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# Ectopic expression of a conifer *Abscisic Acid Insensitive3* transcription factor induces high-level synthesis of recombinant human α-L-iduronidase in transgenic tobacco leaves

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**Abstract** We are examining various plant-based systems to produce enzymes for the treatment of human lysosomal storage disorders. Constitutive expression of the gene encoding the human lysosomal enzyme, α-L-iduronidase (IDUA; EC 3.2.1.76) in leaves of transgenic tobacco plants resulted in low-enzyme activity, and the protein appeared to be subject to proteolysis. Toward enhancing production of this recombinant enzyme in vegetative tissues, transgenic tobacco plants were generated to co-express a CaMV35S:*Chamaecyparis nootkatensis Abscisic Acid Insensitive3 (CnABI3)* gene construct, along with the human gene construct. The latter contained regulatory sequences of the *Phaseolus vulgaris arcelin 5–I* gene (5′-flanking, signal-peptide-encoding, and 3′-flanking regions). Ectopic synthesis of the CnABI3 protein led to the transactivation of the arcelin promoter and accordingly high activity (e.g., 25,000 pmol/min/mg total soluble protein) and levels of recombinant IDUA mRNA and protein were induced in leaves of transgenic tobacco, particularly in the presence of 150–200 μM *S*-(+)-ABA. Synthesis of human IDUA containing a carboxy-terminal ER retention (SEKDEL) sequence was also inducible by ABA in leaves co-transformed with the *CnABI3* gene. As compared to the natural *S*-(+)-ABA, two persistent ABA analogues, (+)–8′ acetylene ABA and (+)–8′methylene ABA, led to greater levels of β-glucuronidase (GUS) reporter activities in leaves co-expressing the *CnABI3* gene and a vicilin:GUS chimeric gene. In contrast, (+)–8′ acetylene ABA and natural ABA appeared to be equally effective in stimulating the CnABI3-induced expression of an arcelin:GUS gene, and of the human IDUA gene, the latter also driven by arcelin-gene-regulatory sequences. Various stress-related treatments, particularly high concentrations of NaCl, had an even greater effect than ABA in promoting accumulation of human IDUA in co-transformed tobacco leaves. This strategy provides the means of enhancing the yields of recombinant proteins in transgenic plant vegetative tissues and potentially in cultured plant cells. The human recombinant protein can be readily induced in the presence of chemicals such as NaCl that can be added to cell cultures or even whole plants without a significant increase in production costs.

**Keywords** Molecular pharming · Recombinant human α-L-iduronidase · *Abscisic Acid Insensitive3* gene · Transactivation · Transgenic tobacco

**Abbreviations**

| | |
|---|---|
| ABA | abscisic acid |
| CnABI3 | Chamaecyparis nootkatensis Abscisic Acid Insensitive3 gene |
| GUS | β-glucuronidase |
| HIDUA | human α-L-iduronidase |

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|---|---|
| MPS | mucopolysaccharidosis |
| TSP | total soluble protein |
| UTR | untranslated region |

## Introduction

Plant expression systems have several potential advantages for large-scale production of recombinant proteins for industrial and pharmaceutical uses (reviewed in Kermode 2006). Production costs may prove to be relatively low, the scale-up capacity is large, and plant cells are not susceptible to contamination by human pathogens as can occur in mammalian expression systems. However, various technical challenges must be met before plant systems can fully emerge as suitable, viable alternatives to current animal-based systems for large-scale production of biopharmaceuticals and other products (Kermode 2006).

Although there are important exceptions, the use of whole transgenic plants and plant culture systems for recombinant protein production can result in low yields of the protein of interest. For example, human protein C is accumulated at levels lower than 0.01% of total soluble protein (TSP); likewise, secretory IgA constitutes only 0.003% of leaf fresh weight (reviewed in Chen et al. 2005; see references therein). For some bacterial, animal, and human proteins expressed in plant systems, yields vary widely and can be as low as 0.0001% TSP. In the field of edible vaccines, some attempts to express a microbial protein (the antigen) in edible parts of transgenic plants (e.g., maize, tomato, and potato) have also resulted in low-expression levels (but see Richter et al. 2000; Streatfield et al. 2003; Lamphear et al. 2002). Thus, one of the key challenges in the area of molecular pharming is the employment of viable strategies to enhance expression levels and to improve the stability of the protein of interest in plant cells (Fischer et al. 2004; Stoger et al. 2005). Consistent dosing, mechanisms for effective antigen delivery, and potential problems associated with the development of oral tolerance to the antigen, are persistent issues in the field of edible vaccines (Hood et al. 1997; Daniell et al. 2001; Fischer et al. 2004; Stoger et al. 2005).

One seed-specific/restrictive promoter that has successfully yielded high-level expression of transgenes in dicot seeds is the 5′ regulatory region of the arcelin–5a-encoding gene, *ARC5-I* isolated from *Phaseolus vulgaris L.* (genotype G02771) (De Jaeger et al. 2002; Downing et al. 2006). This promoter has been used to successfully express a single-chain antibody in *Arabidopsis thaliana* seeds; in comparison to protein accumulation driven by the CaMV35S promoter (1% TSP), the bean *ARC5-I* gene promoter results in antibody levels in excess of 36% TSP in homozygous seeds. The antibody is synthesized in a functional form and retains its antigen-binding activity and affinity (De Jaeger et al. 2002). Likewise, the *ARC5-I* promoter effects a high level of accumulation of the human lysosomal enzyme, α-L-iduronidase (IDUA) in *Arabidopsis cgl* seeds (Downing et al. 2006), although in this case, other regulatory elements of the arcelin gene likely act in a synergistic manner to yield high expression (Kermode 2006).

Although seeds have numerous advantages as hosts for production of human recombinant proteins (reviewed in Stoger et al. 2005), there are also benefits of using plant-cultured cells for human recombinant protein production, for which constitutive promoters or modified constitutive promoters may be most suitable. The advantages of plant cell cultures include the precise control over growth conditions, the ease of application of various agents (e.g., inducers of gene expression, enhancers of protein secretion, and glycan processing inhibitors), batch-to-batch product consistency, a high level of containment, and the ability to produce recombinant proteins in compliance with good manufacturing practice (GMP) (reviewed in Hellwig et al. 2004). However, as noted earlier, vegetative tissues and plant cell cultures may suffer the drawback of low levels of accumulation of the recombinant protein of interest.

Mucopolysaccharidosis (MPS) I is a lysosomal storage disease characterized by the deficiency of IDUA, an enzyme involved in the stepwise degradation of glycosaminoglycans; in severely affected humans this genetic disease leads to death in early childhood because of profound skeletal, cardiac and neurological disturbances (Neufeld and Muenzer 2001). Lysosomal storage diseases, which collectively represent over 50 disorders, are generally amenable to enzyme replacement therapies in which the recombinant lysosomal enzyme is delivered intravenously (reviewed in Desnick and Schuchman 2002; Brady 2003). Receptor-mediated uptake via a salvage pathway allows the enzyme to be sequestered intracellularly and subsequently targeted correctly to the human cell lysosome. Some of these enzymes have been produced as recombinant proteins in plants (Cramer et al. 1999; Downing et al. 2006; Reggi et al. 2005).

In the present study, we examined a strategy to enhance the production of the recombinant human lysosomal enzyme, IDUA (EC 3.2.1.76) in vegetative tissues, as a prelude to developing a cell culture system

for its production. We tested the ability of a plant B3 domain transcription factor (ABI3; ABscisic acid Insensitive3) to enhance expression levels of human IDUA (hIDUA) in transgenic tobacco leaves. ABI3 transcriptional factors play critical roles in the regulation of ABA responsive genes in developing seeds, particularly those required for reserve deposition, dormancy inception, and the acquisition of desiccation tolerance (reviewed in Bonetta and McCourt 1998; Finkelstein et al. 2002). This family of proteins is able to transactivate the expression of ABA-responsive genes in developing seeds or embryos, and under some conditions, in seedlings/plants. Moreover, ectopically expressed ABI3 protein (effected by stable transformation with a chimeric 35S:*ABI3* gene) leads to the reactivated expression of seed-specific genes in vegetative tissues and seedlings (Parcy and Giraudat 1997; Parcy et al. 1994; Zeng et al. 2003).

