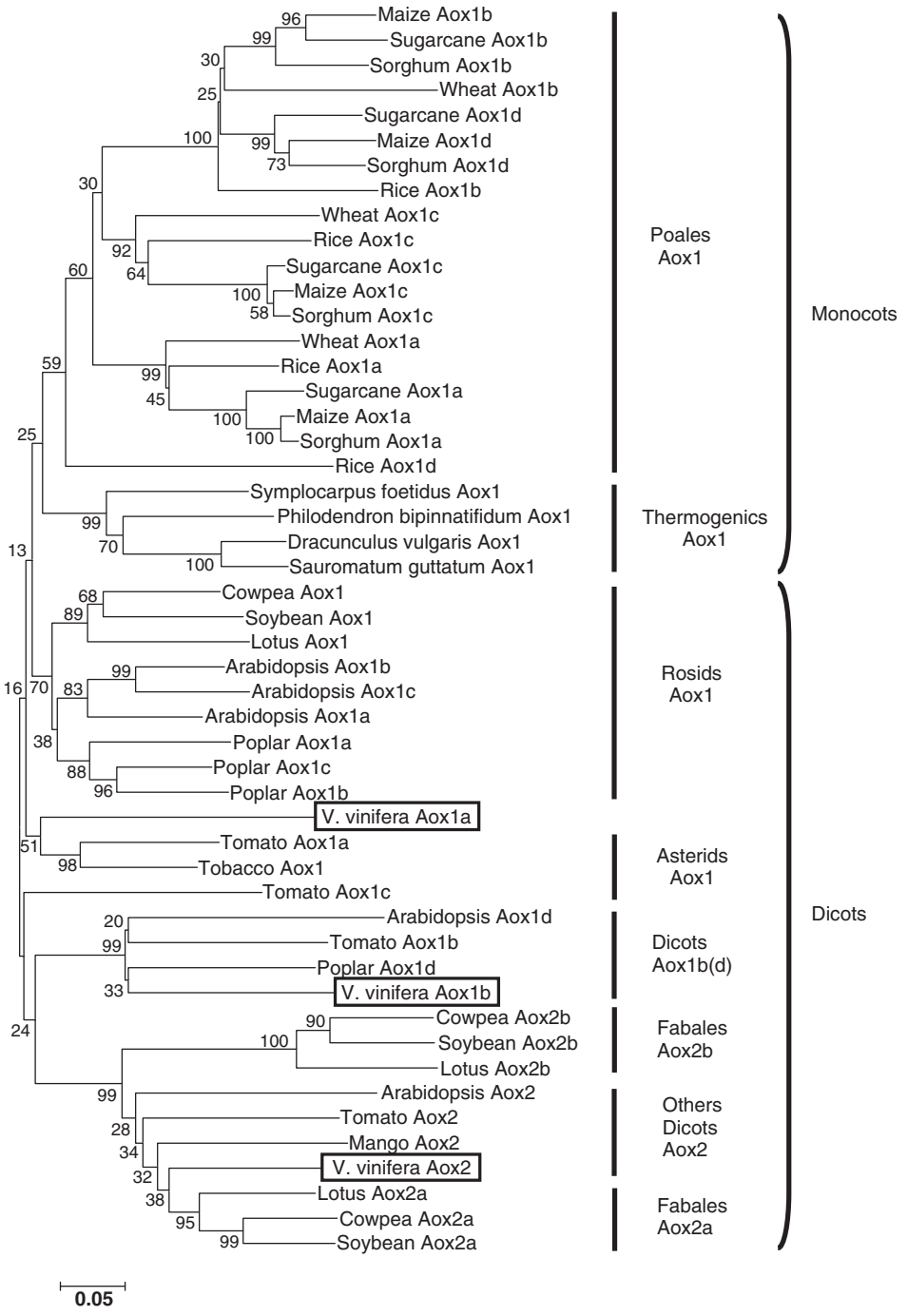

Fig. 1. Phylogenetic tree of 50 Aox proteins from several plants highlighting the positions of Aox1a, Aox1b and Aox2 of *V. vinifera*. The tree was obtained by the *neighbor-joining* method (Saitou and Nei 1987). Classification of Aox proteins is according to Considine et al. (2002). Horizontal distances are proportional to evolutionary distances according to the scale shown on the bottom. The tree was displayed with the MEGA4 program showing bootstrap values (from 1000 replicates).

