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Comparative characterization of the *Arabidopsis* subfamily a1 β-galactosidases

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ABSTRACT

The *Arabidopsis* genome contains 17 predicted β-galactosidase genes, all of which belong to glycosyl hydrolase (GH) Family 35. These genes have been further grouped into seven subfamilies based on sequence similarity. The largest of these, subfamily a1, consists of six genes, *Gal-1* (At3g13750), *Gal-2* (At3g52840), *Gal-3* (At4g36360), *Gal-4* (At5g56870), *Gal-5* (At1g45130), and *Gal-12* (At4g26140), some of which were characterized in previous studies. We report here the purification and biochemical characterization of recombinant Gal-1, Gal-3, Gal-4 and Gal-12 from *Pichia pastoris*, completing the analysis of all six recombinant proteins, as well as the isolation and characterization of the native Gal-2 protein from *Arabidopsis* leaves. Comparison of the relative expression levels of the subfamily a1 β-galactosidases at the mRNA and protein levels uncovered evidence of differential regulation, which may involve post-transcriptional and post-translational processes. In addition, this study provides further support for the proposed function of the subfamily a1 β-galactosidases in cell wall modification based on analysis of the organ-specific expression and subcellular localization of Gal-1 and Gal-12. Our study suggests that, despite some differences in individual biochemical characteristics and expression patterns, each member of the family has the potential to contribute to the dynamics of the *Arabidopsis* plant cell wall.
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1. Introduction

β-Galactosidases (EC 3.2.1.23) are enzymes that cleave substrates containing galactosyl moieties, such as lactose, glycolipids, proteoglycans, oligosaccharides, and polysaccharides. β-Galactosidases are of interest because of their use in the dairy and food industry, agriculture, and biotechnology (Betancor et al., 2008; Callahan, 1999; Miezeliene et al., 2000; Oehmig et al., 2007). Based on amino acid sequence similarities, β-galactosidases have been found to fall into four of the 113 current glycosyl hydrolase (GH) families, GH-1, GH-2, GH-35 and GH-42 (Coutinho and Henrissat, 1999); http://www.cazy.org/). β-Galactosidases belonging to GH-1, GH-2, and GH-42 are found predominantly in microorganisms, whereas the GH-35 enzymes are found in both prokaryotes and eukaryotes. In higher plants, the GH-35 family contains 15–20 predicted β-galactosidases, which can be further grouped into subfamilies based on sequence similarity (Ahn et al., 2007; Perez-Almeida, 2004; Tanthanuch et al., 2008). In other eukaryotes, the total number of predicted β-galactosidases in GH-35 is much smaller. While *Arabidopsis* has 17, rice 15, and poplar about 20, the human genome appears to encode four, fruit fly two, and *Chlamydomonas* and budding yeast none (http://www.cazy.org; http://genome.jgi-psf.org/Poptr1/Poptr1.home.html).

Plant β-galactosidases play important roles in the metabolism of galactosyl conjugates during carbohydrate reserve mobilization, cell wall expansion and degradation, and turnover of signaling molecules (de Alcantara et al., 2006; Esteban et al., 2003; McDougall and Fry, 1990). A correlation between β-galactosidase expression and cell wall disassembly has been reported in fruit ripening of tomato (Carey et al., 1995), apple and kiwi (Ross et al., 1993), pear (Tateishi et al., 2001), and papaya (Lazan et al., 2004). Besides fruit ripening, β-galactosidases have been shown to participate in seed germination in *Arabidopsis* (Dean et al., 2007), radish (Kotake et al., 2005; Sekimata et al., 1989), and rice (Chantarangsee et al., 2007). β-Galactosidase activities associated with various physiological processes have been observed in different plant tissues, including the cotyledon of lupin (Buckeridge et al., 2005), floral tissue of Sandersonia flower (O'Donoghue et al., 2002), seedling of mung bean (Li et al., 2001), and pollen of tobacco (Hruba et al., 2005). The analysis of individual β-galactosidases purified from different plants has led to better understanding of their functions and natural substrate specificities. Further genetic and bioinformatics studies are necessary to understand the interrelationships among β-galactosidase isoforms and the mechanisms by which they participate in the regulation of growth and development.

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For a systematic characterization of β-galactosidases, the most attractive organism is a model plant with a completely sequenced genome. In *Arabidopsis thaliana*, a gene family of 17 β-*galactosidases* has been identified (Ahn et al., 2007). This family has been further subdivided into nine subgroups based on sequence similarity (Perez-Almeida, 2004; Tanthanuch et al., 2008). Although most of these groups contain one to three members, subfamily a1 consists of six genes, *Gal-1* (At3g13750), *Gal-2* (At3g52840), *Gal-3* (At4g36360), *Gal-4* (At5g56870), *Gal-5* (At1g45130), and *Gal-12* (At4g26140). The presence of such a large subfamily raises the question of whether these isozymes have either redundant or distinct functions within the plant. Although many orthologs of the subfamily a1 genes and their protein products have been studied in other plant species (Buckeridge et al., 2005; Carey et al., 1995; Chantarangsee et al., 2007; Lazan et al., 2004; Tateishi et al., 2001; Trainotti et al., 2001), a characterization of the entire subfamily from a single species has not yet been carried out. We recently reported the biochemical characterization of Gal-4 (Ahn et al., 2007) and Gal-2 and Gal-5 (Gantulga et al., 2008), showing that these enzymes have hydrolase activity with cell wall-derived pectic polysaccharides. We also demonstrated the presence of Gal-2 and Gal-5 proteins in the cell wall of rosette leaves by dot-immunoblot analysis. Our results suggest that the sequence similarities of the genes in the subfamily translate into functional similarities of the proteins in cell wall modification.

