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Tetrahedron: *Asymmetry* 16 (2005) 143–147

Resolution of (+)-abscisic acid using an *Arabidopsis* glycosyltransferase

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Received 27 October 2004; accepted 24 November 2004
Available online 23 December 2004

Abstract—Abscisic acid (ABA) can exist as two enantiomers, with (+)-ABA as the naturally occurring form. Typically, both enantiomers occur in chemical preparations and both can be modified in the plant to their respective glucose esters. To identify glycosyltransferases capable of discriminating between the different forms of ABA, the Family 1 enzymes of *Arabidopsis thaliana* were screened for activity towards (±)-ABA. Eight enzymes were found to recognise the plant hormone, with one UGT71B6 showing enantioselective glucosylation towards (+)-ABA. UGT71B6 was used in a whole-cell biocatalysis system as a means of separating (+)- and (−)-ABA, thereby offering an alternative to chemical synthesis for the production of pure (+)-ABA.

1. Introduction

Abscisic acid (ABA) is a plant hormone involved in the regulation of a wide range of developmental and stress-related events.[1–3] ABA is chemically synthesised as a racemic mixture of (+)- and (−)-stereoisomers,[4] although when extracted from plant tissues, the (+) enantiomer of ABA is recovered, indicating that this is the natural form of the hormone.[5,6] Whilst (−)-ABA is the 'unnatural' form, nevertheless when applied to plants or plant cell culture, the molecule has been reported to have effects on certain known ABA responses.[7–9]

In planta, glucosylation of ABA is one recognised means of controlling hormonal homeostasis,[5,6,10] and interestingly, when (±)-ABA racemic mixtures are applied, both enantiomers are converted to their respective glucose ester.[5] Conjugated ABA is thought to be inactive, but there is some evidence suggesting it represents a long-distance transport form of the molecule.[11–13]

As part of our on-going studies into the Family 1 glycosyltransferases of *Arabidopsis*,[14] we have identified a number of genes encoding enzymes that glucosylate plant hormones in a regioselective manner.[15–17] It was of interest to determine whether the enzymes from this family were also capable of stereoselective glucosylation and whether we would identify glycosyltransferases that could discriminate between the enantiomers of ABA. The results described herein show that there are eight enzymes capable of glucosylating ABA in vitro, but only one that recognises the naturally occurring enantiomer. We use this glycosyltransferase in a whole-cell biocatalysis system to resolve (+)-ABA.

2. Results

2.1. Identification of the *Arabidopsis* UGTs capable of glucosylating (±)-ABA in vitro

Members of the *Arabidopsis* UGT multigene family were expressed in *Escherichia coli*, purified as fusion proteins and screened for activity in vitro against (±)-ABA. The entire family[18,19] was analysed with the exception of two sequences (UGTs 76E9 and 90A1) which did not form soluble proteins. The reaction mixture was analysed individually using reverse-phase HPLC. An authentic (±)-ABA glucose ester, available commercially, was used as the reference for the chromatography. Of the

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doi:10.1016/j.tetasy.2004.11.062

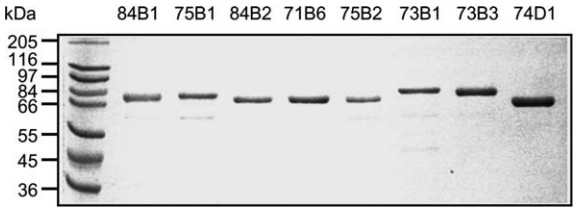

Figure 1. SDS-PAGE analysis of the recombinant UGTs capable of glucosylating (±)-ABA in vitro. The UGTs were analysed in a 10% (w/v) polyacrylamide gel and visualised with Coomassie Brilliant Blue staining.

