

Isolation of alternative oxidase (AOX) gene sequences of *Hypericum perforatum* L. reveals intron polymorphism

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ABSTRACT

The present work is linked to the EU Marie Curie Chair project 'Stress adaptation in plants - A molecular approach of socio-economic interest'. This larger project is related to understanding the role of AOX as a general marker for the initiation of adaptive stress reactions in plant physiology. In this study, we started to isolate AOX gene sequences of *Hypericum perforatum* L. using the P1 and P2 primers to amplify a preserved region within exon3 (1). We identified four distinct AOX sequences, three belong to AOX1 and one belongs to the subfamily AOX2. The existence of two AOX subfamilies in *H. perforatum* L. is in accordance with current observations in other dicots (2). Our results strongly suggest that *H. perforatum* L. specific AOX expression is regulated by a multigene family, as it is already known for other dicot species, such as *Olea europaea* (3), *Vigna unguiculata* (4), *Arabidopsis thaliana* (1), *Glycine max* (5), and *Nicotiana tabacum* (6). Additionally, the use of degenerated primers that were originally designed to isolate genomic sequences in *Daucus carota* L., permitted the isolation of three genomic AOX gene sequences with introns. Two sequences belong to the AOX1 and one to the AOX2 subfamilies. Between these two AOX1 sequences we found total homology in the exon regions, however, we observed length polymorphism in both included introns, being one sequence larger than the other (1349 to 1408 pb). The intron length polymorphism (ILP) was identified in several plants of a wild *H. perforatum* collection.

MATERIAL AND METHODS

Young leaves of *in vitro* cultured plants of *H. perforatum* L. were used for DNA extraction using the DNEasy kit (Qiagen). RAPD fingerprints were performed with six plants from different accessions in Portugal using 20 primers of the random primers Kit D (Roth). AOX multigene fragments were amplified with the degenerate primers P1 and P2 for a conserved region within exon 3 (1) and degenerate primers originally designed to isolate genomic sequences in *Daucus carota* L. 41aox1, 40aox2, 41aox2 and 44aoxR (Costa et al., in prep.). All fragments were inserted in the pGEM®-T Easy Vector (Promega). Competent *E. coli* JM109 cells (Promega) were transformed with the reaction product. Plasmid DNA was extracted using the alkaline lyses protocol (7) and was characterized by the restriction enzymes *Eco*RI, *Hpy*F3I and *Alu*I (Fermentas). One hundred and twenty six recombinant clones were analysed and 55 polymorphic clones were selected for sequencing. The identification of sequences considered as AOX genes were based on high homology between the isolated sequences and AOX sequences available in the NCBI GenBank. The search of homologue sequences was done on the NCBI data base using the BLAST algorithm (8) (<http://www.ncbi.nlm.nih.gov/>). The alignments among *H. perforatum* DNA sequences and homologous sequences from data base were performed using Clustal W method in the BioEdit software. MEGA 3.1 software was applied for dendrogram construction. For the analysis of the polymorphism in the introns were designed specific primers in the first and third exons. The PCR results were analyzed in an electrophoresis gel (2%) stained with ethidium bromide (2ng/ml).

RESULTS

AOX genes identification

The dendrogram shows three *H. perforatum* sequences belonging to AOX1 and one belonging to the AOX2 subfamily



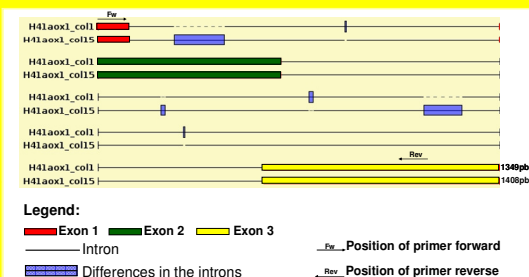
Dendrogram constructed of putative peptides from part of exon 3 (444pb) of AOX from plants and fungi (GenBank of NCBI and Swiss-Pro/TrEMBL). The dendrogram is Neighbor-Joining (1000 bootstrap). The bar indicates 0,05 substitutions per site.

RAPD fingerprint analyses identified three different subspecies genotypes among six plants from different *H. perforatum* accessions.

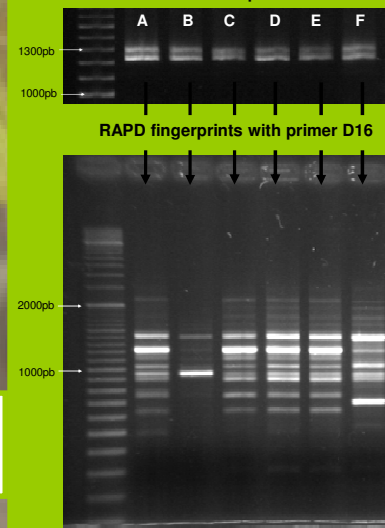
All six plants of the wild *H. perforatum* accessions demonstrated identical ILPs (AOX1)

Intron lenght polymorphisms (ILPs)

In two genomic AOX1 sequences, showing total homology between corresponding exons, ILPs were found in introns 1 and 2



ILPs in six plants



CONCLUSIONS

- Four different AOX gene sequences were identified, three belong to AOX1 and one belongs to the AOX2 subfamily;
- Two genomic AOX1 sequences with identical exons demonstrated length polymorphism in both included introns, being one sequence larger than the other (1349 to 1408 pb);
- The same ILP was identified in diverse genotypes of a wild collection suggesting this ILP a molecular marker candidate for the species *H. perforatum*.

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