genomic and EST sequences. Fig. 4 shows the primary transcript lengths of the different *Aox* subfamilies of *V. vinifera* cultivars PN40024 and Pinot Noir compared with those of five dicots (cowpea, soybean, lotus, Arabidopsis and Poplar) and three monocots (rice, maize and Sorghum). The primary transcript length profile of the *Aox1* subfamily was similar among all species, ranging from 1450 bp in the *Arabidopsis Aox1b* to 2809 bp

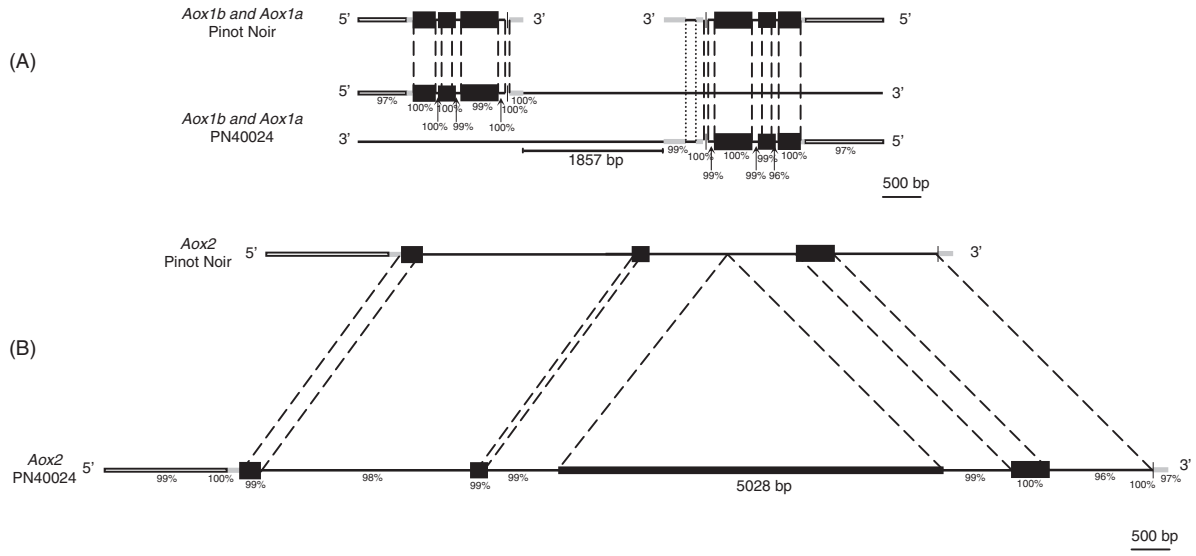

Fig. 2. Comparison of the *Aox* genes in PN40024 and Pinot Noir cultivars of *V. vinifera* using a scale diagram of the intron/exon structure of the *Aox1* (A) and *Aox2* (B) genes. Filled boxes represent exons and lines among exons represent introns. Promoters are represented by a narrow rectangle in the 5′ end. The 5028 bp sequence inserted in intron 2 of the *VvAox2* of the PN40024 cultivar is shown. Bar corresponds to 500 bp of chromosomal DNA.