The objectives of our study were threefold: (1) To examine whether ectopic (constitutive) expression of a yellow-cedar, *Chamaecyparis nootkatensis* (*Cn*)*ABI3* gene effects the transactivation of a human gene driven by a seed-restrictive promoter and other expression-enhancing regulatory sequences; (2) To examine the synergistic effects of ABA and of metabolism-resistant ABA analogues on the ability of CnABI3 to transactive the human gene and reporter gene constructs; and (3) To examine the effects of various stress treatments, designed to enhance the endogenous ABA level, on accumulation levels of the human recombinant protein.

## Materials and methods

### Generation of gene construct for constitutive expression of human IDUA in tobacco

In addition to the various gene constructs that were generated for the co-expression studies using the *CnABI3* gene (see below), a construct was made to constitutively express the human IDUA gene (alone) in tobacco (Construct 35S-hIDUA; Fig. 1). This construct contained the cauliflower mosaic virus 35S 5′ upstream region with a duplicated enhancer element, a 5′-untranslated region (UTR) from alfalfa mosaic virus RNA 4 (AMV), the full-length coding sequence of the hIDUA gene (Scott et al. 1991; GenBank Accession no. M74715), and a nopaline synthase (Nos) gene 3′ flanking region. The *db35S-AMV-hIDUA-Nos* gene construct (referred to as 35S-hIDUA; Fig. 1), was generated as follows: a modified pBI121 plasmid containing the coding region of β-glucuronidase (GUS) flanked by the 35S 5′ upstream region with duplicated enhancer element, AMV 5′ UTR and Nos 3′ flanking sequences (Datla et al. 1993) was digested with *NcoI* and *SstI* to remove the GUS coding region, and the vector fragment containing the 5′ flanking- and Nos–3′ sequences was isolated. Mung bean nuclease was used to remove the nucleotides specifying an extra start codon in the *NcoI* restriction site, following the manufacturer's instructions (Invitrogen Canada Inc., Burlington, ON, Canada). *HindIII* and *EcoRI* were used to cleave the hIDUA gene coding region from plasmid pLC1 (kindly provided by L.A. Clarke, University of British Columbia, BC, Canada); the isolated fragment was blunt-ended using Klenow fragment. Following ligation of the IDUA- and gene-regulatory-fragments, the resultant plasmid DNA was digested with *HindIII* and *PstI* to verify the correct orientation. The *db35S-AMV-hIDUA-Nos* fragment was cloned into the binary vector pCambia with *HindIII* and *EcoRI* sites and the resultant construct was transferred into *Agrobacterium* strain LBA4404 by electroporation in order to carry out stable transformation of tobacco plants.

### Generation of gene constructs for co-expression studies

Generation of the human gene construct for the co-expression studies, denoted ARC-hIDUA (Fig. 1, construct d), and containing the 5′-flanking, 5′ UTR, signal-peptide-encoding, and 3′ flanking sequences from the *arc5-I* gene, was as described in Downing et al. (2006) (therein described as ARC5s3).

For one gene construct (Fig. 1, construct e), the sequence encoding the amino acids S-E-K-D-E-L, an ER-retention signal, was fused to the 3′ end of the hIDUA cDNA sequence. This was achieved as follows: Two sequence-specific primers (forward primer: 3′ CTCTGCAGCCCCGACGG 5′ and a reverse primer that included a SEKDEL coding sequence, stop codon and *XbaI* restriction site:

5′ GCTCTAGATCAGAGCTCATCCTTCTCAGATGGATTGCCCGGGGATGG 3′) were used to amplify a ~500 bp portion of the 3′ end of the hIDUA cDNA using a plasmid (pAK12) containing the full-size hIDUA gene coding region (Scott et al. 1991) as the template. The PCR fragment was subsequently digested with *PstI* (part of the IDUA cDNA) and *XbaI* to replace the original 3′ end of the cDNA encoding hIDUA with the fragment generated by PCR containing the 3′ end of the hIDUA, the SEKDEL sequence and a stop codon (IDUA/SEKDEL), generating the plasmid pAK31. This plasmid was then used to generate the plasmid pAK81 consisting of the

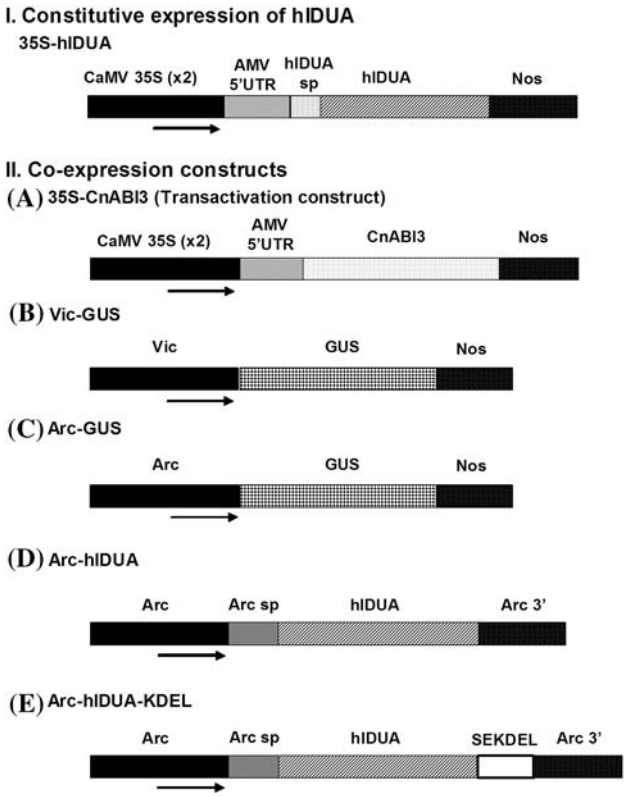



**Fig. 1** (1) Gene construct for constitutive expression of human α-L-iduronidase (*hIDUA*) in transgenic tobacco leaves. (2) Gene constructs for co-expression of GUS or hIDUA in transgenic tobacco along with the *CnABI3* gene. Constructs (b–e) include two for the synthesis of the bacterial reporter protein GUS (Vic-GUS and Arc-GUS; containing promoter and 5′ UTR sequences of the pea vicilin and *arc 5-I* genes, respectively), and two for synthesis of the human lysosomal enzyme α-L-iduronidase (Arc-hIDUA and Arc-hIDUA-KDEL). Construct **a** ('transactivation construct') is one for the ectopic expression of a conifer (yellow-cedar, *Chamaecyparis nootkatensis*) *CnABI3* gene. Co-expression of the construct encoding the transcription factor CnABI3 and any one of the other constructs is designed to cause the ''ectopic'' activation of the chimeric (GUS or hIDUA) genes driven by the seed-restrictive gene promoters (vicilin and arcelin gene promoters). The desired effect is the high-level accumulation of the recombinant protein (bacterial GUS or human IDUA) in the vegetative tissues of transgenic tobacco. Transformants expressing constructs (**b**), (**c**), (**d**), or (**e**) served as controls for comparison. *SP* signal peptide

*ARC5-I* gene 5′ flanking region, 5′UTR and signal peptide-encoding sequences fused in-frame to the sequence encoding the mature IDUA/SEKDEL protein and the *ARC5-I* gene 3′ UTR (Downing et al. 2006).