105 enzymes analysed, eight UGTs (Fig. 1) were found to form ABA glucose ester in vitro; the chromatograms of these reaction mixes are shown in Figure 2. Amongst

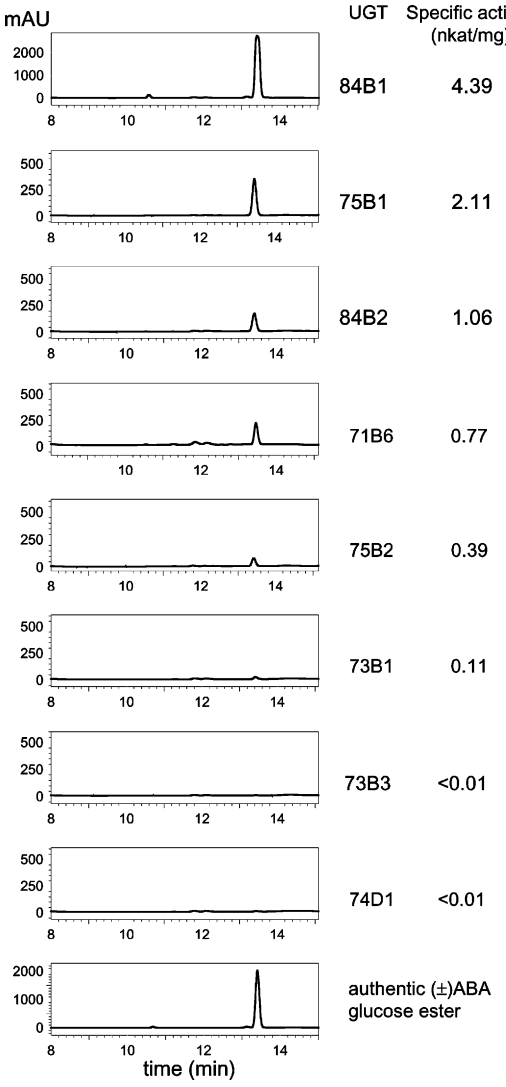

Figure 2. Reverse-phase HPLC analysis of (±)-ABA glucose ester formed in the reaction mix of different recombinant UGTs. The chromatograms (monitored at 270 nm) are shown at the same scale, except from that of UGT84B1 due to the high level of glucose ester produced in the in vitro reaction. The product peaks were quantified using the extinction coefficient of an authentic (±)-ABA glucose ester. The specific enzyme activity was expressed as nanomoles of (±)-ABA glucosylated per second (nanokatal, nkat) by 1 mg of protein.

these UGTs, UGT84B1 was found to possess the highest catalytic activity. UGTs 73B3 and 74D1 showed only trace activity toward (±)-ABA and were therefore not characterised further.

2.2. Enantioselectivity of the UGTs towards (±)-ABA

Since the racemic substrate used in the initial screen consisted of two enantiomers, (+)-ABA and (−)-ABA, the activity of the UGTs were further analysed using chiral HPLC to determine their enantioselectivity. As described in Figure 3, the (±)-ABA glucose esters produced by UGTs in vitro were purified and deglucosylated by β-glucosidase. The resultant enantiomers were then analysed by chiral HPLC using authentic (+)-ABA and (−)-ABA as references. Results are shown in Figure 4. Analysis of the authentic enantiomers indicated that (−)-ABA had less retention time on the chromatogram than (+)-ABA. All of the glucose esters from the reaction mixtures yielded both (+)-ABA and (−)-ABA, with the exception of the mix from one UGT. Thus, UGT71B6 produced a glucose ester from (±)-ABA that following glucosidase treatment yielded only the (+)-ABA, indicating the enzyme had only recognised a single enantiomer. In contrast, both enantiomers were found in the reaction mixtures from the other UGTs, showing the enzymes had glucosylated both (+)-ABA and (−)-ABA. The enantiomeric excess of (+)-ABA glucose ester in the product fraction of UGT71B6 was 92%.

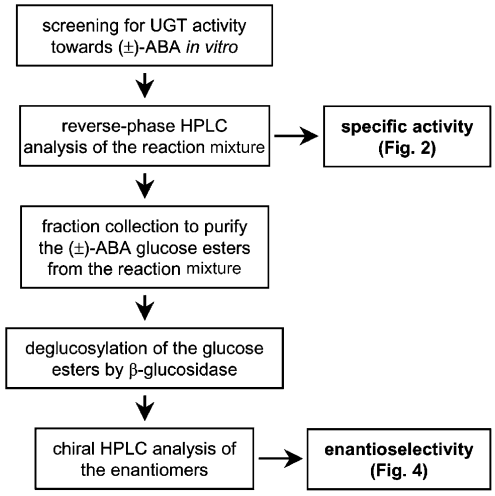

Figure 3. The steps involved in the analysis of UGT enantioselectivity.

