Molecular and physiological analysis of drought response in the Mediterranean conifer *Pinus pinaster* Ait.

Nuria de María, María Ángeles Guevara, José Antonio Cabezas, Enrique Sáez-Laguna, Marina de Miguel, Luis Manuel Díaz, Alberto Pizarro, Carmen Collada, David Sánchez-Gómez, Estrella Cadahía, Ismael Aranda, M. Teresa Cervera

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**Background**

Drought is a major environmental stress factor that determines the growth, development and survival of plants living in water scarce environments. Climate change predictions point at increasing dryness over the Mediterranean region. *Pinus pinaster* Ait. is an important Mediterranean conifer subjected to recurrent drought periods. Notwithstanding its relatively small geographical range this species, which is found along a rainfall cline, is characterized by a significant genetic and adaptive diversity. Different morphological and physiological responses appear to play an important role in drought adaptation of this conifer [1]. These complex responses to drought, from perception to transcriptional, metabolic and physiological changes, need to be considered at a global systems biology level to study the multiple interactive components [2]. Integration and analysis of multidisciplinary datasets will likely increase our understanding of molecular mechanisms controlling *Pinus pinaster* response to drought [1].
Methods

Different approaches were designed to analyze the processes involved in *Pinus pinaster* response to drought, all of them based on a progeny from an *ad-hoc* designed full-sib cross (Gal1056xOria6), that segregates for the response to this environmental factor:

*Transcriptome and miRNA characterization:* cDNA and miRNA libraries were constructed using RNAs extracted from different tissues from Gal1056xOria6 individuals vegetatively propagated and subjected to different treatments. cDNA and miRNAs libraries were sequenced using GS FLX Titanium and Illumina, respectively (3, Sáez-Laguna & Cervera, unpublished results, de Maria et al, unpublished results). Assembled reads were annotated and differentially expressed genes selected as potential candidates for qRT-PCR studies. SNPs were identified in the transcriptome.

*Construction of genetic maps:* Genetic maps of different INIA’s progenies were developed mainly based on SNP segregation using Illumina SNP arrays (i.e. Infinium, Golden Gate; 3, 4). Additionally, in the frame of the ProCoGen project, an exome capture system is being developed to study segregation on a set of thousands sequences in different conifers and perform conifer comparative mapping.

*QTL analysis:* Identification of genome regions involved in the genetic control of *Pinus pinaster* response to water stress was carried out searching for associations between morpho-functional and molecular variants in the corresponding linkage maps [3]. This analysis allowed identification of positional candidate genes.

*Study of cytosine methylation:* Cytosine methylation during drought response was study at different scales: total cytosine methylation (HPLC analysis), genome-wide methylation changes at anonymous CCGG motives (MSAPs; 5) as well as detailed methylation pattern of a collection of candidate genes in response to the stress (collaboration with Dr. Díaz-Sala, UAH).

*Global metabolomic analysis:* A set of samples were also used to conduct global metabolomic profiling in order to analyze changes associated with drought stress
in different tissues as well as to analyze metabolite composition associated to the response to combined stresses.

*Phenotypic analysis:* Growth and different morpho-functional traits related to water and carbon plant economy were analyzed for contrasted genotypes in response to the interaction of water stress and different concentrations of atmospheric CO₂ (6, Sanchez-Gómez & Aranda unpublished results).

**Results and Conclusions**

More than 20 cDNA libraries were constructed representing different tissues and growing conditions. Differential expression analysis is ongoing to identify potential candidate genes that are further subjected to qRT-PCR. Additionally, a 1,536 SNP array has been developed based on SNPs associated to the reference mapping population and used to construct, together with additional SNPs, dense genetic maps [3]. These maps will be further saturated using an exome capture system designed by ProCoGen consortia in collaboration with Dr. Kirst (UFL, USA) for conifer comparative mapping.

Clones from Gal1056xOria6 showed high variability in their response to drought [6], i.e osmotic adjustment capacity differing between clones. Heritability values for stomatal conductance and intrinsic water use efficiency were moderate.

