A photoimmobilisation strategy that maximises exploration of chemical space in small molecule affinity selection and target discovery

Supplementary Information

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The three classes of photochemical[1] 'Tags' (diazirine,[2-7] two azides,[8] two benzophenones[9-11]) were linked to aminopropyl derivatised polystyrene 96-well plates using high yielding routes from readily available starting materials (Figure 1 and Scheme 1). In the case of the azide and benzophenone reagents, two very different functional linker groups (alkane and thiourea; ether and amide respectively) were employed, as we were conscious of the significant electronic effects these could have on photoreactivity. In order to enhance the biocompatibility of the supported photoreagent on both the amino-functionalised plates, a short PEG chain was incorporated.

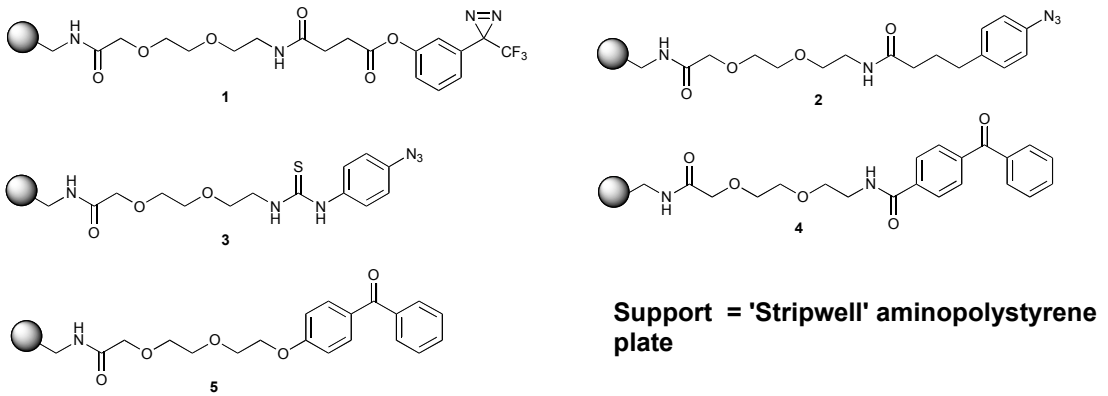
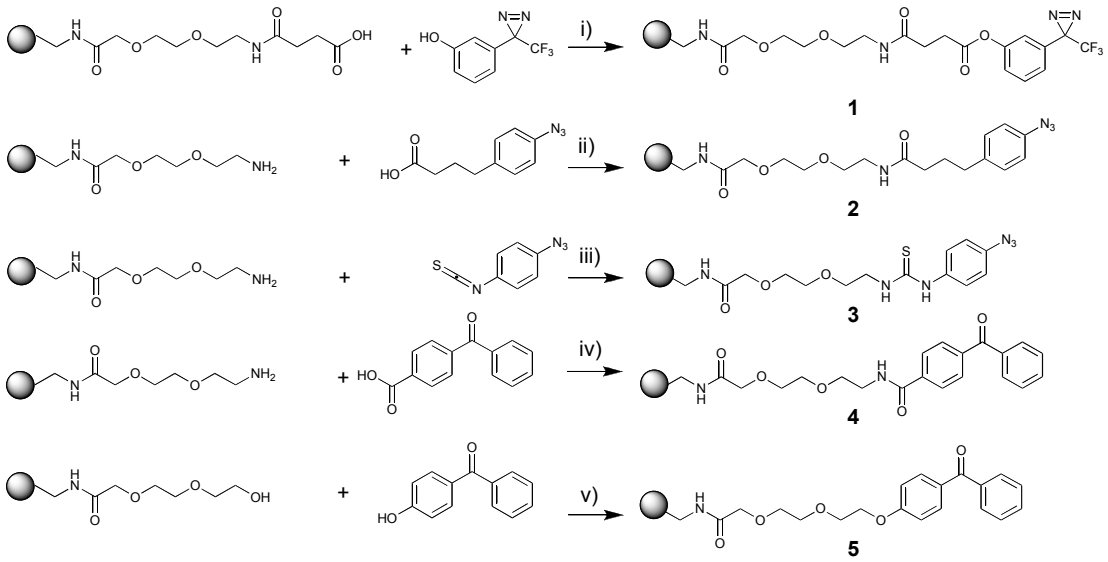


Figure 1. Aminopolystyrene Stripwell™ Plate supported photoreactive reagents ('Tags') incorporating PEG spacers.