The *db35S-AMV-CnABI3-Nos* gene construct for co-expression studies (denoted as 35S-CnABI3, the 'transactivation construct'; Fig. 1, construct a) was generated as described in Zeng et al. (2003). The gene construct containing the vicilin (seed-storage-protein) gene promoter and 5′ UTR (Higgins et al. 1988) linked to the GUS gene coding region and containing a Nos 3′ end (Fig. 1, construct b, Vic-GUS) was produced as described in Jiang et al. (1995).

To generate the Arc-GUS construct (Fig. 1, construct c), the 5′ flanking sequences of the arcelin 5-I gene (isolated from *Phaseolus vulgaris* L., genotype G02771; Goossens et al. 1995, 1999; Downing et al. 2006) were PCR amplified (Genbank Z50202; nucleotides 697–1836; forward primer: GCGAAGCTTGTTATTTCCTCATCACCAGACAAA; reverse primer: CGCGTCTAGAGATCATGCATTCATGCATTAACCA). The resultant PCR product was digested with *HindIII* and *XbaI*. Plasmid pBI121 containing the *HindIII*–35S–5′-*XbaI*-GUS-Nos–3′ construct was treated with the same enzymes and following isolation of the double-digested fragments, the two were ligated. This replaced the 35S 5′ sequences with the arcelin 5′ sequences (promoter and 5′ UTR). The plasmid was sequenced to confirm the correct sequence and orientation prior to electroporating the plasmid into Agrobacterium LBA4404.

## Generation of transgenic tobacco plants

Stable transformation of tobacco was carried out using the leaf disc method (Higgins et al. 1988; Jiang et al. 1995). Transgenic tobacco plants were generated that expressed the 35S-hIDUA gene construct (alone) in a constitutive manner; production of the human protein in leaves was monitored by Western blotting.

Dually transformed tobacco plants were generated by co-expressing the *35S-CnABI3* gene (Fig. 1, construct a, the transactivation construct) (Zeng et al. 2003) and one of four gene constructs containing a seed-restrictive gene promoter (derived from the vicilin or arcelin–5-I genes), and either the GUS reporter gene coding region or the hIDUA gene coding region (Fig. 1, constructs b–e). Note that construct e of Fig. 1, Arc-hIDUA-KDEL, is the same gene construct as in (d), except that the coding region of the hIDUA gene has been modified so that the synthesized protein will contain a C-terminal ER-lumen-retention motif, SEKDEL. Stably transformed plants were cultured in magenta boxes at 25°C and sub-cultured every 3 months. Healthy, fully expanded leaves from 4-week plants were used in all experiments.

PCR was used to confirm both the single transformants, and the co-transformants expressing the transactivation construct (Fig. 1, construct a) and one of constructs b–e (Fig. 1) (data not shown).

### Effects of ABA and ABA analogues on GUS and IDUA expression

The effects of ABA and ABA analogues on the expression of GUS reporter- and hIDUA-genes were tested using the leaves of tobacco plants co-transformed with the appropriate chimeric gene construct and the *CnABI3* gene construct (Fig. 1). GUS was monitored at the activity level and hIDUA gene expression was monitored at the levels of steady-state IDUA mRNA, IDUA protein and IDUA activity. Co-transformed tobacco leaves of 4-week-old tissue culture plants were cut into ~5 $cm^2$ pieces (GUS testing) or cut symmetrically into four pieces (IDUA testing), and placed in liquid Murashige and Skoog (MS) medium (with 3% of sucrose; 2.5 ml MS medium per $60 \times 15$ $mm^2$ Petri dish) containing various concentrations of *S*-(+)-ABA (PBI 58), *R*-(-)-ABA, (+)–8′-acetylene ABA (PBI 425) and (+)–8′-methylene ABA (PBI 359). (+)–8′-Acetylene ABA (PBI 425) and (+)–8′-methylene ABA (PBI 359) were synthesized according to procedures described in Rose et al. (1997) and in Abrams et al. (1997), respectively. The leaf pieces were incubated at 25°C for 5–6 days under dim light, after which GUS and IDUA expression was monitored; this time point was associated with the maximum enzyme activities.

### Stress treatments for induction of human IDUA in tobacco leaves

The expression of hIDUA in co-transformed transgenic tobacco leaves was examined in response to some common stress conditions, all designed to enhance endogenous levels of ABA and/or ABA signaling. Leaves of co-transformed 4-week-old tissue-cultured tobacco plants were exposed to the following treatments in MS liquid medium for 6 days: 3–18% polyethylene glycol (PEG), 60–300 mM NaCl, 100 mM sucrose, and 200–600 mM glucose. The IDUA activities at the pretreatment time (day 0), and after exposure for 6 days in 0 μM ABA and 150 μM ABA in MS medium were used as controls. At the end of day 6, ~100 mg of leaf pieces were used for IDUA activity assays.

### GUS activity assays

Tissue samples were ground on ice in GUS extraction buffer (50 mM $NaPO_4$, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine and 0.1% triton X–100) and centrifuged at 10,000×g for 10 min at 4°C; GUS activities of the supernatants were determined by the fluorometric method of Jefferson (1987) using MUG (4-methyl umbelliferyl β-D-glucuronide) as a substrate (Sigma Chemical Co., St. Louis, MO, USA). Reactions were carried out at 37°C, aliquots were removed at regular time intervals and following the addition of 0.2 M $Na_2CO_3$ to terminate the reaction, fluorescence of the reaction mixture was determined with a Hoefer TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA). GUS activities were expressed as pmol MU/min/mg protein. Protein concentration of the extract was determined using the Bio-Rad Dc protein assay (Alam 1992) with bovine serum albumin fraction V (Bio-Rad, Mississauga, ON, Canada) as a standard. Activities represent the average of three determinations ± SD.

### Protein extraction, Western blot analysis and IDUA activity assays

Procedures for extraction of TSP from tobacco leaves, Western blot analyses, and analyses of IDUA activities were as described in Downing et al. (2006). Activities were expressed as pmol MU/min/mg TSP. Unless stated otherwise, activities represent the average of three determinations ± SD.

### RNA extraction

Transgenic leaves were ground in liquid $N_2$ with a minipestle then add 0.5 ml of ''Concert'' plant RNA reagent (Invitrogen Life Technologies) and 0.1 ml of phenol (Invitrogen) in a 1.5-ml Eppendorf tube. After thawing on ice, samples were ground more thoroughly. Isolation of RNA by subsequent centrifugation was according to the supplier's protocol. The final RNA pellet was dissolved in sterile $H_2O$.

### Generation of [$^{32}$P]-radiolabeled DNA probes

*IDUA probe*: A GC-rich PCR system (Roche, Mississauga, ON, Canada) was used to PCR-amplify and label the IDUA probe with α-[$^{32}$P]-dCTP (PerkinElmer-NEN, Montreal, PQ, Canada) according to the supplier's instructions. A 0.54-kb *Pst*I-*Xba*I fragment (3′ end of the IDUA cDNA) isolated from pAK12 was used as template, with forward primer oAK3 (5′-CTCTGCAGCCCCGACGG-3′) and reverse primer oAK4a (5′-TGGATTGCCCGGGGATGG-3′). The labeled probe was purified using a PCR purification kit (QIAGEN, Mississauga, ON, Canada). *18S rDNA probe*: The 1.5-kb *Eco*RI 18S rDNA insert of clone ''G'' from radish (Delcasso-Tremousaygue et al. 1988) was

labeled with α-[$^{32}$P]-dCTP using the Rediprime II random prime labeling system (Amersham Biosciences, Baie d'Urfé, PQ, Canada). The labeled probe was purified by using a MicroSpin S–200 HR column (Amersham Biosciences).

