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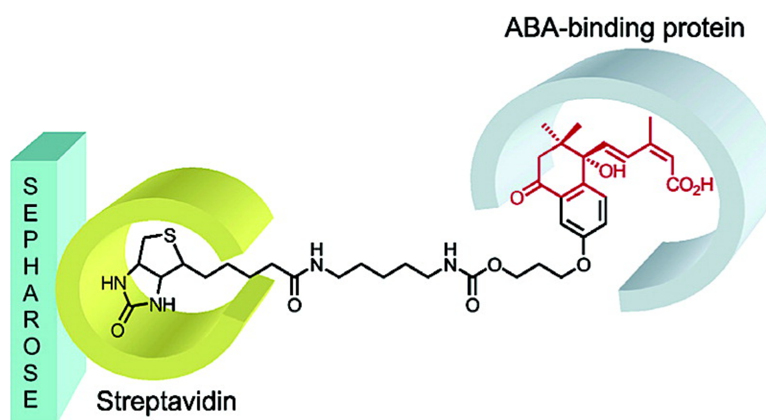

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An Affinity Probe for Isolation of Abscisic Acid-Binding Proteins

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The sesquiterpene plant hormone abscisic acid *S*-(+)-ABA **1** (Figure 1) regulates many agronomically important processes in plant growth and development.[5] For example, in developing seeds ABA mediates accumulation of storage proteins and lipids, tolerance of desiccation, inhibition of precocious germination, and maintenance of dormancy.[6] Stomatal aperture and plant responses to diverse environmental stresses such as drought, salinity, and extremes of temperature are regulated by ABA.[7] Screens for ABA-sensitive mutants have identified genes coding for transcription factors involved in ABA signal transduction processes and have been successfully employed for improving stress tolerance of plants.[8]

Genetic and biochemical approaches to identify ABA receptors have thus far proved unsuccessful despite considerable effort.[9] Several ABA-binding proteins involved in ABA metabolism have been identified recently. ABA 8′-hydroxylase, the key enzyme in the principal pathway of ABA catabolism in plants, a cytochrome P450 monooxygenase that oxidizes ABA at the 8′-carbon atom, was identified by screening genes homologous to gibberellin hydroxylases.[10,11] A glucosyltransferase from Adzuki bean seedlings has been also isolated and reported to be responsible for glycosylation of ABA at the C-1 position.[12] An ABA-binding protein of unknown function has recently been isolated by Razem et al.[13] by using an anti-idiotypic antibody. This protein was found to bind ABA and the biosynthetic precursors abscisyl aldehyde and alcohol. Receptor proteins involved in the initial perception of ABA are still unknown.

Affinity-based methods employing a biologically active derivative of ABA have the potential to identify ABA binding proteins including receptors. To date, probes constructed from derivatives of ABA, linked through either the carboxyl group or the ketone, have been employed to isolate proteins from plant extracts.[14−16] An inherent problem with these probes is that alterations of ABA at C-1 and C-4′ result in substantial loss in hormonal activity, presumably because of reduced affinity for ABA receptors. Zhang et al.[14] reported the isolation of a 42-kDa ABA-binding protein from the epidermis of broad bean leaves by affinity chromatography with ABA linked to sepharose through C-1. The function and sequence of the isolated protein have not yet been determined.

Yamazaki et al.[16] localized ABA-binding protein sites on stomatal guard cells by using microscopic methods with a C-4′-linked probe and fluorescence-labeled avidin. This approach is problematic because hydrazone derivatives of ABA have been reported to be inactive in stomatal responses.[17]

Our objective has been to design probes for ABA binding proteins specifically to target elusive ABA receptors. Such a probe must incorporate all the functional groups of ABA required for binding and activity. Structure−activity studies to determine the requirements of the ABA molecule for perception and action in

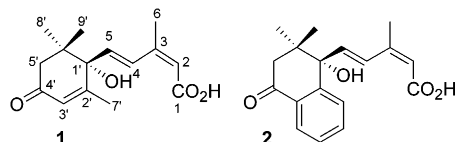

Figure 1. ABA (**1**) and its bicyclic analogue (**2**).

assays in a variety of plant tissues had led to the conclusions that a number of features of the ABA molecule should be maintained. The essential features are the stereochemistry at C-1′, the presence of the 7′ carbon, the 2-*Z*-enoic acid on the side chain and the ketone on the ring.[18] These requirements are met in a biologically active bicyclic analogue **2** in which an aromatic ring replaced the planar vinyl methyl group.[19] It was anticipated that an affinity probe could be developed with a biotin group attached through a tether to the aromatic ring, without sacrificing biological activity. In this communication we report the synthesis and validation of a bioactive, biotinylated ABA probe with the tether on the aromatic ring. The C-1 carboxylic acid group, vinyl carbon, and the C-4′ carbonyl group, which are crucial for ABA's activity, remain unmodified.