2.3. Enantioselective synthesis of (+)-ABA glucose ester in whole-cell biocatalysis

Previous studies have shown that bacterial cells expressing plant UGTs can be used as whole-cell biocatalysts to glucosylate the substrate added to the culture medium in the absence of supplementary UDP-glucose.[20,21] In this study, *E. coli* cells expressing UGT71B6 were incubated with 0.1 mM (±)-ABA. Under the conditions used, 20% of the aglycone was converted within 24 h and the glucose esters were recovered in the culture medium (Fig.

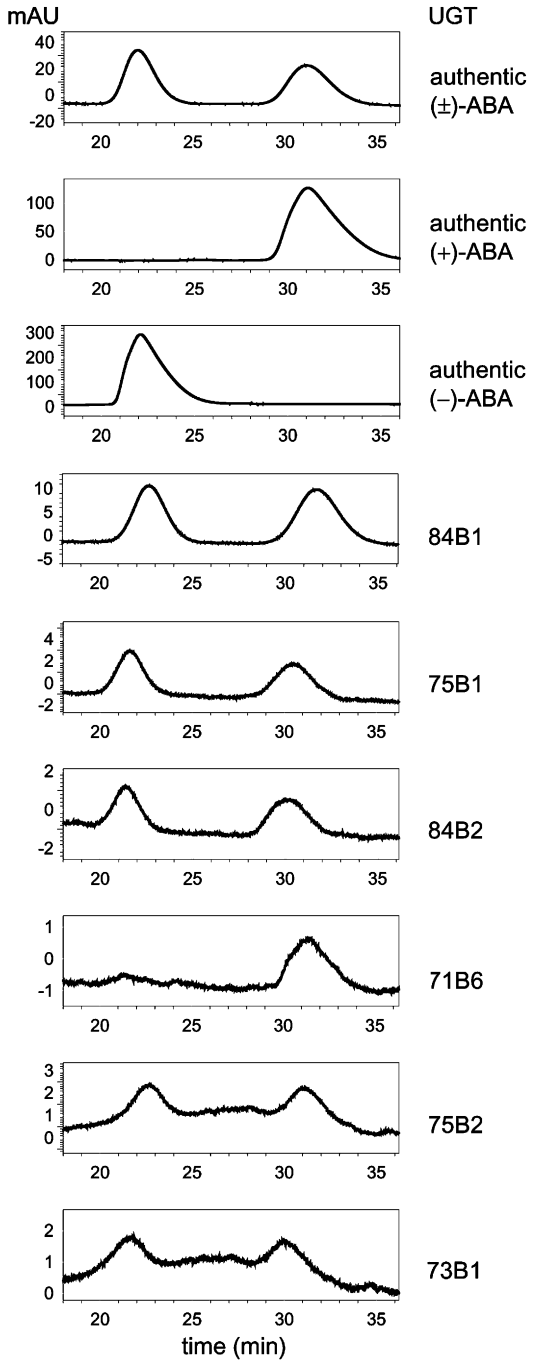

Figure 4. Chiral HPLC analysis of the (±)-ABA enantiomers released from the glucose esters generated by different UGTs. Authentic (+)-ABA and (−)-ABA enantiomers were used as references.

5A and B). The glucose ester peak (Fig. 5B) was purified and deglucosylated as described before and the products analysed by chiral HPLC, as shown in Figure 5B (insert). As expected, (+)-ABA was the major enantiomer present, indicating that the whole-cell biocatalysis system could be used to separate (±)-ABA enantiomers. The enantiomeric excess of (+)-ABA glucose ester was slightly lower (84%) than that observed for the in vitro system, probably reflecting a trace activity of UGT71B6 towards the (−)-ABA, when the enzyme was used in bacterial cells.

