Development of yeast-based methods to screen for plant cytokinin-binding proteins

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ABSTRACT

Cytokinin is an important plant hormone that controls many aspects of plant growth and development. A great amount of research has been done regarding its function and recently some significant process made on its signal transduction pathway. However much remains to be discovered and in particular the identity of cytokinin-binding proteins that function as signal transducing receptors would be valuable information. This study aimed to develop two yeast-based methods, to search for plant cytokinin-binding proteins.

To establish the yeast three-hybrid (Y-3-H) system and fluorescence activated cell sorting (FACS) screening, four different novel 6-benzylamino purine (BAP)-dexamethasone conjugates and two kinds of biotinylated BAP conjugates were synthesized, respectively. The suitability of these specially designed cytokinin derivatives for use in the study of cytokinin-binding proteins were demonstrated by their activities in competing with the proven cytokinin signal molecule BAP in inducing a response in vivo in the Amaranthus bioassay.

The design of the Y-3-H system was based on that reported by Licitra and Liu (1996), with modifications that included use of a different yeast host strain to allow positive selection for the LEU2-containing fish plasmid and creation of a new hook plasmid to make the expression of HIS3 in yeast host L40 useable as a reporter gene. Hybrid plant-animal hormone molecules (baits) created in Chapter II allow the animal hormone moiety to be anchored to a fusion of a DNA binding domain (DBD) with an animal hormone receptor produced by hook plasmid. The DBD can then be held in juxtaposition with a transcriptional activation domain (AD) as part of fusion protein produced by fish plasmids to create a functional bipartite transcription factor, if the
AD is fused with a plant hormone receptor protein. The novel hybrid hormone thus acts as a bridge between the DBD and AD and allows transcription from reporter genes that allow the identification of individual cells in a population transformed with a plant fusion library. Individuals that have received a receptor able to bind plant hormone can then transcribe genes that allow prototrophic growth while the majority of cells are auxotrophs. Such cells can also transcribe the LacZ gene that allows hydrolysis of the substrate 5-Bromo-4-chloro-3-indolylb-D-galactopyranoside (X-gal) to a blue product. The genes that encode the cytokinin-binding proteins can then be recovered from cells with those properties. All the components, as far as could be tested, were verified for their functions before assembling together for test.

A quite different approach, based on the capacity of FACS to recognize and separate microscopic particles, was also used to recover yeast cells that were expressing Arabidopsis cDNAs that increased cytokinin binding. In this screen, the plant proteins did not need to be fusions with a transcriptional AD domain. If any yeast cell expressing cytokinin-binding protein on its outer membrane, FACS should be able to identify the cell from the whole population of cells expressing plant cDNAs by the increased retention of biotinylated BAP (synthesized in Chapter II) and its consequent binding of the fluorescent streptavidin-phycoerythrin (PE). Then the sorted cell can be tested to confirm that its properties are altered by the introduced gene and the identity of the cytokinin-binding protein is revealed by sequencing that gene.

Test screens have been conducted with the two methods. An Arabidopsis cDNA library and a maize cDNA library that were constructed in fusion with a transcription AD have been screened with the Y-3-H method, while some His-independent clones were observed, none of them could activate the other reporter gene LacZ. A non-
fusion cDNA library of *Arabidopsis* screened by the FACS method has resulted in some primary positives that warrant further testing. These results together with possible future improvements of the screen methods have been discussed.
DECLARATION

I certify that this thesis is submitted in accordance with the regulations of the University of Wollongong in fulfillment of the degree of Master of Science by Research. It does not include any material published by any other person, except where due reference is given in the text. The experimental work described in this thesis is original with all collaborations acknowledged and has not been submitted for a degree in any other university.

You Wang
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I really thank my parents for their strong supports and encouragement when I was in the difficult time. Due to these, I persisted in completing my study.
ABBREVIATIONS

A$_{542}$ absorbance at 542 nm
A$_{620}$ absorbance at 620 nm
ABRC Arabidopsis Biological Resource Center
AD activation domain
Ade adenine
AHK Arabidopsis histidine protein kinase
AHP Arabidopsis histidine phosphotransfer proteins
APRT adenosine phosphoribosyl transferase
ARR Arabidopsis nuclear response regulator
Asp aspartate
3-AT 3-aminotriazole
BAP benzylaminopurine
bp base pair
CBP cytokinin-binding protein
CDK cyclin-dependent kinase
cDNA complementary DNA
CHASE cyclases/histidine kinases-associated sensory extracellular
C-terminal carboxyl terminal
DBD DNA binding domain
DCHC 1,3-dicyclohexylcarbodiimide
dH$_2$O sterilized water
DMF dimethylformamide
DMSO dimethylsulfoxide
DNA deoxyribonucleic acid
DTT dothiothreitol
EB ethidium bromide
EDTA ethylenediamine tetraacetic acid
EI electron impact
ELISA enzyme-linked immunosorbent assay
ES-MS electrospray mass spectroscopy
FACS fluorescent-activated cell sorting
FITC fluorescein isothiocyanate
g gram
g gravity
GARP glutamic acid-rich protein
GEF guanyl-nucleotide exchange factor
GC-MS gas chromatography-mass spectroscopy
Glu glutamate
<table>
<thead>
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<tr>
<td>h</td>
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<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>Hpt</td>
<td>histidine-containing phosphotransferase</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>Ile</td>
<td>isoleucine</td>
</tr>
<tr>
<td>IPA</td>
<td>isopentenyl adenosine</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kV</td>
<td>kilovolt</td>
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<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>Leu</td>
<td>leucine</td>
</tr>
<tr>
<td>LSD</td>
<td>least significant difference</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>mAbs</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>min</td>
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<td>mRNA</td>
<td>messenger-ribonucleic acid</td>
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<td>MS</td>
<td>mass spectroscopy</td>
</tr>
<tr>
<td>N</td>
<td>nitrogen</td>
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<td>Na</td>
<td>sodium</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NLSs</td>
<td>nuclear localization signals</td>
</tr>
<tr>
<td>n-PrOH</td>
<td>n-propyl alcohol</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino terminal</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
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<td>pH</td>
<td>hydrogen ion exponent</td>
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<tr>
<td>PRPP</td>
<td>5-phospho-1-ribosyl-1-pyrophosphate</td>
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<tr>
<td>rGR</td>
<td>rat glucocorticoid receptor</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>rpm</td>
<td>cycle per minute</td>
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<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SAM</td>
<td>shoot apical meristem</td>
</tr>
<tr>
<td>SAP</td>
<td>shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylammonium</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
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<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>u</td>
<td>unit</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
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<td>Ura</td>
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UV
wol
X-gal
X-gal
Y-2-H
Y-3-H
µF
µg
µL
µM
Ω
ultraviolet
wooden leg
5-Bromo-4-chloro-3-indolylb-D-galactopyranoside
yeast two-hybrid
yeast three-hybrid
microfarad
microgram
microliter
micromole
omega
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