

Characterization of a collection of *Hypericum perforatum* L. plants by intron length and sequence polymorphism in genes of β -Tubulin (*TB*) and the alternative oxidase (*AOX*)



FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Alexandre Oliveira Ferreira, Hélia Guerra Cardoso and Birgit Arnholdt-Schmitt*

EU Marie Curie Chair, ICAAM, Universidade de Évora, 7002-554 Évora, Portugal

* Corresponding author: eu_chair@uevora.pt



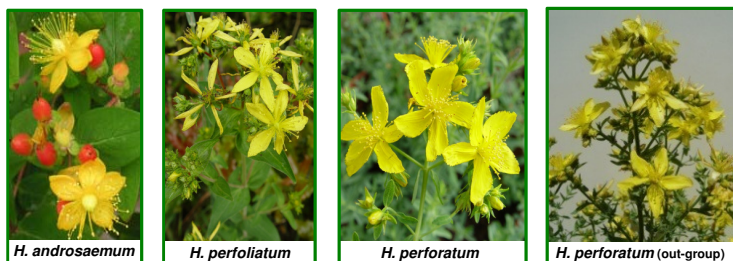
Abstract

A small collection of tetraploid *Hypericum perforatum* L. plants has been established from the region of Alentejo in Portugal. This collection will be used to identify plant genotypes with diverse growth behavior for the development of *AOX* as a functional marker candidate. The alternative oxidase (*AOX*) is recently proposed as a marker for stress tolerance behavior and yield stability. The approach requires near-homogenous genetic backgrounds but diversity in the candidate gene *AOX*. The combined characterization of intron1 and intron2 of β -tubulin by exon-primed intron crossing PCR (EPIC-PCR by degenerated primers) was recently proposed as a rapid and handy methodology to screen genotype diversity across species. Here we report the application of this novel technique to screen the *H. perforatum* collection for homogeneity. In *AOX1b* identity in exon sequences but high sequence polymorphism rates by insertion/deletion events as well as SNPs in intron1 and intron2 were discovered which allow discriminating between individual plants. Prediction of pre-microRNA was affected by an InDel in intron 1.

Materials and Methods

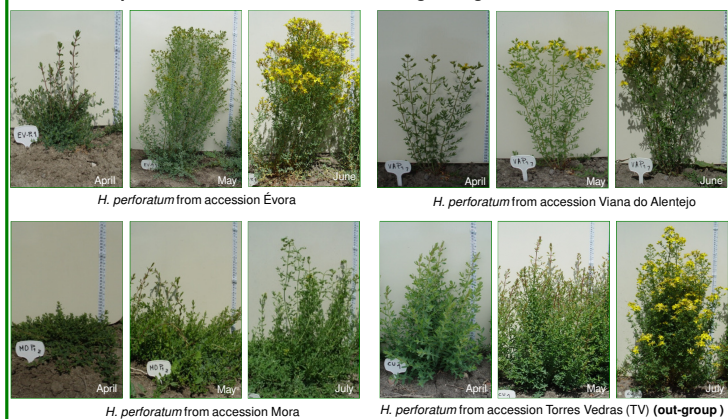
Plant Material and DNA Extraction

Plants of three species from the genus *Hypericum* were used for genomic DNA isolation: one plant of *H. androsaemum* L. (Ha), one plant of *H. perforatum* L. (Hpl) and 31 plants of *H. perforatum* L. (Hp) being 30 from an collection representing the Alentejo-Portugal region and 1 plant (TV) from Extremadura-Portugal considered as out-group in this trials (this plant shows a different phenotype comparing with the other *H. perforatum* plants).



The collection was established using seeds collected in 12 wild populations in Alentejo-Portugal: Mora (MO), Évora (EV), Viana do Alentejo (VA), Nisa (NI), Ponte de Sôr (PS), Elvas (EL), Beja (BE), Serpa (SE), Aljustrel (AL), Odemira (OD), Mértola (ME) and Ourique (OU). A previous study made in field conditions revealed differences in the phenotype of plants provided from different accessions and also in agronomic traits (see figures below).

Plants provided from different accessions growing under field conditions



Total DNA was prepared using a DNAeasy Plant Mini Kit (Qiagen). The genomic DNA integrity was analysed by electrophoresis in 1% agarose after staining in an ethidium bromide solution (0.2 μ g/ml). DNA was quantified by using defined amounts of lambda DNA and the software GeneTools (SynGene).

β -Tubulin Based Polymorphism (TBP/cTBP) Method

Exon Priming Intron Crossing (EPIC)-PCRs were performed using degenerated primers of β -Tubulin genes to amplify intron 1 and 2 (Breviaro *et al.* 2007). The reactions were made using PuReTaq Ready-To-GoTM PCR Beads (GE Healthcare) following the protocol described by Braglia *et al.* (2010).