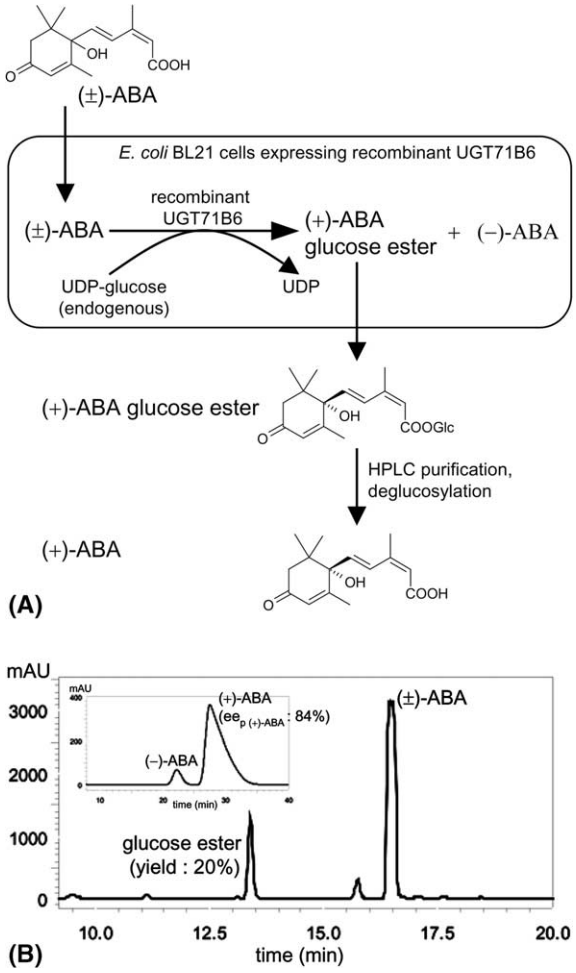

Figure 5. The use of UGT71B6 as whole-cell biocatalyst to purify the (+)-ABA enantiomer. (A) The whole-cell biocatalysis process. (B) Reverse-phase HPLC analysis of the culture medium from the whole-cell biocatalysis process; insert: chiral HPLC analysis of the (±)-ABA enantiomeric composition of the product fraction after glucose ester purification and deglucosylation.

3. Discussion

Our study has revealed that eight Family 1 UGTs from *Arabidopsis thaliana* are capable of glucosylating the plant hormone ABA in vitro. Of these eight, only one UGT, 71B6, recognises the natural enantiomer (+)-ABA. There is one previous study of a recombinant enzyme able to glucosylate ABA in vitro.[22] The gene was cloned from Adzuki bean and the enzyme was found to conjugate 2-*trans*-ABA, a biologically inactive isomer of the hormone.[23,24] The signal from the qualitative TLC analysis of products was found to be 12-fold higher towards 2-*trans*-ABA than that towards (−)-ABA, with a 2-fold selectivity also towards the (+)-ABA compared to (−)-ABA.

There has been interest in the glucosylation of ABA for many years, since the glucose ester is readily recoverable from plant extracts and whilst known to be biologically inactive, it has been implicated in the long-distance transport of the hormone.[11–13] Rapid progress has been made in the identification of genes encoding enzymes

involved in the hydroxylation of ABA,[25–27] the principle means of catabolism,[6] but until now little information has been available of the nature and number of enzymes involved in glucosylation. The UGTs described in this report are clearly capable of recognising (±)-ABA as a substrate, but the data provide no insight into whether the native enzymes function in ABA homeostasis *in planta*.

Interestingly, UGT84B1 showing the highest activity in vitro towards (±)-ABA also glucosylates IAA and can disturb IAA homeostasis when the gene is constitutively over-expressed *in planta*.[15,28] The biological significance of this dual activity is as yet unknown, but it will be of interest to determine whether glucosylation plays a role in balancing the relative cellular levels of ABA and IAA.

4. Conclusion

To our knowledge, this is the first demonstration of a glycosyltransferase from this family recognising a substrate in an enantioselective manner. The glucosylation of (+)-ABA by UGT71B6 offers a novel means of separation of (±)-ABA enantiomers and we have used a whole-cell biocatalysis system to produce the (+)-ABA glucose ester and go on to recover the single enantiomer. This method provides an alternative to chemical synthesis and chromatographic resolution for the large-scale preparation of the natural form, (+)-ABA,[29–34] and establishes a new foundation for using glycosyltransferases for chiral resolution.