Genetic maps have also been used for the dissection of leaf gas exchange (photosynthesis and stomatal conductance to water vapour), chlorophyll fluorescence parameters and water use efficiency in response to drought, detecting QTLs that explained 10-20% of the observed phenotypic variability for each trait. Untargeted analysis of metabolic profiles allowed the discrimination of genotypes with contrasting drought response. Different metabolites, such as glutamate family amino acids, polyols and lipids, were correlated with some of the ecophysiological traits responding to drought.

**Competing interests**

The author declares that they have no competing interests.

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Nuria de María1,2, M. Ángeles Guevara1,2, José Antonio Cabezas1,2, M. Dolores Vélez1,2, Enrique Sáez-Laguna1,2, Marina de Miguel1,2, Luis Manuel Díaz1,2, José Antonio Mancha1, Alberto Pizarro3, Carmen Collada1,4, David Sánchez-Gómez2, Carmen Díaz-Sala3, Brígida Fernández de Simón4, Estrella Cadahia5, Ismael Aranda1, M. Teresa Cervera1,2

1Deparmento de Ecología y Genética Forestal. INIA-CIFOR. Ctra. De La Coruña Km 7.5, 28040 Madrid, Spain (cervera@inia.es).
2Unidad Mixta de Genómica y Ecología Forestal. INIA/UPM, Madrid, Spain.
3Departamento de Ciencias de la Vida. Universidad de Alcalá. Ctra. De Barcelona Km 33,600, 28871 Alcalá de Henares, Madrid, Spain.
4Deparmento de Sistemas y Recursos Naturales. ETSIMI. Ciudad Universitaria s/n, 28040 Madrid, Spain.
5Departamento de Industrias Forestales. INIA-CIFOR. Ctra. De La Coruña Km 7.5, 28040 Madrid, Spain.

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**Materials and experimental design**

- **Elisa clone**: Gal1056 (wood production + drought response + cold response) and Oria 6 (wood production + drought response + cold response)
- **162 F1 segregating progeny**
- **Destructive assays** (vegetative propagation, non-destructive field assay)

**Genetic mapping and trait dissection**

- **Gal1056 × Oria 6**: 28 significant QTLs and 27 suggestive QTLs
- **Coca × Coca**: 1,662 cm² in 12 linkage groups
- **1636 SNPs (1,204 genes)**
- **28 SAMSs; 5 SSRs**

**QTL analyses**

- **Phenotypic variance explained by a single TQT**: 4.6% (WUE), 20.9% (FV:FMR)
- **Phenotypic variance explained by all QTLs detected for a trait up to 64% (FV:FMR)**

**Morphological and ecophysiological characterization**

- **Traits**: Photosynthesis (Aₑ), Stomatal conductance (gₛ), Chlorophyll Fluorescence (WUE = Aₑ / gₛ), S/LA = leaf area / leaf biomass

**Phenotypic characterization**

- Accurate phenotypic estimates based on replicated genotypes
- High variability in Gal1056 x Oria6
- F1 full-sib family

**Transcriptomics**

- cDNA Libraries: Different tissues (roots, stems, needles)
- Different treatments (drought, hormones, control)
- 454 GS FLX sequencing
- 22,477 isoforms
- 16,335 isoforms

**Functional Annotation**

- 70% annotated
- Multi-data integration

**Metabolomics**

- Metabolite profiling of Gal1056 X Oria6 genotypes with contrasting drought stress response
- Clonal variability in the metabolic response

**Cytosine methylation status during drought response under different [CO2] have been analyzed using MSAP (Methylation-Sensitive Amplified Polymorphism) analysis**

**Comparative mapping**

- 5 *P. pinaster* progenies from INRA
- Comparative mapping**
- species composite maps**

**On going work**

- 19 mapping progenies (5 additional conifer spp.)**

**Study of cytosine methylation**

- The PCA based on PMS loci clearly differentiated genotypes, but any effect associated treatments was observed

**Conclusion**

- Drought is a major environmental stress factor that determines the growth, development and survival of plants living in water scarce environments. *Pinus pinaster* Ait. is an important Mediterranean conifer subjected to recurrent drought periods, which will be increased according to climate change predictions (IPCC, 2014). *P. pinaster* shows a significant genetic and adaptive diversity despite its reduced geographical range of distribution. Different morphological and physiological responses appear to play an important role in drought adaptation of this conifer. A better understanding of these complex responses to drought requires a multidisciplinary approach for data generation, analysis and integration, which is partially described in this work.