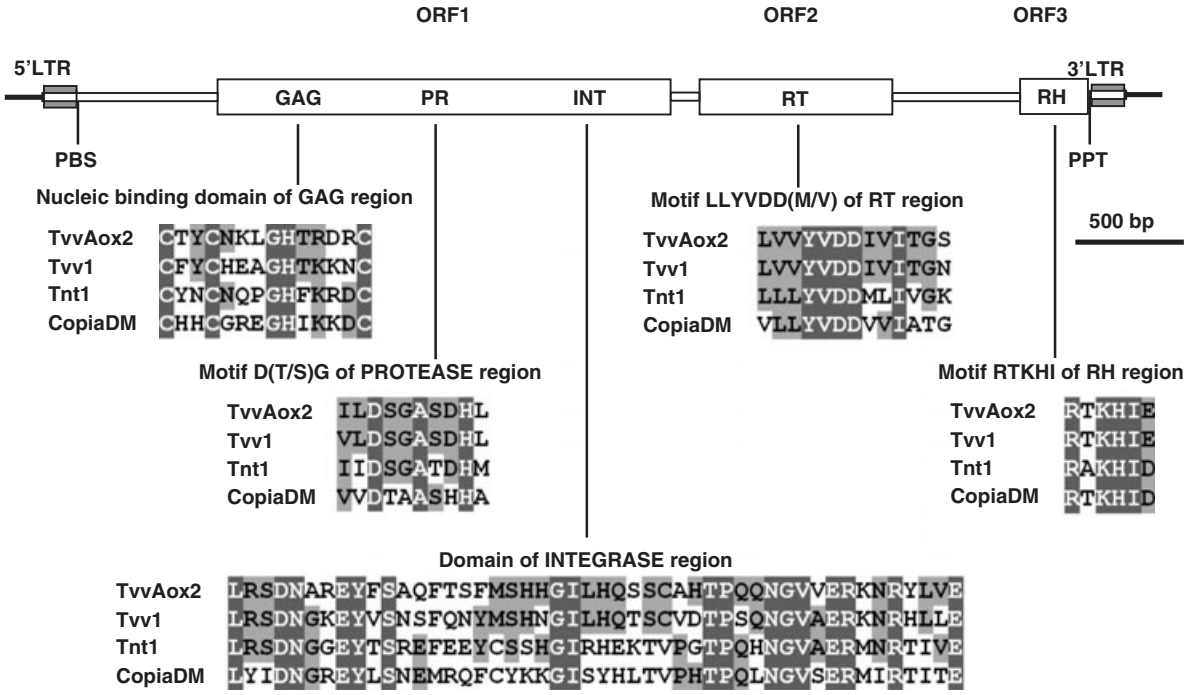

Fig. 3. *TvvAox2*, a *copia* retrotransposon inserted in intron 2 of the *VvAox2* of *V. vinifera*. The structure of the *TvvAox2* element is schematically shown at the top of the figure. Alignment of amino acid sequences of relevant domains corresponding to GAG, protease, integrase, RT and RNase H proteins of *Tvv1* of *V. vinifera* (Pelsy and Merdinoglu 2002), *Tnt1* of tobacco (Grandbastien et al. 1989), and *copia* of *Drosophila* (Mount and Rubin 1985) retrotransposons are shown together with those encoded by the *TvvAox2* element.

in the soybean *Aox1*. However, the *Aox2* subfamily of PN40024 and Pinot Noir presented a distinct profile compared with other dicots and with each other. The *Aox2* primary transcript length ranged from 1960 bp in the *Arabidopsis Aox2* to 3097 bp in the soybean *Aox2a* among other dicots. In cv. Pinot Noir, the *Aox2* transcript length was 7279 bp and for cv. PN40024 it was 12 329 bp.

Table 1. BLAT search of similar Ty1/*copia*-retroelement gene inserted at the PN40024 cultivar in Grape Genome Browser (http://www.genoscope.cns.fr/externe/ GenomeBrowser/Vitis) using the identified *TvvAox2*. The most similar sequences with *TvvAox2* ranging from 97 to 98.4% of identity were analyzed. Repeated sequences not gene inserted are indicated by (–).

