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Induction of acid phosphatase transcripts, protein and enzymatic activity by simulated herbivory of hybrid poplar

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ABSTRACT

Herbivory and wounding upregulate a large suite of defense genes in hybrid poplar leaves. A strongly wound- and herbivore-induced gene with high similarity to *Arabidopsis* vegetative storage proteins (VSPs) and acid phosphatase (AP) was identified among genes strongly expressed during the poplar herbivore defense response. Phylogenetic analysis showed that the putative poplar acid phosphatase (PtdAP1) gene is part of an eight-member AP gene family in poplar, and is most closely related to a functionally characterized soybean nodule AP. Unlike the other poplar APs, PtdAP1 is expressed in variety of tissues, as observed in an analysis of EST data. Following wounding, the gene shows an expression profile similar to other known poplar defense genes such as protease inhibitors, chitinase, and polyphenol oxidase. Significantly, we show for the first time that subsequent to the wound-induction of PtdAP1 transcripts, AP protein and activity increase in extracts of leaves and other tissues. Although its mechanism of action is as yet unknown, these results suggest in hybrid poplar PtdAP1 is likely a component of the defense response against leaf-eating herbivores.

1. Introduction

Phosphorus (P) is a key element for plant metabolism and is often a limiting nutrient in crop plants (Vance et al., 2003). Phosphatases (EC 3.1.3.2) hydrolyse the phosphate anions (P_i) from orthophosphate monoesters, and are thus important enzymes of plant metabolism at many levels (Duff et al., 1994). Phosphatases are traditionally classified as either acidic or basic depending on their pH optima. Alkaline phosphatases typically show absolute specificity and take only single substrates, while the acid phosphatases (APs) usually have broad substrate preferences and can accept a variety of phosphorylated substrates (Duff et al., 1994). This low substrate specificity of APs makes it difficult to assign physiological roles to specific enzymes based on substrate preferences. For example, a purified AP from potato tubers was able to release P_i from a wide variety of substrates, such as small molecules and model substrates including phosphoenol pyruvate, pyrophosphate, *p*-nitrophenyl phosphate (pNPP), ATP, as well as phosphotyrosine and several phosphorylated potato proteins (Gellatly et al., 1994).

The expression of AP genes is often associated with P_i limitation, and active AP enzymes are commonly secreted into the rhizosphere in response to P_i starvation (Tadano et al., 1993; Vance et al., 2003). Therefore, a major role of the extracellular root APs appears to be in P_i acquisition, i.e., facilitating the release of P_i from various organic compounds in the soil (Fernandez and Ascencio, 1994; Vance et al., 2003). Extracellular secretion of AP into the soil from roots has been investigated in many species, and is considered an indicator of P stress. Intracellular APs, which may be either vacuolar or cytoplasmic, are also induced by P_i starvation, sometimes in aerial plant organs (Duff et al., 1991; Baldwin et al., 2001; Tian et al., 2003; Veljanovski et al., 2006). Such leaf APs may contribute to plant nutrition by mobilizing internal stores of P_i from senescing tissues and cells. However, Yan et al. (2001) found no correlation of induced intracellular APs with improved nutrition, and thus the significance of these APs for P_i metabolism is still unclear.

In some plants, proteins with strong similarity to APs have been found to be vegetative storage proteins (VSPs), defined by their temporal patterns of synthesis and degradation that reflect the tissue-specific nitrogen supply in vegetative tissues. For example, soybean VSPα and VSPβ accumulate dramatically in stems and other vegetative tissues of soybean plants following removal of seed pods. Their function is presumed to be temporary nitrogen storage in vegetative tissues when the plant is deprived of its normal nitrogen sink (Staswick et al., 1994). These VSPs have signifi-

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cant sequence similarity to APs, yet the gene product demonstrated only low levels of AP enzymatic activity (Leelapon et al., 2004). This confirms that their primary role is for nitrogen/amino acid storage, rather than P_i metabolism. Synthesis of the soybean VSPs can be stimulated by the wound and defense signal methyl jasmonate (Franceschi and Grimes, 1991), an observation that first provided a link of AP-like proteins to defense responses.

In *Arabidopsis*, two genes (*AtVSP1* and *AtVSP2*) with similarity to the soybean VSPs and APs are strongly induced by herbivory, wounding, and jasmonate (Berger et al., 1995, 2002). These expression patterns are most consistent with a function in direct defense rather than in temporary nitrogen storage. The *Arabidopsis* VSP genes are regulated via the JA signaling defense pathway, and mutants without the capacity to synthesize JA do not accumulate VSPs (Berger et al., 1996). The link of VSPs to plant defense was strengthened via the analysis of *Arabidopsis* mutants, in which VSP expression is correlated with defense capacity against lepidopteran herbivores (discussed by Liu et al., 2005). Significantly, recombinant AtVSP2 protein demonstrated anti-insect activity in bioassays with coleopteran pests as well as a dipteran species (Liu et al., 2005). AtVSP2 with a mutated active site lost its inhibitory activity against the test insects, thus linking AP activity to its biological effects. The mechanism of action against pest insects is not known, however.