An efficient route for the synthesis of biotinylated bicyclic ABA probe (+)**-17** was developed employing 14 steps starting from commercially available 6-methoxy-1-tetralone (**3**) (Scheme 1). The reaction of **3** with methyl iodide in the presence of sodium hydride afforded the dimethyl analogue **4** in near quantitative yield.[20] Refluxing **4** with 48% hydrobromic acid overnight gave the phenol derivative **5**.[21] Introduction of a short three-carbon silyl-protected tether by alkylation of derivative **5** with 1-bromo-3-propanol followed by protection of the resulting alcohol **6** with TBDMSCl and imidazole gave analogue **7**. The side chain was introduced by direct addition of the lithium dianion of (*Z*)-3-methyl-2-penten-4-yn-1-ol to **7** to give alcohol **8**. Red Al reduction of the triple bond, followed by sequential oxidation to the aldehyde and then to the ester gave compound **11** (54% over three steps). Benzylic oxidation with PDC and *tert*-butyl hydroperoxide[22] afforded the ketone **12**, which was subsequently resolved on a preparative (*R*,*R*)-Whelk O1 HPLC column using 3% 2-propanol in hexane. To attach the biotinylated tether, the silyl group on the bicyclic analogue **13** was first removed with tetrabutylammonium fluoride (TBAF). The carbamate linkage was synthesized by reacting the primary hydroxyl group in **14** with carbodiimidazole (CDI) to give water-sensitive intermediate **15**, which was coupled in DMF with commercially available 5-(biotinamido)pentylamine to give the biotinylated ester **16**. Hydrolysis of the methyl ester (1.0 N KOH in methanol at 40 °C for 3 h) afforded the acid **17**. The stereochemistry of the enantiomers of **13** was assigned by comparison of elution order and specific rotation with the enantiomers of **2** (the absolute configuration of which had been determined). The resolved isomers

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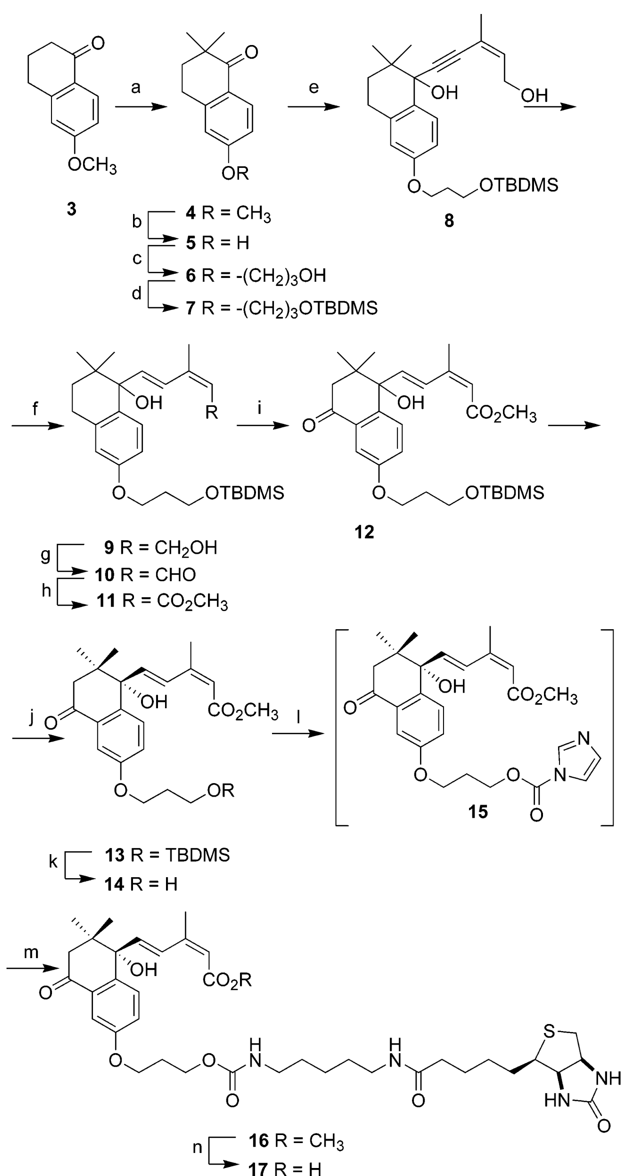
Scheme 1 [a]