The suite of supported photoreactive Tags was used to capture the test biological ligand, *S*-(+)-abscisic acid by brief UV irradiation (>254 nm) from dilute phosphate buffered saline solution. The supports were then incubated as described with polyclonal antibodies raised to *S*-(+)-abscisic acid haptens in two positions and, after washing, further treated with a fluorescently labelled (FITC) anti-idiotype antibody. The fluorescence of the treated supports was compared with that of supports treated with analogous blank solutions (see Experimental section). Further comparison was also made with the commercially available 'Universal BIND' plates.



Scheme 1 *Reagents and conditions*: i) EDC, DMAP, PBS; ii) DIPEA, EtOH; iii) EDC, DMAP, PBS; iv) Sodium ethoxide, EtOH; v) EDC, DMAP, PBS.

Experimental Section

General Stripwell™ amine functionalised plates were obtained from Corning, filtration plates with Univac® system were obtained from Whatman and all other chemical, solvents and reagents were obtained from Acros, Aldrich and Lancaster Synthesis and used without further purification. ^{1}H-NMR spectra were recorded on a Bruker DPX300 spectrometer. Chemical shifts are reported in ppm for chloroform-*d* solution using TMS as an internal standard unless stated otherwise. Infrared spectra were recorded using an Avatar 320 FT-IR spectrometer fitted with a "Golden Gate" attenuated total reflection attachment. Elemental analysis was performed by Warwick Analytical Services using a Leeman Labs CE44U elemental analyser.

Dark procedures indicates that reactions were performed with light excluded. If necessary certain manipulations were carried out under a standard photographic darkroom safelight.

Procedure for antibody binding assay to immobilised *S*-(+)-ABA

On each Stripwell™, four wells were treated with a solution of *S*-(+)-ABA (1 mg/ml in PBS, 0.2 ml); four wells with PBS only (0.2 ml) and incubated at room temperature for 1 h. The plate was then irradiated under a handheld 254 nm source for 5 min. The solution was tapped out and the plate was treated with a 2% solution of BSA (0.2 ml) for 1 h. The solution was removed, and the plate was then treated with a solution of appropriate *S*-(+)-ABA antibody (50 μl/well; MAC252 or Olchemim 0052707). After incubation for 1 h at room temperature the plate was then washed with: PBS (x 6). An FITC labelled anti-idiotype antibody (0.1 ml) was added to each well and incubated at room temperature for 1 h. The plate was then washed with: PBS (x 3) and water (x 3) and following addition of water (0.3 ml) the fluorescence (λ_{ex} = 485 nm; λ_{em} = 520 nm) was recorded on a Tecan GENios plate reader. An average was taken of the fluorescence reading from each four well segment containing *S*-(+)-ABA and the corresponding value from the wells containing PBS only was subtracted. Error bars represent the resulting standard deviation.

Procedure for immobilisation and phage screening of *S*-(+)-ABA.

