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Research article

Organ-specific metabolic responses to drought in *Pinus pinaster* Ait.

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ABSTRACT

Drought is an important driver of plant survival, growth, and distribution. Water deficit affects different pathways of metabolism, depending on plant organ. While previous studies have mainly focused on the metabolic drought response of a single organ, analysis of metabolic differences between organs is essential to achieve an integrated understanding of the whole plant response. In this work, untargeted metabolic profiling was used to examine the response of roots, stems, adult and juvenile needles from *Pinus pinaster* Ait. full-sib individuals, subjected to a moderate and long lasting drought period. Cyclitols content showed a significant alteration, in response to drought in all organs examined, but other metabolites increased or decreased differentially depending on the analyzed organ. While a high number of flavonoids were only detected in aerial organs, an induction of the glutathione pathway was mainly detected in roots. This result may reflect different antioxidant mechanisms activated in aerial organs and roots. Metabolic changes were more remarkable in roots than in the other organs, highlighting its prominent role in the response to water stress. Significant changes in flavonoids and ascorbate metabolism were also observed between adult and juvenile needles, consistent with previously proven differential functional responses between the two developmental stages. Genetic polymorphisms in candidate genes coding for a Myb1 transcription factor and a malate dehydrogenase (EC 1.1.1.37) were associated with different concentration of phenylalanine, phenylpropanoids and malate, respectively. The results obtained will support further research on metabolites and genes potentially involved in functional mechanisms related to drought tolerance in trees.

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1. Introduction

Drought is an important abiotic stress that influences the survival, growth and distribution of plant species. Water deficit is especially relevant in Mediterranean ecosystems, which are characterized by dry summers. In addition, drought effects are expected to be exacerbated in the face of climatic change predictions (Carnicer et al., 2010). Considering that forest trees are subjected to recurrent drought periods due to their long lifetime, it is especially relevant to understand the mechanisms of tolerance to water limiting conditions in these organisms at different biological scales. In this respect, it is important to unravel the drought response of forest tree species focusing on functional and molecular mechanisms and on the inter-play between them (Chaves et al., 2003).

Since metabolites are end products of cellular regulatory processes, they exemplify the final response of individuals to environmental or genetic changes. Metabolomics contribute significantly to the study of stress biology in plants by identifying changes in compounds of primary and secondary metabolism, in

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response to abiotic stresses or stress signal transduction as part of the acclimation responses. In the last years, high-throughput metabolic identification technologies have been implemented and applied to analyze drought response in several species but only a small number of studies have been performed in forest trees (e.g: (Warren et al., 2011, 2012; De Diego et al., 2013). In addition, just a few studies have evaluated changes in metabolic profile in different organs of the same tree (Piper, 2011; Merchant et al., 2006; Arndt et al., 2008).

Species-specific metabolic response to drought has been described (Warren et al., 2012; Piper, 2011), but intra-specific differences between genotypes or populations have been observed (Davey et al., 2008; Foito et al., 2009). However, it is difficult to understand the plant strategy used to cope with stress by studying the response of a single organ, as done in most drought experiments. Since drought affects different pathways of plant metabolism according to tissue, it is essential to identify metabolic changes in different organs in order to achieve a more integrated understanding of the whole plant response (Pinheiro et al., 2004). Plants have aerial and underground organs, highly specialized in different functions, and consequently differences in gene expression and metabolic pathways can be expected.

As a consequence of drought, carbon and nitrogen metabolism are altered. Depending on stress length and intensity, concentration of different compounds change according to organ (Pinheiro et al., 2011). Carbon compounds, such as sugars or polyols, and nitrogen storage metabolites, such as some amino acids or quaternary ammonium compounds, have been reported to accumulate under drought (Pinheiro et al., 2004; Atkinson et al., 2012). Roots and stems constitute the main storage reservoirs of these primary metabolites in most woody plants (Chapin et al., 1990). If the stress is long-lasting or severe, carbohydrate reserves can become depleted due to reductions in photosynthetic activity (Piper, 2011). Energy metabolism is also adjusted in response to drought to maintain carbon balance (Pinheiro and Chaves, 2011). While drought usually decreases photosynthesis, its impact on respiration and its interaction with other environmental factors such as light is still unclear, with different results depending on the species (Pinheiro and Chaves, 2011). Metabolic changes in response to drought also play an important role in osmotic adjustment, an important drought tolerance mechanism in leaves and roots (Nguyen and Lamant, 1988). Several metabolites may act as compatible solutes with osmoprotectant functions, such as sugars, amino acids, cyclitols or methylated quaternary ammonium compounds (Hare et al., 2002). In addition, antioxidant molecules usually increase in photosynthetically active tissues in response to drought as mechanisms of Reactive Oxygen Species (ROS) scavenging (Noctor and Foyer, 1998).