### Northern hybridization analysis

Total RNA (7 μg) was fractionated, with an RNA ladder (high range molecular weight; Fermentas Canada Inc., Vancouver, BC, CANADA) on a 1% agarose–0.64 M formaldehyde gel (Sambrook et al. 1989). RNA species were transferred to Hybond-XL membrane (Amersham Biosciences) according to the manufacturer's protocol, using the downward blotting method adapted for RNA transfer (Koetsier et al. 1993). After UV-crosslinking (GS GeneLinker UV Chamber, Bio-Rad), the membrane was prehybridized in modified Gilbert–Church buffer and hybridized with [$^{32}$P]-labeled IDUA probe at 65°C according to the supplier's instructions in a Hybaid incubator (Bio/can Scientific, Montreal, PQ, Canada). The membrane was washed briefly at room temperature with 2XSSC, 0.1% SDS; twice with 2XSSC, 0.1% SDS at 65°C for 15 min, and twice with 0.1XSSC, 0.1% SDS at 65°C for 25 min. The membrane was exposed to X-OMAT LS X-ray Kodak film. The membrane was rehybridized with the [$^{32}$P]-labeled rDNA probe and treated as above.

## Results

### Constitutive expression of the human IDUA gene in transgenic tobacco yields low-protein levels and activity in leaves

Transgenic tobacco plants (15 independently transformed lines) were generated expressing the 35S-hIDUA gene (Fig. 1) and the IDUA protein was monitored (Fig. 2). Western blot analysis of selected tobacco lines (five lines exhibiting IDUA activities of 0.04 pmol/min/mg TSP or greater), shows that the different lines contain variable amounts of IDUA protein in leaves (Fig. 2). Very low amounts of the ~79-kDa IDUA protein were detected in the leaves, and in some lines the protein appeared to be subject to proteolysis yielding lower molecular weight species. Perhaps most importantly, in contrast to the dually transformed plants (see below), the activities of hIDUA in the leaves were very low among the population of transformants. For example, in the five lines shown in Fig. 2, they ranged from 0.04 to 0.34 pmol/min/mg protein.

### Ectopic expression of the CnABI3 gene induces expression of human recombinant IDUA in transgenic tobacco leaves

For these studies, we used a gene construct that yields high-level expression of the hIDUA in seeds (e.g., those of Arabidopsis *cgl* plants) (Downing et al. 2006). This construct is denoted Arc-hIDUA in Fig. 1, and contains the 5′-flanking, 5′ UTR, signal-peptide-encoding, and 3′ flanking sequences from the *arc5-I* gene. The conifer *CnABI3* gene used for co-transformation studies contained sequences for enhanced transcription/translation, i.e., a modified 35S cauliflower mosaic virus promoter containing a duplicated 400-bp enhancer element; a 5′-UTR from the AMV RNA 4 (Datla et al. 1993) and a 3′ end of the Nos gene (Depicker et al. 1982) (Fig. 1, construct a). This construct yields ectopic (constitutive) expression of the *CnABI3* gene in tobacco leaves and other vegetative tissues (Zeng et al. 2003). Sequences encoding the carboxy-terminal ER retention motif, KDEL (as SEKDEL) were added onto the coding region of one of the IDUA constructs (construct e, Fig. 1), since localization of recombinant proteins to the ER lumen often promotes their enhanced stability in vegetative tissues.

As shown in Fig. 3a, b, within the population of 20 transgenic lines containing constructs Arc-hIDUA or Arc-hIDUA-KDEL alone (Fig. 1, construct d or e alone; black bars), the levels of IDUA activities in leaves were similar to that exhibited by the control (i.e., in the leaves of untransformed tobacco plants, wt). However, in the co-transformed plants expressing either Arc-hIDUA or Arc-hIDUA-KDEL, along with the 'transactivation construct'—the *CnABI3* gene, IDUA activities were induced (Fig. 3, shaded bars). In the highest lines, IDUA activities were 1,180 and 360 pmol/min/mg TSP, respectively.

### ABA and metabolism-resistant ABA analogues show strong synergistic effects with CnABI3 in the ectopic activation of reporter gene expression driven by seed-restrictive gene promoters

Because of the mechanism of action of the ABI3 transcription factor in transactivating seed protein gene promoters, it is expected that the phytohormone ABA will act in a synergistic manner, promoting a further increase in gene transcription (see discussion) (Zeng et al. 2003; also see references therein). Indeed, we know from previous studies that CnABI3 is able to activate the promoters of the seed-restrictive genes vicilin and napin, and induces the ectopic expression of

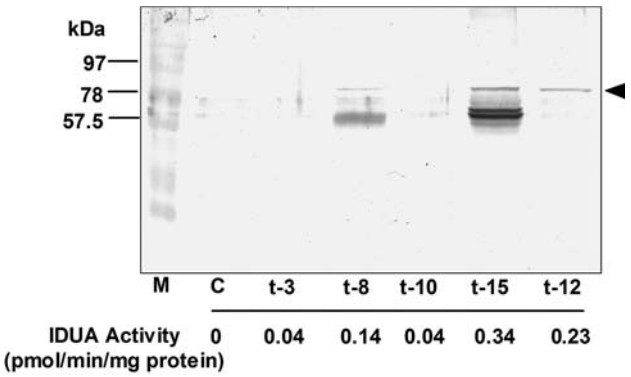



**Fig. 2** Western blot analysis of hIDUA protein in leaves of transgenic tobacco expressing the gene construct for constitutive expression shown in Fig. 1(1). Soluble protein was extracted from tobacco tissue culture plants using the fully expanded leaves. Fifty microgram of soluble protein was added in each lane. The hIDUA activities are shown on the lower panel and are expressed as pmol/min/mg TSP. The leaves of the independent transformants contained variable amounts of hIDUA protein (~79 kDa) and low-hIDUA activities

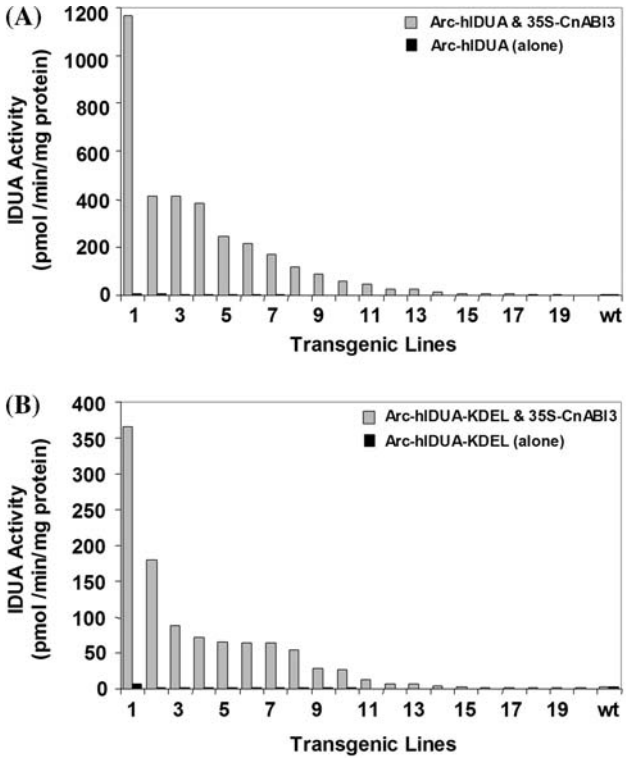
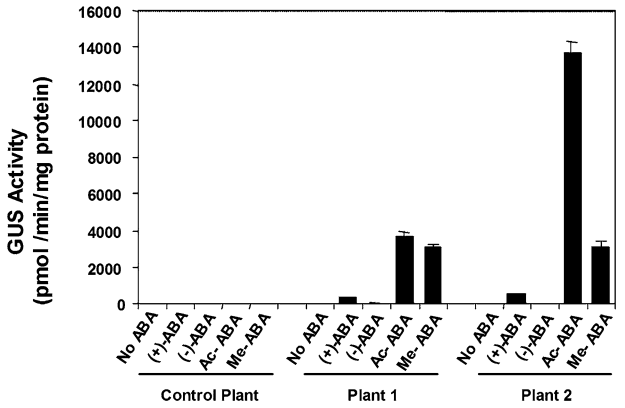