Identity with *TvvAox2* (%)	Chromosome	Aligned sequence length (bp)	Intron length (minus inserted repeated sequence)/involved intron	Gene involved with retroelement insertion	Gene length/intron number
100	chr12_random	5028	1857 bp/intron 2	Aox	12 561 bp/3 introns
98.4	chr4	5074	3919 bp/intron 19	Phosphatidylinositol 4-kinase	46 835 bp/20 introns
98.3	chr7	5040	–	–	–
98.3	chrUn_random	4931	4978 bp/intron 20	Hypothetical protein nuclear matrix protein-related	119 806 bp/22 introns
98.2	chrUn_random	5021	–	–	–
98.1	chr13	5051	6823 bp/intron 4	Hypothetical protein	16 879 bp/7 introns
98.1	chr17_random	4989	–	–	–
98.1	chr14	4784	–	–	–
97.9	chr7	4992	–	–	–
97.9	chr14	5008	26 988 bp/intron 5	Dehydroascorbate reductase	53 580 bp/5 introns
97.8	chr5	5052	–	–	–
97.8	chr18_random	5031	–	–	–
97.6	chr9	5034	–	–	–
97.5	chr16_random	5056	–	–	–
97.5	chr12	5025	12 897 bp/intron 5	Hypothetical protein	36 573 bp/14 introns
97.5	chr19	5041	2480 bp/intron 6	Diacylglycerol kinase	23 418 bp/11 introns
97.5	chr11	5042	11 832 bp/intron 4	CD2-binding protein-related	28 247 bp/9 introns
97.4	chr5	5041	5214 bp/intron5	Na^+/myo-inositol symporter	32 914 bp/5 introns
97.4	chr18	5037	9978 bp/intron 6	Voltage-gated potassium channel	33 888 bp/10 introns
97.4	chr11	5035	5110 bp/intron 11	Nucleotidase-like protein	69 225 bp/11 introns
97.4	chr8	5038	9420 bp/intron 3	Voltage-gated potassium channel	17 815 bp/6 introns
97.4	chr1	5087	9997 bp/intron 15	Plasmalemma Na^+/H^+ antiporter (*sos1* gene)	62 459 bp/22 introns
97.4	chr14	5045	913 bp/intron 2	Nucleic acid binding	10 003 bp/5 introns
97.4	chr1_random	5040	–	–	–
97.3	chr4	5025	6050 bp/intron 11	Translation activator	78 455 bp/57 introns
97.3	chr1	5038	–	–	–
97.2	chr1	4992	13 079 bp/intron 5	U2 small nuclear ribonucleoprotein A	23 262 bp/6 introns
97.2	chr8	5047	8575 bp/intron 2	Hypothetical protein regulation of pre-mRNA	49 550 bp/7 introns
97.2	chr11	5020	3439 bp/intron 1	Methyltransferase	9422 bp/1 intron
97.2	chr1_random	5026	5366 bp/intron 9	Mannosyl-oligosaccharide glucosidase	27 257 bp/21 introns
97.0	chrUn_random	5018	2787 bp/intron 6	Ubiquinone biosynthesis protein AarF	65 009 bp/12 introns

EST frequency of V. vinifera *Aox* genes

The EST frequency of *VvAox1a, VvAox1b* and *VvAox2* was obtained from a BLASTn search using each gene sequence against 35 3706 sequences from the *V. vinifera* EST database at NCBI. The percentages of ESTs for each gene were: *Aox1a* (26.2%:11 ESTs), *Aox1b* (11.9%: 5 ESTs) and *Aox2* (61.9%: 26 ESTs) (Fig. 5A). *Aox1a* was detected in fruit pulp (5 ESTs from 11), berry (3 ESTs from 11) and roots (3 ESTs from 11) while *Aox1b* was confined to flowers (5 ESTs from 5). *Aox2* was found in roots (3 ESTs out of 26), leaves (2 ESTs out of 26), fruit pulp (6 ESTs out of 26), berry (10 ESTs out of 26), flowers (3 ESTs out of 26), seeds (1 EST out of 26) and cell suspension (1 EST out of 26) cDNA libraries (Fig. 5B).

Discussion

The strongest challenge for 'wet' biology related to stress research is to link molecular bottom-up and whole plant top-down approaches in favor of crop improvement strategies (Arnholdt-Schmitt 2005, Hammer et al. 2004). The gap between genomics and phenomics must be overcome that fundamental knowledge in stress research can be applied directly to plant breeding. This is still a difficult task (Collins et al. 2008, www.generationcp.org). Grapevine offers a good possibility to advance current knowledge, because the genomes of two grapevine varieties, PN40024 (ca. 93% homozygous) and Pinot Noir (heterozygous), have been recently sequenced (Jaillon et al. 2007, Velasco et al.