5. Experimental

5.1. Glycosyltransferase activity assay

The construction of the UGT expression plasmids has been described previously.[35] The plasmids were transformed into *E. coli* XL-1 Blue individually for recombinant protein expression. Recombinant UGTs were purified as fusion proteins with glutathione-*S*-transferase attached to the N-terminus of the UGTs.[34] 105 UGTs out of the total 107 UGTs were subjected to the initial screen. UGTs 76E9 and 90A1 were not included due to the inability to recover soluble recombinant proteins. The glucosyltransferase assay was carried out following the conditions described by Schwarzkopf and Miersch with modifications.[36] Each assay mix (200 μL) contained 1 μg of recombinant protein, 50 mM Tris–HCl, pH 7.0, 2.5 mM UDP-glucose and 0.5 mM (±)-ABA. The reaction was carried out at 30 °C for 1 h, and was stopped by the addition of 20 μL of trichloroacetic acid (240 mg/mL), quick-frozen and stored at −20 °C prior to the reverse-phase HPLC analysis.

5.2. Reverse-phase HPLC analysis

Reverse-phase HPLC (SpectraSYSTEM HPLC systems and UV6000LP Photodiode Array Detector, ThermoQuest) analysis was carried out using a Columbus 5 μ C18 column (250 × 4.60 mm, Phenomenex). (±)-ABA glucose ester (retention time, R_t = 13.4 min) was separated from (±)-ABA (R_t = 16.5 min) by a linear gradient of 10%–90% methanol in H_2O (all solutions contained 2.5 mL/L of acetic acid and 0.4 mL/L of triethylamine) at 1 mL/min over 20 min. The chromatogram was monitored at 270 nm. Authentic (±)-ABA glucose ester purchased from OlChemIm Ltd (Czech Republic) was used as reference to confirm the (±)-ABA glucose ester produced in the in vitro reactions. An extinction coefficient for quantification of reaction products was obtained using authentic (±)-ABA glucose ester.

5.3. Sample preparation for chiral HPLC

The (±)-ABA glucose ester produced by recombinant UGTs was purified using reverse-phase HPLC equipped with a Gilson FC 240 fraction collector. The sample was freeze-dried in vacuo and dissolved in 75 mM sodium phosphate, pH 5.0. The glucose ester was then treated with β-glucosidase (Sigma) at 37 °C for 1 h to release the aglycone (±)-ABA. The completion of deglucosylation was confirmed using reverse-phase HPLC. Water–saturated butanol was used to extract the aglycone. The organic extract was then dried in vacuo, dissolved in 75 mM sodium phosphate, pH 5.0, and subjected to chiral HPLC analysis.

5.4. Chiral HPLC analysis

Chiral HPLC was performed using a Chiral-AGP column (100 × 4.0 mm, ChromTech). (±)-ABA enantiomers were separated by an isocratic mobile phase, 75 mM sodium phosphate, pH 5.0, at a flow rate of 0.9 mL/min. The chromatogram was monitored at 270 nm. (−)-ABA (R_t = 23.1 min) and (+)-ABA (R_t = 32.2 min) was confirmed by comparing the chromatogram with that of the authentic enantiomers purchased from Sigma.

The enantiomeric excess (ee_p) of the product fraction (glucose esters) was calculated from the equation $ee_p = (P - Q)/(P + Q)$ where P and Q are the (+)-ABA glucose ester and (−)-ABA glucose ester produced in the process.[37]

5.5. Whole-cell biocatalysis with UGT71B6

The *E. coli* BL21 culture expressing UGT71B6 was grown in 100 mL 2 × YT medium at 37 °C until the A600 reading reached 1.0. The bacterial cells were then exchanged to 50 mM sodium phosphate (pH 7.4) containing 10 g/L glucose, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and 1 mM (±)-ABA. After 24 h incubation at 28 °C, the culture was centrifuged at 5000*g* for 5 min. The supernatant fraction (culture medium) was collected for HPLC analysis described in the Section 5.4.

Acknowledgements

The research was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) grant

87/EGA16205 and funding from The Garfield Weston Foundation.

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