**Fig. 3** Enhancement of recombinant hIDUA activities in transgenic tobacco in the presence of the CnABI3 protein. Transgenic tobacco leaves expressing constructs Arc-hIDUA or Arc-hIDUA-KDEL alone (*black bars*, **a** and **b**) have very little α-L-iduronidase activity. However, in the presence of the ABI3 protein (i.e., in tobacco leaves co-expressing the transactivation construct, 35S-CnABI3) and one of the human gene constructs (Arc-hIDUA or Arc-hIDUA-KDEL) (*shaded bars*, **a** and **b**), there is a major increase in the yield (activity) of the recombinant protein. Activities represent the average of two determinations; plants were previously screened by PCR to reveal only positive transformants or co-transformants. *Wt* non-transformed tobacco leaves

GUS in tobacco leaves; ABA has a further enhancing effect on gene expression (Zeng et al. 2003).

*S*-(+)-ABA is the natural enantiomer of ABA; in studies of exogenous application, *S*-(+)-ABA generally exhibits differential effects on gene expression, and is a stronger inhibitor of dicot germination, than is the unnatural enantiomer of ABA, *R*-(-)-ABA (reviewed in Zaharia et al. 2005; Lin et al. 2005). Enzymatic hydroxylation of ABA by plant tissues (the first step in its metabolism) involves cleavage of a C–H bond at the 8′ position, a reaction carried out by 8′-hydroxylase. Chemically modified analogues have been synthesized that are metabolized more slowly, i.e., are more persistent in plant tissues and possess activity greater than that of the unmodified hormone, *S*-(+)-ABA. Two such analogues are (+)–8′-methylene ABA, and (+)–8′-acetylene ABA; the latter is an inhibitor of the ABA 8′-hydroxylase. In some plant tissues (e.g., corn suspension cultures), in vivo oxidation rates are slower for these analogues than for *S*-(+)-ABA, and the biological activities of the analogues are higher with respect to delaying/inhibiting seed germination (Abrams et al. 1997; Cutler et al. 2000; reviewed in Zaharia et al. 2005).

In the present case, we studied the effects of natural ABA and the ABA analogues on gene expression in the co-transformed tobacco plants. We first determined the ability of the ectopically expressed conifer *ABI3* gene to trans-activate the expression of the common GUS reporter gene (driven by seed-restrictive promoters) in the presence of natural ABA, unnatural ABA and the two metabolism-resistant ABA analogues. For this analysis, two of the lines previously demonstrated to co-express 35S-CnABI3 and Vic-GUS genes (Zeng et al. 2003) were selected. As shown in Fig. 4, while the natural ABA has an enhancing effect on vicilin-promoter-driven GUS reporter activities in leaves of co-transformed tobacco plants (in comparison to no ABA and the unnatural *R*-(-)-ABA), the two metabolism-resistant ABA analogues showed stronger effects. We cannot make conclusions about the relative effectiveness of one analogue over the other, because of the variability between different transgenic lines in their responses to (+)–8′-acetylene ABA versus (+)–8′-methylene ABA, in promoting GUS reporter activities. Used at the same concentration (20 μM) on independent transgenic lines co-expressing the 35S-CnABI3 gene and Vic-GUS, (+)–8′-acetylene ABA had variable effects on the corresponding GUS activi-




| Treatment | GUS activity (pmol MU/mg protein/min) | | |
|---|---|---|---|
| | Control plant | Plant 1 | Plant 2 |
| No ABA | 10 ± 2 | 3 ± 1 | 8 ± 1 |
| (+)-ABA | 8 ± 1 | 355 ± 16 | 494 ± 31 |
| (-)-ABA | 8 ± 1 | 24 ± 10 | 3 ± 2 |
| 8'-Acetylene-ABA | 11 ± 1 | 3651 ± 261 | 13719 ± 632 |
| 8'-Methylene-ABA | 5 ± 2 | 3055 ± 220 | 3056 ± 349 |

**Fig. 4** Effects of 20 μM ABA and ABA analogues on GUS activities in leaves of transgenic tobacco plants co-expressing 35S-CnABI3 and Vic-GUS genes. Shows the effects of *S*-(+)-ABA, *R*-(-)-ABA, (+)–8′-acetylene ABA (PBI 425), and (+)–8′-methylene ABA (PBI 359) on GUS activities of the singly transformed (Vic-GUS control) plants and of the dually transformed (Vic-GUS/35S-CnABI3) plants. Plants 1 and 2 represent two independent (co-transformed) lines. The activities are also shown in a Table since some of the lower values are not illustrated well in the figure. *Ac-ABA* (+)–8′-Acetylene ABA (PBI 425), *Me-ABA* (+)–8′-methylene ABA (PBI 359)

ties, but the effect of (+)–8′-acetylene ABA was always stronger than that of the natural ABA (data not shown).

The same transgenic line co-expressing *Vic-GUS* and 35S-*CnABI3* genes (line 2 of Fig. 4), was selected for more detailed examination using different concentrations of natural ABA and (+)–8′-acetylene ABA (Fig. 5). Surprisingly, higher concentrations of both *S*-(+)-ABA and (+)–8′-acetylene ABA led to stronger enhancement of GUS activities. After 5 days of incubation, the effects of *S*-(+)-ABA showed a maximum at 200 μM; at higher concentrations (e.g., 300 μM), toxicity effects in the cultured leaves were observed. In contrast to the natural ABA, the effects of (+)–8′-acetylene ABA showed a maximum at 100 μM and the effect on GUS activity was similar to the effect of natural ABA at 200 μM. Leaf tissues showed severe stress characteristics at 150 μM (+)–8′-acetylene ABA and the majority of the cells died in 200 and 300 μM (+)–8′-acetylene ABA.

When comparing the effects of natural ABA and the metabolism-resistant analogue at the same (non-lethal) concentrations) (e.g., 100 μM), (+)–8′-acetylene ABA consistently showed stronger effects than natural *S*-(+)-ABA on the induction of GUS activities. This is particularly obvious when concentrations are at the relatively lower range; for example, at 10 μM, GUS activity of leaf tissues incubated with (+)–8′-acetylene ABA was 16 times higher than that of leaves incubated with ABA. These results initially suggested that ABA turnover (even in leaves maintained in dim light) limits the effectiveness of the natural ABA in the induction of GUS reporter gene expression monitored at the activity level (see below and discussion).

PCR identified 15 lines of tobacco transformants co-expressing 35S-ABI3 and Arc-GUS genes (Fig. 1, constructs a and c) (data not shown). Three lines with GUS activity higher than 200 pmol/min/mg protein were used for subsequent analyses. In this case, both (+)–8′-acetylene ABA and *S*-(+)-ABA showed similar effects on the enhancement of GUS activities over the control (i.e., no ABA) (Fig. 6). It is unclear whether the differences in GUS activities in response to the natural ABA versus the ABA analogue in Figs. 4 and 6 are a consequence of the different promoters used (those of the vicilin and arcelin genes, respectively).