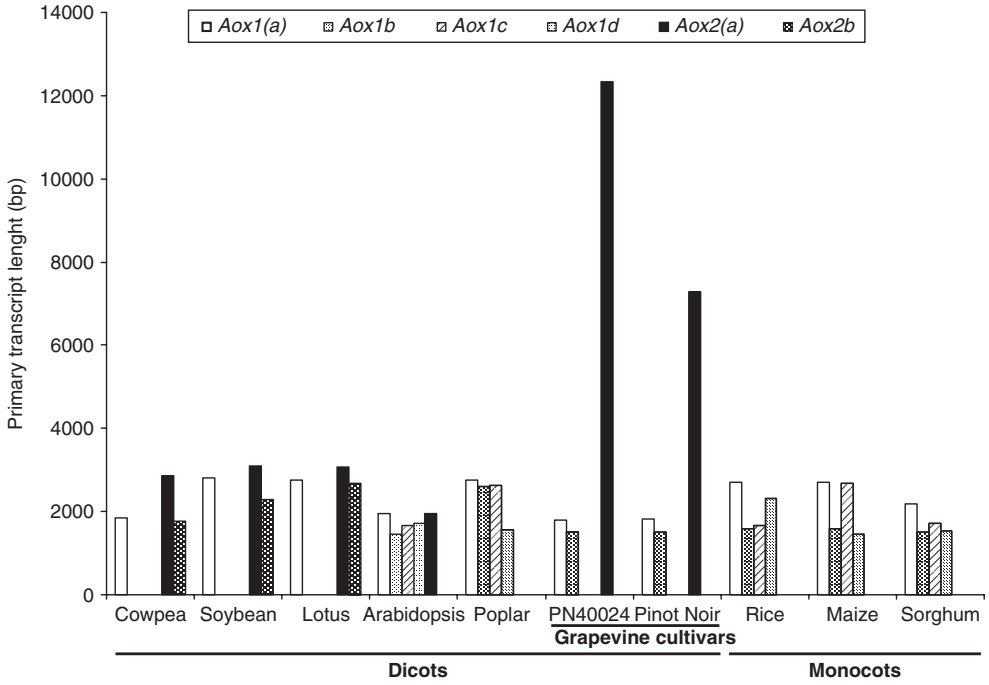

Fig. 4. Comparison of the primary transcript lengths of available Aox gene family members between dicot and monocot species. The 5′- and 3′-UTRs (untranslated regions) of primary transcripts were estimated by full-length cDNAs or EST data.

2007). Thus, expression data that have been obtained in laboratories can be studied by bioinformatics at wider genome level to get novel insights into general rules for genome regulation and the importance of 'genomic design' (Arnholdt-Schmitt 2004, Vinogradov 2004). This strategy can lead to a new class of 'structural' markers for breeding. Highlighting structural markers such as intron length in genes that are involved in the adaptive behavior of plants is of special interest in this context. Results from the grapevine genomes in addition to the 'wet' identification of *Aox* genes from different *V. vinifera* cultivars lead us to conclude that Aox is encoded by a small family composed of three genes: *VvAox1a*; *VvAox1b* and *VvAox2*. The clear difference in *VvAox2* gene size (Figs 2 and 4) in comparison to other published plant Aox genes is striking. Thus, we identified complete plant Aox families from several angiosperms using public genome sequence databases in order to compare these with *VvAox2* and to attempt to understand the length peculiarity of this gene.

In our comparative search, we identified previously unknown Aox gene families in Poplar, lotus and Sorghum. According to the phylogenetic analysis (Fig. 1), the Poplar *Aox* family retrieved from the complete genome (Tuskan et al. 2006) was closely related to that of *Arabidopsis*. These genes are described here for the first time. *Aox2* was not found in the Poplar genome, making this the first eudicot species that reveals an absence of *Aox2*, suggesting that this gene was lost during or after the speciation event. Furthermore, distinct Aox1 clades are revealed through our analyses. In both species, three typical *Aox1* genes (*Aox1a*, *Aox1b* and *Aox1c*) and one *Aox1d* were found, suggesting that there were two *Aox1* ancestors before speciation of Poplar and *Arabidopsis*. After speciation, the typical *Aox1* ancestor was apparently duplicated originating in *Aox1a, Aox1b* and *Aox1c* because paralogous genes of Poplar and *Arabidopsis* are in adjacent clades. Another Aox family that reveals an interesting evolutionary insight is lotus. Although sequencing of the genome is not yet completed, analysis of this Aox family demonstrated the presence of one *Aox1* gene on chromosome 2 (AP009156) and *Aox2a* and *Aox2b* genes located in tandem on chromosome 4 (AP007304) revealing a similar profile of orthologous genes in comparison with cowpea (Costa et al. 2004) and soybean (Thirkettle-Watts et al. 2003). Both belong to the order of Fabales. As monocot models, Sorghum *Aox* genes are orthologous to four sugarcane *Aox1*-type (*Aox1a–1d*), which were previously identified by Borecky et al. (2006). We have also found a fourth *Aox* (*Aox1d*) in the closely related species maize, previously reported as a small family of three members (Karpova et al. 2002). In fact, *Aox1d* appears to be a duplication of *Aox1b* in Sorghum,