Each well of a Corning Stripwell™ plate derivatised with one of Tags **1**–**5** was treated with a solution of *S*-(+)-ABA (1mg/ml solution in 1X phosphate buffered saline, PBS, 0.1 ml) and irradiated under a handheld 254 nm source for 10 minutes. The support was then washed with water (x 3), methanol (x 3) and PBS (x 3). Bacteriophage λ were amplified in *E. coli* TG1 to a titre of 2.2 x 10^{11} pfu/ml. Phage (0.1 ml) were then diluted with tris-buffered saline, *p*H 7.5. (TBS; 0.1 ml) and added to each of 5 Tag wells treated with *S*-(+)-ABA as above and 5 corresponding control Tag wells. The

phage were incubated at room temperature with gentle shaking for 1 h. The wells were then washed six times for 3 minutes each with TBS with 0.5% Tween (v/v; 0.3 ml). The wells were incubated with a 1 mg/ml solution of *S*-(+)-ABA for two hours, and the resulting solution added to a logarithmically growing culture of *E. coli* Q526 (50 ml). After overnight incubation, the phage were precipitated with PEG 8000 (3.3M in sat. brine) and stored in TBS. For rounds one and two of biopanning a portion of this stored solution was then re-amplified in TG1 for direct use in the subsequent round. In the third round, the solution was plated on NZY agar to allow selection of individual phage clones.

Procedure for secondary screening of selected phage clones

Each freshly amplified clone was incubated with immobilised *S*-(+)-ABA in each of the appropriate 5 wells. After washing with TBS containing 0.5% Tween, the phage were specifically eluted with TBS, or TBS solutions of *S*-(+)-ABA, *R*-(-)-ABA, indole acetic acid or DMSO (one each per well) at concentrations of either 1 mg per ml TBS or 1 μl per ml TBS in the case of DMSO. The phage retrieved during this elution were titred and the plaque forming units (pfu)/ml are reported as a percentage of those eluted only with TBS.

Preparation of derivatised Stripwell™ plates

Supported 2-(2-[2-acetimidoethoxy]ethoxy)chloride

A solution of 2-(2-[2-chloroethoxy]ethoxy) acetic acid (0.11 g, 0.6 mmol), EDC (0.24 g, 1.2 mmol and DMAP (10 mg, 0.08 mmol) in PBS (40 ml) and DMF (0.4 ml) was added to amine functionalised Stripwell™ plates (2 plates, 24 strips, 188 wells, 0.2

ml/well). The plate was shaken for 24 h, then washed with PBS (x 3), water (x 3) and methanol (x 3). Drying at 50 ^{o}C yielded the Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)chloride.

Supported 2-(2-[2-acetimidoethoxy]ethoxy)phthalimide

A solution of 2-(2-[2-phthalimidoethoxy]ethoxy) acetic acid (0.09 g, 0.3 mmol), EDC (0.12 g, 0.6 mmol) and DMAP (5 mg, 10 mol%) in PBS (30 ml) was added to an amine functionalised Stripwell™ plate (1 plate, 12 strips, 96 wells, 0.3 ml/well). The plate was shaken for 72 h, then washed with water (x 3), PBS (x 3) and methanol (x 3). Drying at 50 ^{o}C yielded the Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)phthalimide.

Supported 2-(2-[2-acetimidoethoxy]ethoxy)amine

A solution of hydrazine monohydrate (0.01 ml, 0.2 mmol) in water (0.01 ml) and ethanol (20 ml) was added to Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)phthalimide (1 plate, 12 strips, 96 wells, 0.2 ml/well). The plate was heated to 50 ^{o}C for 2 h, then shaken for a further 2 h, and washed with PBS (x 3), water (x 3) and methanol (x 3). Drying at 50 ^{o}C yielded the Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)amine.

Supported 2-(2-[2-acetimidoethoxy]ethoxy)acetamidopropanoic acid

A solution of succinic anhydride (25 mg, 0.25 mmol) and diisopropylethylamine (0.04 ml, 0.1 mmol) in acetonitrile (4 ml) was added to Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)amine (1 plate, 12 strips, 96 wells, 0.05 ml/well). The plate was shaken for 20 h, then washed with PBS (x 3), water (x 3) and methanol (x 3).

Drying at 50 ^{o}C yielded the Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)acetamidopropanoic acid.

Supported 3-(3-acetoxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine 1

Dark procedures followed.