## ABA and the metabolism resistant ABA analogue (+)–8′-acetylene ABA act synergistically with CnABI3 to activate human IDUA gene expression, protein levels and activity

Similar to the results using the GUS reporter gene constructs, the ectopic induction of hIDUA activity in tobacco leaves by CnABI3 was strongly enhanced in the presence of *S*-(+) ABA (Fig. 7). The induction of activity occurred in a concentration-dependent manner; 150–200 μM *S*-(+) ABA elicited the greatest increase in IDUA activity in co-transformed leaves. This was true for the activities of both the unmodified hIDUA protein (Fig. 7a) and the IDUA-KDEL protein (Fig. 7b). In the latter case, 150 μM *S*-(+) ABA stimulated an approximate 40-fold enhancement of IDUA activity as compared to transgenic leaves incubated in medium containing no ABA. For the unmodified IDUA protein, the fold enhancement was lower (Fig. 7a). However, the results of Fig. 7a were obtained using a leaves of a single co-transformed line; for other co-transformed lines, the level of enhancement of IDUA activity by150 μM ABA was variable, and as high as 50-fold (data not shown).

Similar to the Arc-GUS construct, and in contrast to Vic-GUS, the *differential effects* of the natural ABA versus the metabolism-resistant ABA analogue, (+)–8′-acetylene ABA, with respect to promoting hIDUA activities, were much reduced (Fig. 7a);

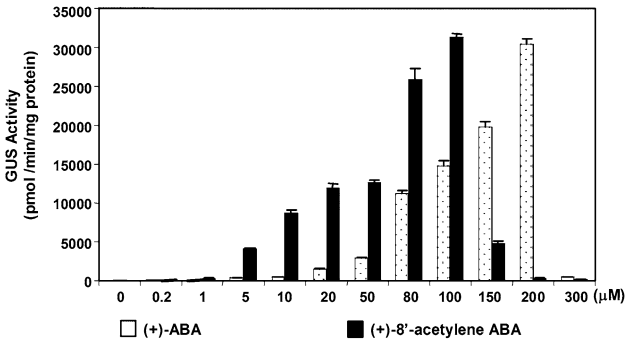



**Fig. 5** Effects of different concentrations of *S*-(+)-ABA and (+)–8′-acetylene ABA (PBI 425) on GUS activities in leaves of transgenic tobacco plants co-expressing 35S-CnABI3 and Vic-GUS genes. The leaves incubated on some of the higher concentrations of ABA (e.g., 300 μM of *S*-(+)-ABA and 150–300 μM (+)8′-acetylene ABA) showed severe stress characteristics and turned brown; most of the cells had died at the end of the culture period

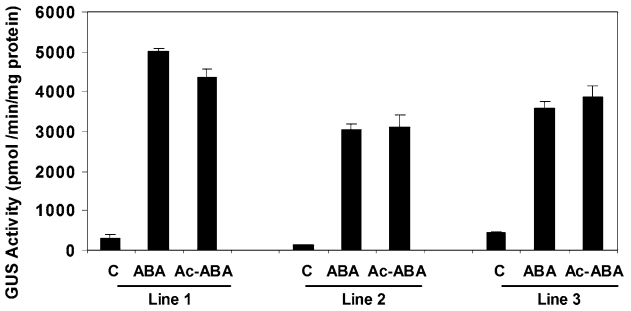



**Fig. 6** Effects of 150 μM *S*-(+)-ABA and (+)–8′-acetylene ABA (PBI 425) on GUS activities in leaves of three independent lines of transgenic tobacco plants co-expressing 35S-CnABI3 and Arc-GUS genes. *C* control: leaves incubated in culture medium containing no ABA. *Ac-ABA* (+)–8′-Acetylene ABA (PBI 425)

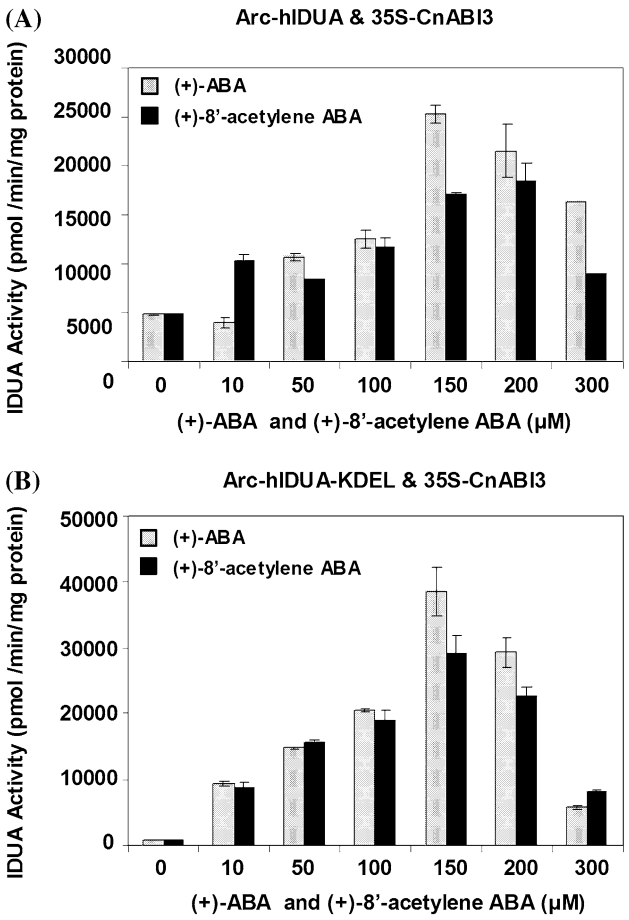



**Fig. 7** Effects of different concentrations of *S*-(+)-ABA and (+)–8′-acetylene ABA (PBI 425) on the enhancement of human α-L-iduronidase activity in leaves of plants co-expressing 35S-CnABI3 and hIDUA genes. When leaves of selected tobacco co-transformants (plants co-expressing 35S-CnABI3 and either Arc-hIDUA (**a**) or Arc-hIDUA-KDEL (**b**), are treated with *S*-(+)-ABA or (+)–8′-acetylene ABA, the α-L-iduronidase activities increase, reaching a maximum at 150 μM. Note that the leaves of one or two plants from the same line were used for these comparisons; leaves of the same stage/maturity were divided as described in the Materials and Methods section and incubated in ABA or ABA analogue at the different concentrations as indicated. Control leaves (i.e., co-transformed leaves that were not treated with exogenous ABA during the 5-day culture period) contained approx. fourfold higher human α-L-iduronidase activities than those present in freshly harvested leaves (compare the 0 ABA of Fig. 7 with transgenic line 1 of Fig. 3); this may have been due to an increase in the endogenous ABA contents of leaves during the culture period

enhancement of hIDUA activities occurred to a similar extent in the presence of both ABA molecules, and, as was the case for the *S*-(+) ABA, 150–200 μM (+)–8′-acetylene ABA yielded a maximal enhancement of IDUA activity. At 150–200 μM, the natural ABA appeared to promote slightly greater IDUA activity of IDUA-KDEL than did (+)–8′-acetylene ABA (Fig. 7b).