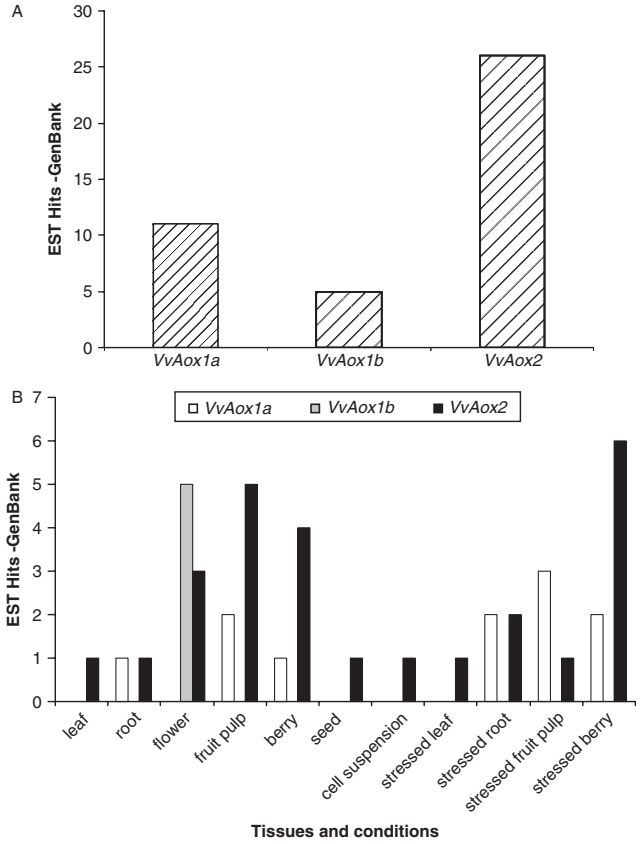

Fig. 5. EST frequency of *V. vinifera* Aox genes in the NCBI database. (A) Total EST number for each Aox gene; (B) EST number of each Aox gene from particular tissues and seedling conditions.

sugarcane and maize, which is not the case in rice (Fig. 1).

The structure of the *VvAox1b* and *VvAox2* genes consists of four exons interrupted by three introns as previously noted in the majority of plant *Aox* genes (Considine et al. 2002). However, *VvAox1a* revealed an additional intron in the 3′-UTR, and this is the first example of an Aox gene with this structure. The existence of UTR introns remains quite mysterious; whereas the removal of introns from coding regions is clearly necessary for accurate translation of full-length proteins, the necessity of splicing in non-coding regions is less obvious (Roy et al. 2007). A possible significance for gene regulation might come through the fact that the presence of introns in the 3′-UTR can affect post-transcriptional expression levels as demonstrated for the EF1*a*-A3 gene of *Arabidopsis* (Chung et al. 2006). In addition, an intron in the 3′-UTR can subject mRNAs to nonsense-mediated mRNA decay (NMD), a mechanism that identifies and eliminates aberrant mRNAs (Kertesz et al. 2006).

V. vinifera Aox genes revealed a peculiarity associated with the atypical *Aox*2 intron length. This is caused by the integration of a repeated element in intron 2 of the *VvAox2* gene of the PN40024 cultivar (Fig. 2). Analysis of the Pinot Noir cultivar sequences AM466432 (*Aox1a*) and AM472072 (*Aox1b*) also indicated that a repeated element appears to be inserted downstream of the 3′ ends of the *VvAox1a* and *VvAox1b* which separates both genes (data not shown).

The repeated element integrated into intron 2 of the *VvAox2* of cv. PN40024 was classified as a Ty1/*copia*-LTR retrotransposon and named TvvAox2 (Fig. 3). This retrotransposon appears to be absent in intron 2 of the *VvAox2* of 'Regent,' 'Touriga Nacional,' 'Gouveio,' 'Trincadeira,' 'Antão Vaz' and 'Aragonês' cultivars. A BLAT search at the PN40024 genome browser revealed several similar sequences allocated in different chromosomes and integrated into the introns of several other genes (Table 1). The more frequently affected genes were involved in membrane transport (Na^+/myo-inositiol symporter;