A solution of EDC (0.08 g, 0.4 mmol) and DMAP (10 mg, 0.08 mmol) in PBS (20 ml) was added to a solution of 3-(3-hydroxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine (0.06 g, 0.3 mmol) in DMF (0.4 ml). The combined solution was then added to Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)acetimidopropanoic acid (1 plate, 12 strips, 96 wells, 0.2 ml/well). The plate was shaken for 20 h, then washed with water (x 3) and methanol (x 3) to yield the Stripwell™ plate-supported acetimidoethoxyethoxy phthalimide.

Supported 1-(4-azidophenyl) 4-amidobutyric acid 2

A solution of 4(4-azidophenyl)butyric acid (0.04 g, 0.2 mmol), EDC (0.08 g, 0.4 mmol) and DMAP (10 mg, 0.08 mmol) in PBS (20 ml) and DMF (0.2 ml) was added to Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)amine (1 plate, 12 strips, 96 wells, 0.2 ml/well). The plate was shaken for 48 h, then washed with PBS (x 3), water (x 3) and methanol (x 3). Drying at 50 ^{o}C yielded the Stripwell™ plate-supported 1-(4-azidophenyl) 4-amidobutyric acid.

Supported 4-isothiocyanophenyl azide 3

A solution of 4-azidophenylisothiocyanate (0.03 g, 0.17 mmol) and diisopropylethylamine (0.06 ml, 0.4 mmol) in ethanol (20 ml) was added to Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)amine (1 plate, 12 strips,

96 wells, 0.2 ml/well). The plate was shaken for 20 h, then washed with methanol (x 3), water (x 3) and methanol (x 3). Drying at 50 ^{o}C yielded the Stripwell™ plate-supported 4-isothiocyanophenyl azide.

Supported 4-amidobenzophenone 4

A solution of 4-benzoylbenzoic acid (0.046 g, 0.2 mmol), EDC (0.08 g, 0.4 mmol) and DMAP (10 mg, 0.08 mmol) in PBS (20 ml) and DMF (0.2 ml) was added to Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)amine (1 plate, 12 strips, 96 wells, 0.2 ml/well). The plate was shaken for 48 h, then washed with PBS (x 3), water (x 3) and methanol (x 3). Drying at 50 ^{o}C yielded the Stripwell™ plate-supported 4-amidobenzophenone.

Supported 4-hydroxybenzophenone 5

Sodium (10 mg) was added to ethanol (20 ml). On cessation of visible reaction, 4-hydroxybenzophenone (0.06 g, 0.5 mmol) was added. The bright yellow solution was then added to Stripwell™ plate-supported 2-(2-[2-acetimidoethoxy]ethoxy)chloride (1 plate, 12 strips, 96 wells, 0.2 ml/well). The plate was shaken for 4 h, then washed with methanol (x 3), water (x 3) and methanol (x 3). Drying at 50 ^{o}C yielded the Stripwell™ plate-supported 4-hydroxybenzophenone.

Additional Bioinformatics Results and Discussion

Selected clones were sequenced using Sanger dideoxy chain termination automated methods with BigDye Terminator.[12] When clone 3 was analysed using the gapped BLAST search algorithm[13] against the NCBI nonredundant (nr) database, two regions of high sequence identity were congruent with a single gene known as *ATE1* from the *A. thaliana* genome (Figure 7). However, a sequence of 9 b.p. (TGATGATGA) was identified as absent from clone 3 that was present in the database within a region of low complexity. Such regions are known to lead to base deletions and the deletion could represent natural genetic variation, or have been generated by slippage during PCR amplifications that were used in library construction and prior to sequencing.