The effects of different ABA and (+)–8′-acetylene ABA concentrations on the enhancement of IDUA activity levels in co-transformed tobacco leaves correlated well with changes in corresponding accumulation of IDUA protein in the leaves, as shown by Western blot analyses (Fig. 8). In contrast to the constitutively produced IDUA (Fig. 2), there were no lower molecular weight products indicative of potential proteolysis of the human protein on the Western blots of Fig. 8 (data not shown). Northern hybridization analysis (Fig. 9) shows that ABA and (+)–8′-acetylene ABA, in this case applied at 100 μM, elicited a strong increase in the steady-state levels of hIDUA transcripts. Thus, as expected, the ABA-CnABI3 transactivation of human gene expression driven by arcelin-gene-regulatory sequences, appears to occur primarily at the level of transcription (and/or post-transcriptional mRNA sta-

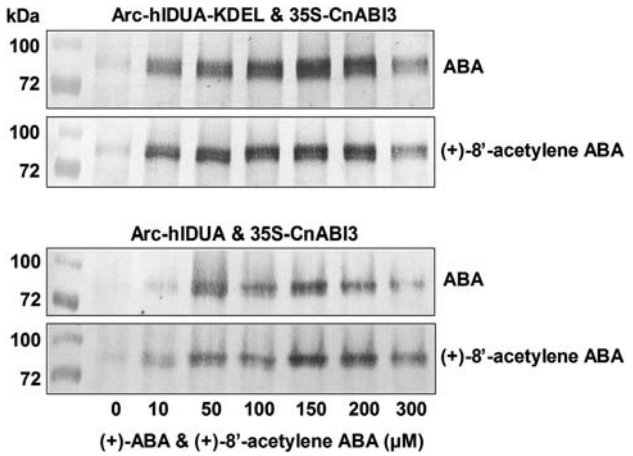



**Fig. 8** Western blot showing the effects of *S*-(+)-ABA and (+)–8′-acetylene ABA (PBI 425) at different concentrations on the levels of human IDUA protein. As with the activity data (Fig. 7), leaves of selected tobacco co-transformants treated with increasing concentrations of either *S*-(+)-ABA or (+)–8′-acetylene ABA, show the maximum hIDUA protein accumulation levels at 150–200 μM

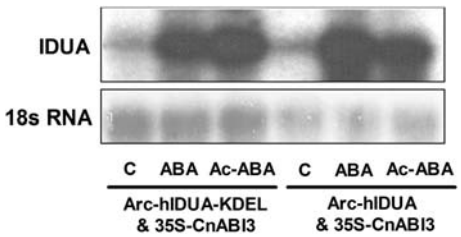



**Fig. 9** ABA enhances the steady-state levels of human IDUA mRNAs. Shows Northern blot analysis of tobacco leaves co-expressing CnABI3 and Arc-hIDUA genes or CnABI3 and Arc-hIDUA-KDEL treated with 100 μM *S*-(+)-ABA, 100 μM (+)–8′-acetylene ABA (PBI 425), or no ABA (“C”). When either ABA is present, the leaves show enhanced steady-state mRNA levels encoding α-L-iduronidase as compared to transgenic control leaves (leaves placed in culture without ABA). Leaves were treated with 100 μM ABA (instead of 150 or 200 μM) because the tissues were more green and fresh at the end of treatment with the lower concentration. *Ac-ABA* (+)–8′-Acetylene ABA (PBI 425)

bility); this enhancement effect not only leads to greater transcript levels, but also to greater accumulation of protein and activity levels in the transgenic leaves of co-transformed plants. Interestingly, the addition of the ER-lumen-retention (SEKDEL) motif onto the C-terminus of hIDUA appeared to promote a greater accumulation of IDUA protein levels and activity in the presence of ABA (Figs. 7, 8). This occurred despite no apparent enhancement in the steady-state levels of IDUA mRNAs (Fig. 9).

### Stresses treatments are effective in enhancing CnABI3-activation of human IDUA in tobacco leaves

Due to regulatory (e.g., containment) concerns, plant cell cultures derived from vegetative tissues may be a superior system to host high-level human recombinant protein production, as compared to whole transgenic plants (Kermode 2006). The present system to induce high-level production of hIDUA in vegetative tissues relies upon the presence of exogenous ABA or ABA analogue to maximize human gene expression. This system could be readily adapted to suspension cultures co-expressing a human gene and the *ABI3* gene; however the requirement of adding ABA would add considerable cost, especially when applied to a large-scale production system. Thus, toward developing more appropriate and cost-effective means of stimulating target human gene expression in this system, various stress-related treatments were imposed on cultured co-transformed tobacco leaves in an effort to stimulate higher endogenous concentrations of ABA and/or ABA signaling. These treatments included exposing leaves to mild water stress (PEG treatment), sugars, salt and chilling (Fig. 10); some, particularly the exposure of co-transformed tobacco leaves to 300 mM NaCl, had an even greater effect than exogenous 150 μM *S*-(+) ABA in promoting hIDUA activities. This strategy provides the means of enhancing the yields of recombinant proteins in transgenic plant vegetative tissues and potentially in cultured plant cells. The human recombinant protein can be readily induced in the presence of chemicals such as NaCl that can be added to cell cultures or even whole plants without a significant increase in production costs.

## Discussion

When expression of the human gene was geared for constitutive synthesis in the vegetative tissues of transgenic tobacco, levels of hIDUA activity were low and the protein appeared to be subject to proteolysis, a frequent problem in the use of plant vegetative tissue-based systems for human recombinant protein production.

The ABI3 family of transcriptional factors plays a role in regulating the expression of ABA-responsive genes in developing seeds or embryos. When expressed in an ectopic manner, (e.g., effected by stable transformation with a chimeric 35S:*ABI3* gene), the ABI3 protein from Arabidopsis re-activates expression of seed-specific genes in vegetative tissues and seedlings

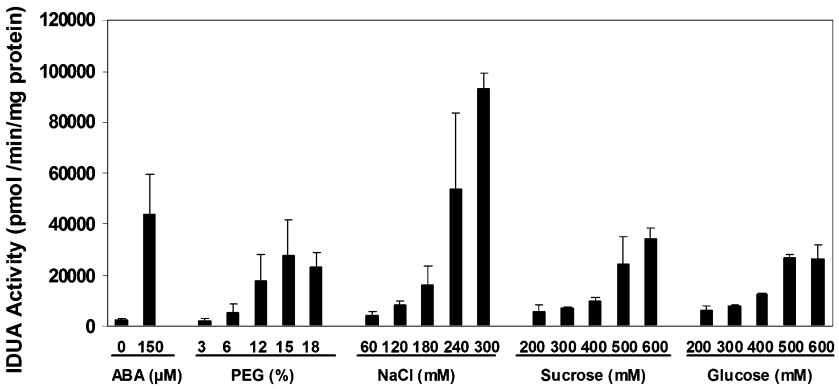



**Fig. 10** The effects of different treatments designed to enhance endogenous ABA levels and/or ABA signaling on human α-L-iduronidase activities. Leaves of selected tobacco co-transformants (plants co-expressing 35S-CnABI3 and Arc-hIDUA genes) were incubated in media containing the various chemicals as indicated

(Parcy and Giraudat 1997; Parcy et al. 1994). The *CnABI3* gene was cloned from the conifer yellow-cedar (*Chamaecyparis nootkatensis*) and is a homolog of *ABI3/VP1* genes of angiosperm species (Lazarova et al. 2002). This transcription factor exhibits some of the common characteristics of its angiosperm counterparts, including the ability to ectopically transactivate seed storage-protein gene promoters in tobacco leaves particularly in the presence of ABA (Zeng et al. 2003). The *CnABI3* gene is able to complement a severe *Arabidopsis abi3* mutant (*abi3–6*), which further attests to its functional conservation with angiosperm *ABI3* genes (Zeng and Kermode 2004).