[a] Reaction conditions: (a) NaH/MeI; (b) 48% HBr, 85% (2 steps); (c) $K_2CO_3/Br(CH_2)_3OH$, 84%; (d) TBDMSCl/imidazole, 92%; (e) (*Z*)-3-methylpent-2-en-4-yn-1-ol, *n*-BuLi, 76%; (f) RedAl; (g) MnO_2, acetone; (h) MnO_2/NaCN/AcOH/MeOH, 54% (3 steps); (i) PDC, *t*-BuOOH, 49%; (j) chiral HPLC; (k) TBAF; (l) CDI; (m) 5-(biotinamido)pentylamine, 53% (3 steps); (n) KOH(aq)/MeOH, 75%.

of **13** were treated separately giving enantiomerically pure diastereomeric acids (+)-**17** and (-)-**17**, in an overall yield of 7% from **3**.

ABA induces expression of genes involved in storage lipid deposition and modification in *Brassica napus*. Cultured microspore-derived embryos of *B. napus* are responsive to ABA and are a convenient system for probing hormonal regulation of gene expression and lipid synthesis.[23] We compared the ability of analogue (+)-**17** with (+)-ABA to induce 3-ketoacyl-CoA synthase (*FAE*) expression in microspore-derived embryos of *B. napus*. At 10 μM, analogue (+)-**17** was more potent than (+)-ABA in inducing mRNA for the *FAE* gene at 72 h, whereas the untreated control at 72 h no induction was observed (Figure 2).

An affinity column was prepared, using the biotinylated probe (+)-**17** and streptavidin-linked sepharose, and employed in two experiments validating the ability of the conjugated probe to bind ABA-specific proteins. First we tested binding of the probe to an

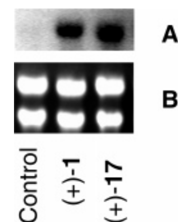


Figure 2. (A) Northern analysis of FAE expression with ABA analogue treatment (B) RNA loading for (+)-**1** and (+)-**17**.

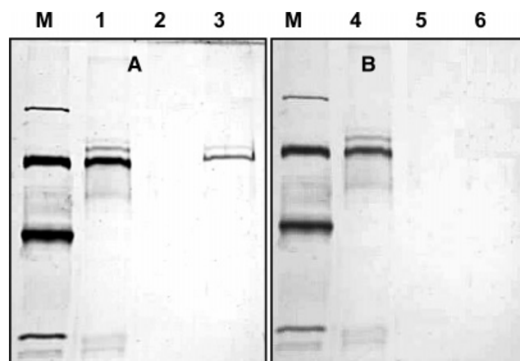


Figure 3. (A) SDS-PAGE (silver-stained) of fractions collected from the column modified with (+)-**17**, (M) protein MW standard (from top: 97.4, 66.2, 45.0, 31.0 KDa), (1) solution of anti-ABA monoclonal antibodies, (2) buffer wash, (3) 5 mM (±)-ABA wash; (B) fractions collected from unmodified column used as control, (4) solution of anti-ABA monoclonal antibodies, (5) buffer wash, (6) 5 mM (±)-ABA wash.

anti-ABA monoclonal antibody (mAb) raised against (+)-ABA.[24,25] The monoclonal antibody did not cross-react with (−)-ABA, but the cross-reactivity with (±)-**2** was found to be 23% relative to those of (+)-ABA (100%) and (±)-ABA (50%).[24] The mAb was retained by the probe-modified column upon washing with buffer and was released when ABA was applied as the eluting agent at the same pH (Figure 3). The unmodified column (without the probe) did not retain the antibody.