In hybrid poplar as in many other species, wounding and leaf damage by herbivory can trigger dramatic changes to the leaf transcriptome, affecting the expression of hundreds to thousands of genes (Ralph et al., 2006; Major and Constabel, 2006). Many of the most strongly upregulated genes typically encode proteins with demonstrated activity against leaf-eating insects. Among these, Kunitz protease inhibitor (PI) transcripts are very prominent in terms of number of genes and proportion of transcripts (Christopher et al., 2004); these were subsequently shown to be active against mammalian and insect proteases (Major and Constabel, 2008). Other induced genes encode chitinases, polyphenol oxidase, and peroxidases, all enzymes for which anti-insect activity has been experimentally demonstrated (reviewed in Constabel and Lindroth, 2010). In addition, wounded leaves of some poplar species can lead to enhanced levels of proanthocyanidins (Peters and Constabel, 2002), which some studies have linked to pest resistance. Therefore, poplar leaves can clearly respond actively to herbivore attack with an active mobilization of defenses targeted to insect pests.

During previous transcript profiling and genomics studies of the hybrid poplar defense response, we identified a strongly wound-inducible AP gene among the top ten most abundant transcripts in a systemically wounded leaf library (Christopher et al., 2004; Major and Constabel, 2006). The strong upregulation of this gene in several, independent differential screening and herbivory experiments suggested that the gene product should be an important component of poplar defense against leaf-eating herbivores. Despite this strong co-expression, as well as independent work showing direct anti-insect effects of *Arabidopsis* AP-like AtVSP2, to date there are no reports for any plant species of either a wound- or herbivore-inducible AP enzyme activity. Here, we directly address this question in hybrid poplar, and further characterize AP induction at the transcript, protein, and enzyme activity levels. Our results demonstrate that wounding induces elevated AP activity in leaves and other tissues of hybrid poplar, supporting a defense role for this AP gene.

2. Results

2.1. Phylogenetic analysis of a wound-induced poplar AP gene

Previous transcriptomic analyses had led to the identification of a strongly wound- and herbivore-inducible gene, corresponding to the JGI protein ID705836, with high sequence similarity to acid phosphatases (Christopher et al., 2004; Major and Constabel, 2006). To confirm this annotation and to identify additional genes belonging to this family in poplar, we searched the genome databases. In total, eight putative poplar AP genes were identified and verified with available ESTs in GenBank at NCBI. The sequences were compared to the ten known *Arabidopsis* APs (Liu et al., 2005) in a phylogenetic analysis. We also included AP genes in GenBank from other species with functional data that could confirm AP activity of the corresponding gene product. In the resulting phylogeny, the poplar and *Arabidopsis* AP gene families showed a similarly wide distribution within the tree (Fig. 1). The new poplar AP gene grouped most closely with *GmACP*, a soybean gene encoding a root nodule-specific protein with demonstrated AP activity and broad substrate specificity (Penheiter et al., 1997, 1998; Leelapon et al., 2004). Two additional genes in the group encode the soybean VSPs (VSPα and VSPβ), proteins with AP activity (DeWald et al., 1992). *Arabidopsis* AtVSP1 and AtVSP2 genes also belong to this clade, and recent evidence indicates that AtVSP-2 has both AP and anti-insect activity. Since the new poplar gene clusters closely with genes whose products have clear enzymatic activity and in some cases are linked to plant defense, it is very likely that the poplar gene also encodes a functional AP with potential to impact insect performance. We therefore named this gene PtdAP1 and undertook experiments to investigate its expression in more detail.

2.2. Constitutive and induced expression of the PtdAP1 gene

To obtain more information on the expression of this candidate poplar defense gene relative to the other poplar APs, we performed a 'digital northern' analysis using the abundance of ESTs deposited in the NCBI GenBank data base in different *Populus* libraries, with an approach demonstrated by Sterky et al. (2004). BLAST searches were used to identify *Populus* AP ESTs, which were then manually matched with the gene models identified in the JGI *P. trichocarpa* genome database. We compared the total numbers of ESTs found in libraries derived from different tissues, including tissues treated with one of several abiotic or biotic stress treatments. These data were used to generate a heat map to represent abundance of transcripts, roughly approximating gene expression, within that tissue (Fig. 2). While this analysis does not easily permit direct comparisons among tissues due to differences in library size, it does allow for a semi-quantitative comparison of expression of poplar AP genes within any one tissue, i.e. within a library or set of libraries. Overall inspection of the heat map showed that PtdAP1 was represented by the largest number of ESTs, and was expressed in the greatest number of tissue types. Thus this gene showed more abundant transcripts than any other poplar AP in libraries derived from shoot tissues including leaf, stem, wood and bark, and roots. Most of the other AP genes were more restricted in expression and ESTs were detected only in a few tissue types and with typically lower inferred expression levels compared to PtdAP1 (Fig. 2). An exception to this pattern was Pt711452, which showed strong representation in the cambial zone and wood libraries, as well as root, and stem, but little expression elsewhere. Of all poplar APs, PtdAP1 showed by far the highest EST representation in the real or simulated herbivory libraries, from both leaf and bark tissues (see arrows, Fig. 2). The low EST counts for most other AP genes in these libraries make their comparison only semi-quantitative, but the much greater abundance of PtdAP1 ESTs is most consistent with a role of this gene in induced defense. None of the other AP genes were as abundant as PtdAP1 in the herbivore-induced tissue libraries, suggesting that herbivore and biotic stress induction is not a general response of APs. The specificity of herbivore-inducibil-