In the present study, we examined whether ectopic (constitutive) expression of the yellow-cedar *CnABI3* gene would yield high levels of expression of a human gene in vegetative tissues in which expression of the latter was driven by a seed-restrictive promoter and other expression-enhancing regulatory sequences. Indeed we found that the constitutive synthesis of the conifer ABI3 transcription factor led to a transactivation of the arcelin promoter in vegetative tissues of transgenic tobacco, and accordingly high activity and levels of the human recombinant protein IDUA resulted, particularly in the presence of the phytohormone ABA. These findings are particularly important in light of our findings that expression of the hIDUA gene in tobacco leaves using a constitutive promoter leads to low-IDUA activities, and potentially, to the non-specific proteolysis of the protein. On a cautionary note, we cannot directly compare the results of leaf expression—in the one case the hIDUA gene was driven by the 35S promoter, and the precursor protein was synthesized with the human (IDUA gene-derived) signal peptide (Fig. 1(1)). In the other case, leaf expression was achieved by a transactivation process—and the precursor protein was synthesized with a plant (arcelin gene-derived) signal peptide (Fig. 1, constructs d and e). We have subsequently found that the level of hIDUA protein accumulation in a seed system (Arabidopsis *cgl* seeds) is influenced by the nature of the signal peptide—i.e., the arcelin signal peptide is superior to the human protein signal peptide (reviewed in Kermode 2006). Thus, this factor could also have played a role in the low-expression levels of constitutively produced hIDUA in tobacco leaves. Nonetheless, the strategy of transactivated-expression appears to be extremely useful for endeavors of molecular pharming in which vegetative tissues of the plant may be chosen to host recombinant protein production, or where cell cultures started from transgenic vegetative cells are used.

There was an unusual phenotype exhibited by many of the CnABI3-IDUA cotransformed tobacco plants; some of these plants exhibited early flowering and seed production. We did not observe this phenotype with the CnABI3-GUS co-transformants; therefore we cannot conclude that the unusual phenotype was due to constitutive expression of the CnABI3 protein. High-level synthesis of IDUA (a hydrolytic enzyme that breaks down the glycosaminoglycans heparin sulfate and dermatin sulfate of human/mammalian tissues; Neufeld and Muenzer, 2001) was likely a contributing factor. Presumably the unusual phenotype would not be a problem if the system were adapted to human recombinant protein production by suspension-cultured cells.

The synthesis of the CnABI3 protein was able to transactivate hIDUA gene expression driven by the arcelin promoter, particularly in the presence of ABA. Human IDUA transcripts were induced under these conditions in the tobacco leaves; the increase in steady-state levels of IDUA mRNAs also led to greater human protein and activity levels. It is possible that both transcriptional and post-transcriptional events led ultimately to the enhanced levels of human protein and activity in the transgenic tobacco leaves. Although the different steps in the pathway leading from the gene to

the functional protein are conventionally viewed as independent events, the various processes are often interdependent and functionally linked. At the transcriptional level, ABI3 is thought to regulate seed storage-protein gene expression by acting synergistically with other transcription factors (e.g., FUS3 and LEC1, LEC2 and others) that participate in combinatorial control (Kroj et al. 2003; Parcy et al. 1997; Finkelstein et al. 2002; Soderman et al. 2000; Nambara et al. 2000). ABI3/VP1 may recruit additional DNA-binding proteins to the promoters of storage-protein genes via its ability to alter chromatin structure (e.g., nucleosome positioning) (Li et al. 2001). Regulation of the expression of an *Arabidopsis* 2S storage protein gene (*At2S3*) appears to involve *FUS3* and *LEC2* that bind directly to promoter elements (RY repeats 1 and 2), while ABI3 acts in an indirect manner (likely via its interaction with bZIP proteins that bind to the G-box) (Kroj et al. 2003). The effects of ABI3 appeared to require ABA for maximal enhancement of gene expression; under these conditions there may be enhanced synthesis/stablization ABI3 and ABI5, the latter a bZIP transcription factor (reviewed in Finkelstein et al. 2002). ABI5 binding to ABREs (ABA Responsive Elements) may tether ABI3 to target promoters and facilitate the interaction of ABI3 with RY elements (a consensus sequence conserved in many seed-specific gene promoters) and transcription complexes (Finkelstein et al. 2002).

All ABI3/VP1 proteins contain four conserved domains: an acidic activation domain and three basic domains, B1, B2, and B3 (Giraudat et al. 1992; McCarty et al. 1991). ABI5 interacts directly via the B1 domain of ABI3 and two of the conserved charged domains of ABI5 that contain putative phosphorylation residues (Nakamura et al. 2001). The B2 domain of ABI3 is required for ABA-regulated gene expression and appears to facilitate the DNA binding capacity of a number of diverse DNA binding proteins (Carson et al. 1997; Hill et al. 1996). Moreover, interactions between the B2 and B3 domains, can mediate activation of target genes by interacting with different *cis*-acting DNA elements on those genes (Ezcurra et al. 2000).

(+)–8′-Acetylene ABA and (+)–8′-methylene ABA are relatively persistent analogues of ABA that exhibit slower oxidation in plant tissues and exhibit stronger effects than the natural unmodified natural ABA, *S*-(+)-ABA, especially with respect to inhibiting seed germination (Abrams et al. 1997; Rose et al. 1997; Qi et al. 1998; Cutler et al. 2000; Zaharia et al. 2005). In the present case, when the vicilin promoter was used to drive expression of the target gene, (+)–8′-acetylene ABA appeared to be superior to *S*-(+)-ABA with respect to inducing much higher GUS reporter activities in the presence of CnABI3 in transgenic tobacco leaves. Generally this was not the case when the arcelin promoter was used to drive expression of GUS or hIDUA. Here the differential effects of the different ABA molecules were much smaller, and at 150–200 μM, the natural ABA appeared to be slightly more promotive than the ABA analogue. It is difficult to conclude the reasons for the differences between these results (e.g., because of variability of expression levels between independent transgenic lines and their responsiveness, different promoters, and other gene regulatory characteristics). It is possible that there are different thresholds for ABA-related events (e.g., ABA signaling components) required for arcelin versus vicilin promoter activation. Both the pea vicilin gene promoter (Higgins et al. 1988) and the *arc 5-I* gene promoter (Goossens et al. 1995) contain ABREs; in the latter, a G-box/ABRE is located at –349 to –344. The rates of metabolism of ABA are expected to be similar in cultured tobacco leaves irrespective of the target transgene expressed. However, these results underscore the importance of not relying upon results from reporter gene systems alone. With few exceptions, the efficacy of strategies used to enhance the accumulation of a protein of interest in transgenic plant cells depends on the nature of the target protein. For this reason, it is inappropriate to rely on studies that are confined to reporter genes/proteins; while convenient, the same enhancement strategies may not work with complex proteins of pharmaceutical interest.

Application of stress treatments (rather than exogenous ABA) to transgenic plant tissues provides a convenient and cost-effective means of inducing recombinant protein expression. The ability of ABI3 to transactivate the arcelin promoter in the presence of high salt or sugars is likely in part to increased endogenous ABA in the tobacco leaves. In addition, novel pathways of ABA signaling may be induced by salt/sugar treatment of leaves. In rice, there is considerable cross-talk between signaling pathways for drought, ABA, and high-salinity stresses (Rabbani et al. 2003; Grennan 2006). Enhanced drought stress tolerance in Arabidopsis involves AREB1, a transcription activator of novel ABRE-dependent ABA signaling (Fujita et al. 2005). In a similar vein, a pathogen- and abiotic stress-inducible transcription factor of pepper, CARAV1 (RAV1: Related to ABI3/VP1), functions as a transcriptional activator triggering resistance to bacterial infection and tolerance to osmotic stresses (Sohn et al. 2006). There is further the possibility that exogenous salt is more effective than

exogenous ABA due to ABA-independent signaling events.

## Summary

Expression of certain recombinant proteins of industrial or pharmaceutical interest in transgenic plants is often problematic and yields can be extremely low, particularly in vegetative tissues. In the present example, we illustrate the enhanced production of enzymatically active hIDUA in transgenic tobacco leaves using co-expression of the transcription factor, CnABI3. The approach can be extended to the production of other recombinant proteins in plant-based systems and can be readily adapted to a plant cell culture-based production system. Transactivation of the gene of interest can be readily induced by addition of ABA, or for lower cost, by the addition of high salt or sugar.

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