voltage-gated potassium channel; phosphatidylinositol 4-kinase and plasmalemma Na^+/H^+ antiporter), or were DNA/RNA-related (nucleotidase-like protein; U2 small nuclear ribonucleoprotein A; nucleic acid binding; regulation of pre-mRNA and translation activator) or involved in ROS defense (Aox and dehydroascorbate reductase). The role of transposable elements (TEs) appears puzzling but it has been suggested that they are involved in gene regulation and contribute to the adaptive fitness of their host (Arnholdt-Schmitt 2004). TE insertion in a gene or a regulatory region in a specific lineage can potentially induce alternative splicing and/or change gene expression patterns, which can result in a relatively rapid change in the function of a gene (Xu and Ramakrishna 2008). Ohtsu et al. (2005) reported the isolation of transposon elements of the *Mu* superfamily in rice that had acquired host genes during evolution and among them *Aox1c* gene sequences. Insertion of the retrotransposon *Tvv*1 or *Gret*1 (grapevine retrotransposon 1) in the promoter sequence of VvMYBA1w in grapevine is involved in an important quality trait for grapevine berries, the fruit color (Kobayashi et al. 2004, Walker et al. 2007). Pereira et al. (2005) found polymorphisms in the element between cultivars, but stability between clones of the same cultivar. Insertion of the TE in stress-inducible Aox genes can be suspected to modify gene regulation and plant behavior related to adaptive traits. Thus, they have the potential to provide a source for functional marker development (Arnholdt-Schmitt et al. 2006).

The integrated Ty1/*copia*-LTR retrotransposon in the second intron of PN40024 *VvAox2* contributed to the longer *VvAox2* primary transcript length when compared with that of the Pinot Noir cultivar. However, it can be seen that both grapevine cultivars presented atypically long *VvAox2* introns when compared with other angiosperm *Aox* genes (Fig. 4). Transcription is a slow and expensive process. In eukaryotes, approximately 20 nucleotides can be transcribed per second at the expense of at least two ATP molecules per nucleotide (Castillo-Davis et al. 2002). Thus, it is surprising that the long *VvAox2* transcript is ubiquitous and present in high abundance compared with the compact *VvAox1a* and 1*b* transcripts (Fig. 5). In fact, *Aox2* genes appear more constitutively expressed and are related to development (Considine et al. 2002) and also to stress responses in some species (Clifton et al. 2005, Costa et al. 2007). In cowpea and soybean, *Aox2b* is ubiquitous while *Aox2a* is confined to photosynthetic tissues (Considine et al. 2002, Costa et al. 2004, Finnegan et al. 1997). However, this cannot be seen as a rule, because the low EST proportions of tomato and tobacco *Aox2* in relation to other paralogous *Aox* genes (data not shown) indicate that this gene is not highly expressed in all species. Thus, it is vital to understand the role of *VvAox2* intron lengths in view of the time and energetic costs of transcription.

In this context, it is of interest that Ren et al. (2006) found that highly expressed plant genes are less compact. However, their results revealed that intron length differences were not relevant for selection based on length. In our particular case, these differences seem to be relevant considering that Pinot Noir *VvAox2* is at least 2.5 times longer than the longest *Aox* gene found to date (Fig. 4). It is known that introns are involved in a variety of regulatory phenomena such as RNA stability (Haddrill et al. 2005, Shabalina and Spiridonov 2004), post-transcriptional gene regulation (Carlini et al. 2001, Shabalina and Spiridonov 2004), nucleosome formation and chromatin organization (Mattick and Gagen 2001, Shabalina and Spiridonov 2004, Vinogradov 2005) and separating the functional domains of proteins (Duester et al. 1986). Thus, it could be hypothesized that any or a combination of the above phenomena could have shaped the structural configuration of *VvAox2* resulting in its ubiquitous expression.

On the basis of our findings, we propose that the Aox family of *V. vinifera* is an attractive model to study the role of genome organization for the control of gene expression. The results together with the recent knowledge on *Aox* intron length polymorphism in other species such as carrot (Cardoso et al. 2009) and St. John's Worth (Ferreira et al. 2009) suggest that research on 'genomic design' is also an important issue in view of future strategies in molecular plant breeding. The described insertion of a retroelement into intron 2 marked the grapevine cultivar PN40024. This insertion was not found in the other cultivars under investigation. Future studies should reveal the importance of this insertion by 'wet' experimentation including expression analyses by real-time PCR, in vivo measurements of Aox activity and association studies.

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