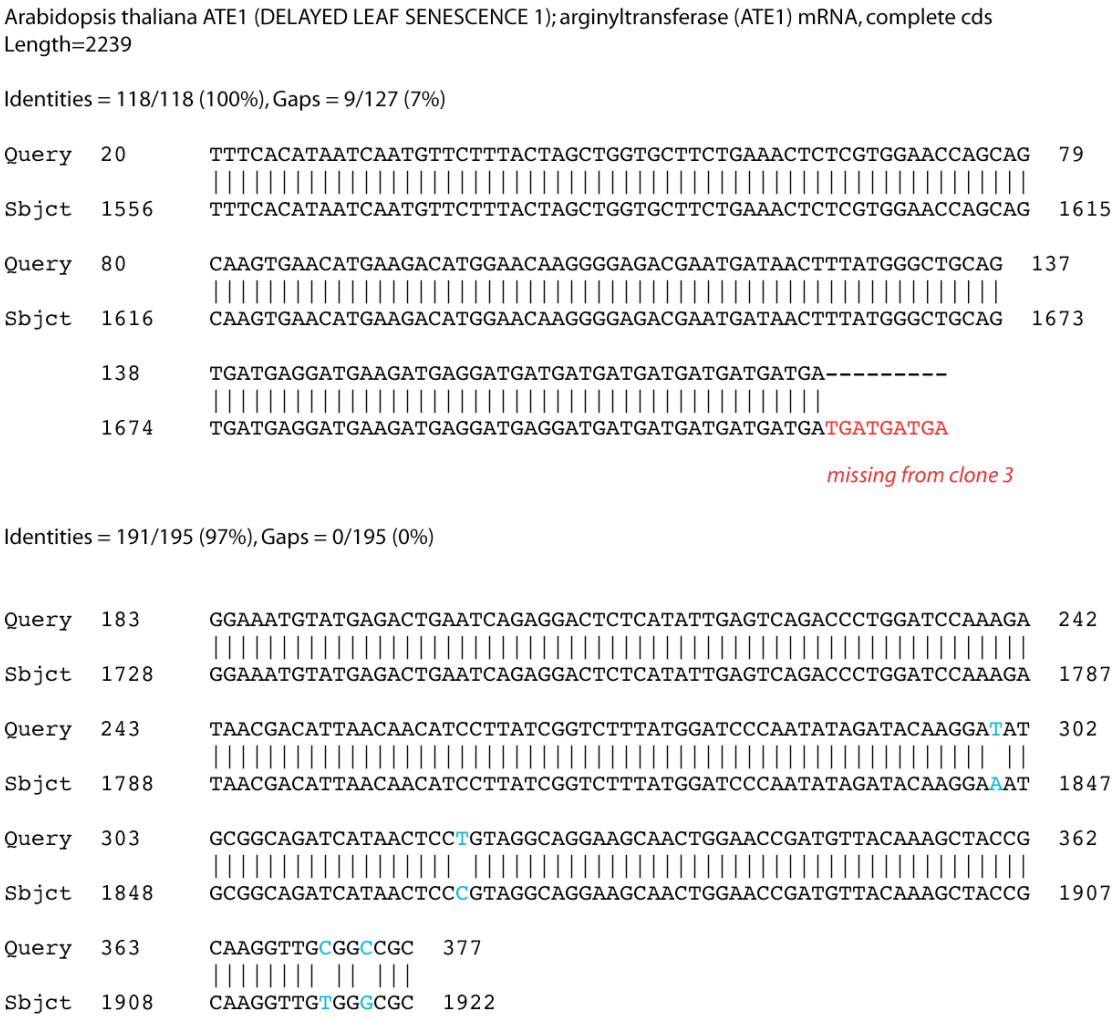


Figure 7 Clone 3 sequence identity and alignments highlighting sequence differences

The translated sequence from the insert is shown below aligned with the ATE1 ORF. It demonstrated no significant identity with solved protein structures in the RCSB protein databank (2-3-07).