HpAOX1b Based Polymorphisms Method

For the analysis of the Intron Length Polymorphism (ILP) in the intron 1 and 2 of the *H. perforatum AOX1b*, specific primers were designed in the first and third exons of that gene. The EPIC-PCRs were carried out as described in Ferreira *et al.* (2009).

References

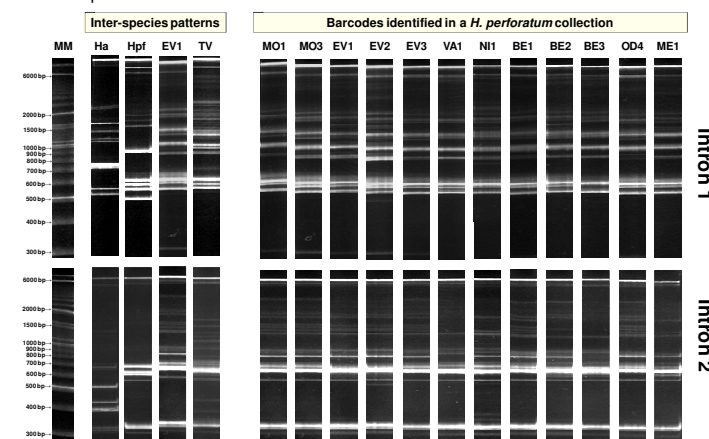
Braglia L., Manca A., Mastromauro F. and Breviaro D. (2010) Diversity 2: 572-585.
Breviaro D., Baird V., Sangoi S., Hilli K., Blumetti P. and Giani S. (2007) Molecular Breeding 20: 249-259.
Ferreira A., Cardoso H. and Arnholdt-Schmitt B. (2009) Physiologia Plantarum 137: 520-531.

Results

Genomic Profile of β -Tubulin Based Polymorphisms (TBP)

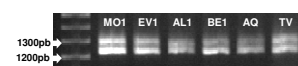
TBP/cTBP barcodes clearly distinguishes plants from three species of the *Hypericum* genus (see figure below at the left). The band patterns indicate the reliability of the method to study phylogenetic relation between species, showing consistent relationship with plant classification. To the contrary, the barcodes show that plants from the same species (both figures) share a high number of bands. The *H. perforatum* plants and the plant of *H. perforatum* show more bands in common than the plant of the species *H. androsaemum*, confirming that the first two species are more closely related.

The analysis made in 30 plants of a *H. perforatum* collection showed high similarity in the genetic background. However, 12 TBP/cTBP band patterns could be discriminated (see figure below at the right). Intron 1 alone distinguishes the 12 genotypes. From these, 8 are also distinguished by different band patterns in intron 2.



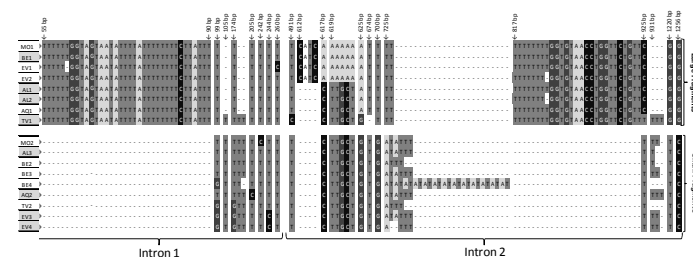
Genomic Profile of *HpAOX1b* Based Polymorphisms

EPIC-PCR revealed natural variability in the region including intron 1 and 2 of *HpAOX1b*. Intron length polymorphisms (ILP) were identified at individual plant level. A common two bands pattern was identified in all plants of the collection.



Large and small fragments of six genotypes were sequenced. Insertions/deletions (InDels), responsible for the ILPs, and single nucleotide polymorphisms (SNPs) were identified (see figure below in which the positions of the nucleotides in the sequence alignment are given on top of the figure representing only the polymorphic sites. Position numbers are related to the alignment of the complete sequences).

All plants were tetraploid. In some genotypes the four putative alleles of *HpAOX1b* were identified, for example in sequences from Évora and Beja [EV1, EV2, EV3, EV4] and [BE1, BE2, BE3, BE4], respectively.



In silico analysis in intron 1 allowed to predict 4 pre-miRNAs in the small fragments, but only one in the larger fragments.

Acknowledgements

This work was supported by the European Commission through the Marie Curie Chair and by Foundation for Science and Technology (FCT). The authors would like to thank Maria Rosário Felix, Laboratory of Plant Virology, University of Évora, for her help to establish polyacrylamide electrophoresis.