In this study, we extend the previous analysis of Gal-2, Gal-4, and Gal-5 to complete the biochemical characterization of the subfamily a1 β-galactosidases. We also describe the purification and characterization of a native β-galactosidase from this subfamily from *Arabidopsis* leaves. Organ-specific expression of all six genes in the subfamily was studied by semi-quantitative RT-PCR and compared with the publicly-available microarray data. We also show that methyl jasmonate treatment, known to arrest growth and control defense responses in plants, down-regulates the expression of several of the genes. In addition, immuno-electron microscopy was used to confirm the predicted localization of Gal-1 and Gal-12 in the cell wall in vascular and epidermal tissues of mature root.

2. Results and discussion

2.1. In silico characterization of the Arabidopsis subfamily a1 β-galactosidases

Previous analysis of the evolutionary relationships among the GH Family 35 β-*galactosidase* genes in 23 eukaryotic genomes showed that the 17 *Arabidopsis* genes fall into two groups and seven subfamilies (Ahn et al., 2007; Perez-Almeida, 2004; Tanthanuch et al., 2008). Fig. 1 shows a phylogenetic tree based on the amino acid sequences predicted for these 17 genes. The largest subfamily, a1, consists of six proteins, Gal-1, Gal-2, Gal-3, Gal-4, Gal-5, and Gal-12, that exhibit 60–81% sequence identity. All are predicted to have signal peptides and basic p*I*s (7.2–8.6), consistent with a cellular destination in the cell wall (Ahn et al., 2007). This localization was recently confirmed for Gal-2 and Gal-5 (Gantulga et al., 2008). The six enzymes differ with regard to the presence of a C-terminal lectin-like domain in Gal-1 and Gal-3, which places these two isoforms with the higher molecular mass Group 2 β-galactosidases (Ahn et al., 2007). It has been suggested that the lectin-like domain may enhance catalytic efficacy by anchoring the enzymes to their polymeric substrates.

2.2. Expression of β-galactosidases in Arabidopsis

Relative RT-PCR analysis previously indicated that *Gal-1*, *2*, *3* and *5* are expressed in diverse tissues, with the highest transcript

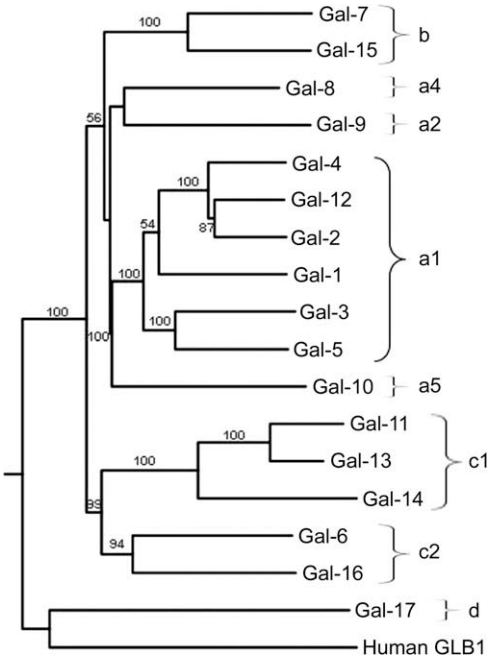

Fig. 1. *Phylogenetic relationships among the Arabidopsis thaliana β-galactosidases*. Amino acid sequences were aligned using ClustalW and a parsimony tree was generated in PAUP 4.0b. Number of bootstrap replicates = 1000. The human GLB1 gene was used as an outgroup. Letters next to the brackets indicate subfamilies designations per Tanthanuch et al. (2008).

levels in roots and flowers, while *Gal-4* transcripts accumulate primarily in roots (Ahn et al., 2007). No expression was detected for *Gal-12* in these experiments. This differential expression is also reflected in the publicly-available microarray data, although in this case all six genes appear to be expressed to some extent at in all tissues that have been examined (Fig. 2A, Gantulga et al., 2008). To confirm these findings and extend the analysis to include such organs as mature root, cauline leaf, petiole of the rosette leaf, and silique, semi-quantitative RT-PCR was performed using a different set of primers than in the experiments of Ahn et al. (2007); Supplementary Table S1). These experiments provided further evidence for temporal and spatial differences in the expression of the six subfamily a1 genes (Fig. 2B). Most of the genes are expressed at some level in all organs, including *Gal-12*, for which we detected two bands for *Gal-12*. This is consistent with the gene model for *Gal-12* in TAIR, which indicates that alternative splicing produces transcripts that differ in size by 100 nt. There are some subtle differences between the outcomes of these experiments (Fig. 2B), those of Ahn et al. (2007), and the microarray data that are likely due to differences in plant age or growth conditions. However, overall it can be concluded that *Gal-1* has essentially constitutive expression, while the other five genes are expressed in most, but not all, tissues. Most striking is that in seedlings only *Gal-1*, *Gal-2*, *Gal-5*, and *Gal-12* are expressed, in the petiole only *Gal-1* and *Gal-3*, and in siliques only *Gal-1*, *Gal-3* and *Gal-4*. These distinct expression patterns may reflect the use of related genes to produce enzymes with subtle functional differences and/or to direct the developmental, tissue-specific, or localized expression of β-galactosidase activity.

We next investigated the effects of a variety of stress conditions and hormone treatments known to regulate plant growth and development (Nemhauser et al., 2006) on expression of the subfamily a1 genes in seedlings. Little or no difference was observed in the transcript levels of the six genes in response to a variety of treatments, including salt, cold, osmotic shock, and treatment with 2,4-dichlorophenoxyacetic acid,(2,4-D; data not shown). This

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