Response to environmental stress can be very different in those species with a marked heterophylly. Heterophylly is defined as the concurrent variation in leaf morphology within a single plant (Zotz et al., 2011). *Pinus* species show a remarkable heterophylly with distinct juvenile and adult vegetative phases. Ontogenic heteroblasty in *Pinus pinaster* Ait. maintains a high genetic control from population to individuals (Climent et al., 2013; Aranda et al., 2010). During the first years of pine seedlings life, juvenile needles are produced and progressively replaced by newly-formed adult needles. The adaptive value of heterophylly remains unclear (Zotz et al., 2011), but a patent differentiation in functional performance according to leaf type has been previously described in relation to heterophylly (Tanaka-Oda et al., 2010). However, it remains unknown whether the functional and morphological differences between juvenile and adult needles translate also in differentiation at the metabolic level.

We hypothesized that metabolic response to water limiting conditions in *P. pinaster*, a Mediterranean tree species, may differ depending on the organ analyzed. Considering the heterophylly of *P. pinaster*, metabolic profiles may also vary between adult and juvenile needles. To test these hypothesis three specific objectives were established: 1) To compare the metabolic response to drought in roots, stems, adult and juvenile needles of four full-sibs individuals; 2) to test the effect of polymorphisms in selected candidate genes on metabolite concentration; 3) to identify ontogeny-related changes in metabolite concentration, as those between juvenile and adult needles.

2. Materials and methods

2.1. Plant material

Plants used in this study were selected from a full-sib family of *P. pinaster* developed via an intraspecific cross involving Gal1056, a female from the wet Coastal North-west provenance (Pontevedra, Spain, 42° 10′N 8° 30′W) and Oria6, a male from Sierra de Oria, a dry natural population (Almería, South-east Spain, 37° 31′N 2° 21′W). Parental trees were selected according to previous studies that showed high tolerance to drought for the male progenitor (Aranda et al., 2010), while the mother progenitor is an elite tree from a regional breeding program for wood production, sensitive to drought. Four clones of the full-sib family (two year old seedlings) were selected for their differential behavior in functional characteristics in response to drought, as previously described (de Miguel et al., 2012). Selected individuals were genotyped for Single Nucleotide Polymorphisms (SNPs) located in two positional candidate genes related to metabolic pathways. Candidate genes were selected from a previous Quantitative Trait Locus (QTL) study developed with the same full-sib family (see de Miguel et al. (2014) for a detailed procedure on SNP genotyping and QTL analysis): i) SNP m747 located in a R2R3 Myb transcription factor that regulates genes of the phenylalanine pathway (Craven-Bartle et al., 2013) and ii) SNP i11178s499 pg located in a gene coding for a malate dehydrogenase (EC 1.1.1.37), an enzyme of cellular metabolism that catalyzes the conversion of malate to oxaloacetate (Musrati et al., 1998). Genotypes for both SNPs in the four analyzed individuals are presented in Supplementary Table S1.

Seven to ten cuttings of each clone were rooted on coir and maintained in the bed rooting for seven months. Temperature in the rooting soil media was 20 °C, and relative humidity over 70% most of the time. Rooted cuttings were transplanted to 3 l containers with a mixture 3:1 v:v peat: sand, and regularly fertilized with a hydro-soluble fertilizer at 1.5 g l^{-1} N:P:K 24:16:12 Abofol (Syngenta, Basel, Switzerland) and 1.5 g l^{-1} Fe Sequestrene (Syngenta, Basel, Switzerland). Fungicide Alliette WG 3 g l^{-1} (Bayer, Leverkusen, Germany) and insecticide Zolone (Bayer, Leverkusen, Germany) were applied regularly to avoid the presence of pathogens. Plant material was maintained in a greenhouse at 20 °C average temperature, 50% relative humidity, natural photoperiod (maximum PAR of 1635 μmol m^{-2} s^{-1}) and watering to full capacity 3 times per week until the beginning of the drought imposition period.

2.2. Experimental setup

Plants were established in a greenhouse following a completely randomized design. Two watering regimes were applied, well watered plants (WW) were irrigated to full holding capacity, and water stressed plants (WS) were irrigated around 50% of full holding capacity. This rendered a moderate water stress for plants in terms of soil moisture availability. Three and four replicates per clone were submitted to WW and WS treatments respectively,

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