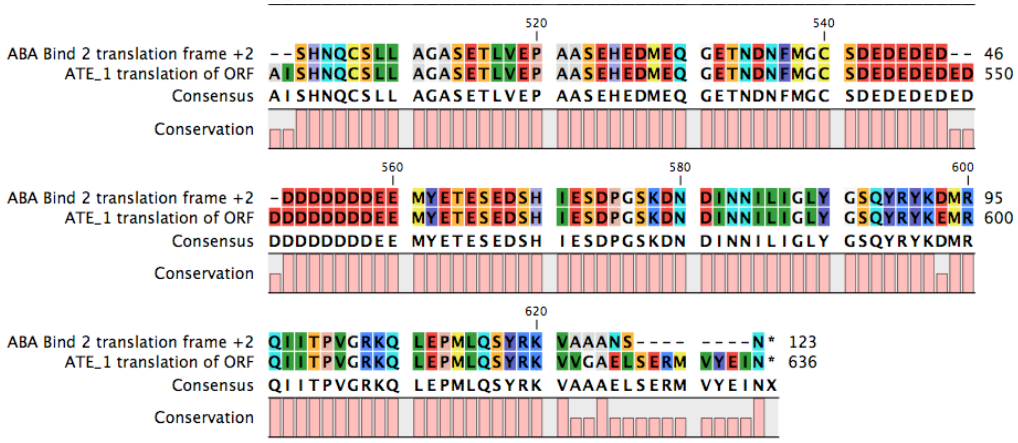


Figure 8 Translated Sequence alignment showing missing Glu Asp Asp (residues 549-551 using ATE1 numbering) within low complexity region.

The insert in clone 9 corresponded to amino acids 203 to 269 in a putative protein kinase (NP_177210). The region corresponds to an unannotated conserved domain known as DUF26 (see Figure 9). These domains, which usually occur in pairs, are only found in plant proteins and are usually associated with protein kinase domains in receptor-like proteins. However, a few are linked to putative reverse transcriptase or nucleotide diphosphate kinase regions. It is not inconceivable that by binding to the DUF26 domains ABA could modulate the activity of the associated kinase or reverse transcriptase segments, although this awaits further experimentation.

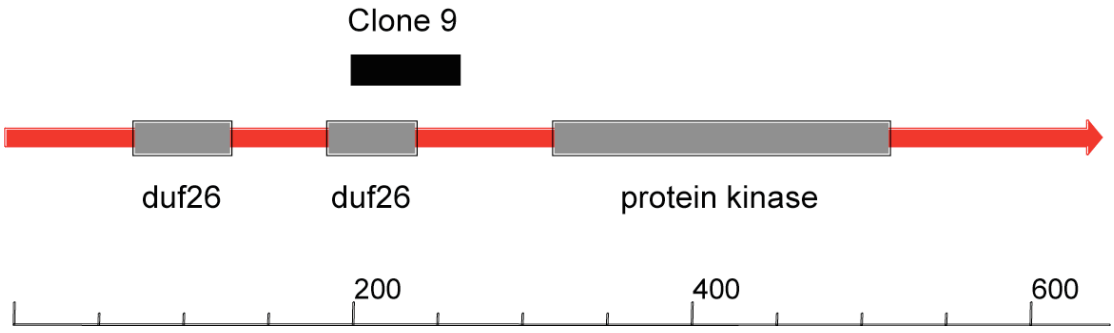


Figure 9 Sequence identity of clone 9 with putative protein kinase NP_177210

Preliminary Protein Expression Work

A feature of many of the clones, including 3 and 9, were that the fusion peptide was out of frame with the *D* gene product. Thus for the fusion protein to be expressed intact, frame slippage would have to occur during translation. This has previously been documented[14] and it was suggested that frame slippage was actually conferring an advantage to the phage, by reducing the requirement to express the fusion protein. Thus, infrequent slippage of an out of frame sequence would reduce the amount of fusion protein in the phage capsid leading to a phage with a selective advantage.