We have also used the affinity probe described above to isolate a membrane-bound protein, ABA 8′-hydroxylase.[11,26] The arabidopsis gene is encoded by CYP707A1, the cDNA open reading frame of which was transferred to pYeDP60 for expression in the WAT11 strain of *Saccharomyces cerevisiae*.[27] The yeast expression vector, pYeDP60, was generously provided by Denis Pompon (CNRS-Centre de Génétique Moléculaire). The solubilized microsomal fraction was applied to the affinity column, and the CYP707A1 enzyme was found to elute with (±)-ABA (Figure 4).[28] We did not observe any specific binding of proteins to the column in protein preparations from yeast transformed with only the pYePD60 (Figure 4). BSA, which is present in the microsomal protein extraction buffer in high quantity, was difficult to remove from the column and was therefore found in all column eluates. Q-TOF mass spectrometry confirmed the presence of the ABA 8′-hydroxylase enzyme in crude yeast microsomal extracts as well as in fractions from the column eluted with ABA. Peptide sequences derived from the purified, trypsinized protein were compared with published sequences and, as expected, corresponded to that of CYP707A1. The peptide coverage map presented in Table 1 represents 69% of the predicted amino acid sequence. This is direct evidence that ABA 8′-hydroxylase bound to the probe (+)-**17** on the affinity column and validated the probe as being recognized by an authentic ABA-binding protein.

In summary, we have synthesized a biologically active ABA probe (+)-**17** and demonstrated its effectiveness for isolating soluble

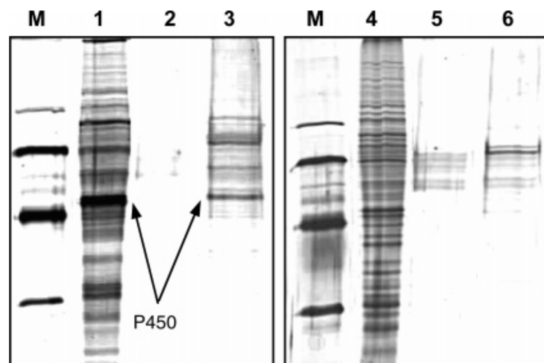


Figure 4. SDS-PAGE (silver-stained) of fractions collected from the column modified with (+)-**17**. (M) protein MW standard (from top: 97.4, 66.2, 45.0, 31.0 kDa), (1) crude microsomal protein extract from yeast cells expressing CYP707A1, (2) buffer wash, (3) 10 mM (±) ABA wash, (4) crude microsomal protein extract from yeast cells with pYeDP60 (control), (5) buffer wash, (6) 10 mM (±) ABA wash.

Table 1. Cytochrome P450 (CYP707A1) Coverage Map of Peptides Resulting from Trypsin Digestion; the Matched Peptides Cover 69% of the Protein (bold, underlined letters)

1	MDISALFLTLFAGSLFLYFLRCLISQRRFGSSKLPLPPGT MGWPYVGETF
51	QLYSQDPNVFFQSKQKRYGSVFK**THVLGCPCVMISSPEAA K**FVLVTK**SHL**
101	**FKPTFPASK**ERMLGK**QAIFFHQGDYHAK**LR KLVLR**AFMPESIRNMVPDIE**
151	**SIAQDSLRSWEGTMINTYQEMKTYTFNVALLSIFGK**DEVLYREDLKR**CYY**
201	**ILEKGYNSMPVNLPGTLFHK**SMKAR**KELSQILAR**ILSERR**QNGSSHNDLL**
251	**GSFMGDKEELTDEQIADNIIGVIFAARDTTASVMSWILKYLAENPNVLEA**
301	**VTEEQMAIRKDKEEGESLTWGDTKKMPLTS R**VIQETLR**VASILSFTFREA**
351	**VEDVEYEGYL IPK**GWKVLPLFR**NIHHSADI FSNPGK**FDPSR**FEVAPKPNT**
401	**FMPFGNGTHSCPGNELAKLEMSIMIHHLTTKYSWSIVGASDGIQYGPFAL**
451	**PQNGLPIVLAR**KPEIEV

and membrane-bound ABA-binding proteins. We plan to focus our future experiments on isolating unknown ABA binding proteins from plant tissues, including the elusive ABA receptors.

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Supporting Information Available: Experimental procedures and characterization data for synthesized compounds. This material is available free of charge via the Internet at http://www.pubs.acs.org.

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