In order to test for expression of the fusion peptide both clone 3 and clone 9 sequences were cloned into the expression vector pET30 (Novagen) in exactly the same context as occurred in the phage, except that a His-tag was added to the *N*-terminus of the glycoprotein D. Thus, the insert was still out of frame with the *D* gene and would produce a truncated protein if frame slippage during translation was not occurring. Expression of both constructs in the amber suppressing strain NovaBlue(DE3 + pSUF) revealed that clone 9 was indeed producing recombinant fusion protein in addition to the wild-type 16.7 kDa His-gpD protein, suggesting slippage was only occurring in this case. No fusion product was observed from this

vector for clone 3. Proteins from the clone 9 strain were purified on a Ni^{2+}- His column alongside protein from a non-suppressing strain (BL21). Figure 10 shows that two proteins were purified from the clone 9 construct in the non-suppressing host. The larger protein shows a molecular mass of around 28 kDa which matches the expected mass of the gpD fused to the peptide. No protein of 21.9 kDa was evident, which is the expected size of the gpD fused to a short nonsense peptide, suggesting that frame slippage was occurring at a high frequency. A protein of around 16.7 kDa was evident in both the suppressing and non-suppressing strain, which represents the His-gpD protein that is a result of termination of translation at the amber stop codon. Further studies using these proteins, including ABA-binding assays, are underway.

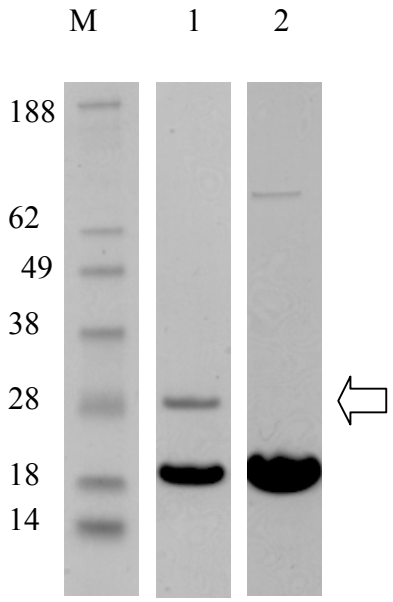


Figure 10 Purification of proteins expressed by pET30-clone9 in suppressing (1) and non-suppressing host (2). The 28 kDa protein in (1) is the size anticipated for the His-gpD-clone3peptide fusion product. M = protein size markers (kDa)

Data demonstrating low similarity of translated sequences with known receptors for ABA.

**********hits-clone3**********

Sequences producing significant alignments: (bits) Value

FCA (Arababdopsis) 18 0.43

>FCA (Arababdopsis)

Length = 747

Score = 18.5 bits (36), Expect = 0.43

Identities = 9/23 (39%), Positives = 10/23 (43%)

Query: 2 HNQCSLLAGASETLVEPAASEHE 24

HNQ +L G V A E E

Sbjct: 182 HNQITLPGGTGPVQVRYADGERE 204

Score E

Sequences producing significant alignments: (bits) Value

MgChel - Arababdopsis 21 0.14

>MgChel - Arababdopsis

Length = 1381

Score = 18.9 bits (37), Expect = 0.67

Identities = 8/20 (40%), Positives = 13/20 (65%), Gaps = 2/20 (10%)

Query: 70 PGSKDNDINNILIGLYGSQY 89

PG+ ++D N+L+ YG Y

Sbjct: 597 PGNLNSDGENLLV--YGKAY 614

**********hits

clone9**********

Sequences producing significant alignments: (bits) Value

MgChel - Arababdopsis 15 5.8

>MgChel - Arababdopsis

Length = 1381

```
 Score = 14.6 bits (26), Expect = 5.8
 Identities = 8/27 (29%), Positives = 13/27 (48%), Gaps =
2/27 (7%)

Query: 5   ECLSKASV--RIGSCLVNEEGRVLSAG 29
           ECL    +   +GS +   EG+ +  G
Sbjct: 944 ECLKLVVMDNELGSLMQALEGKYVEPG 970

***** No hits found ******
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