Identification and Expression Analyses of Genes involved in Early Endosperm Development in *Arabidopsis* and Cereals

by

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DECLARATION

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Journal articles

- Data presented in Chapter Four have been published in the following journal article:

  Li, **M**., Singh, R., Bazanova, N., Milligan, A.S., Shirley, N., Langridge, P. and Lopato, S. (2008). Spatial and temporal expression of endosperm transfer cell-specific promoters in transgenic rice and barley. *Plant Biotechnol J*. 6 (5): 465-476.

- Data presented in Chapter Six have been incorporated into the following journal article:

  Kovalchuk, N., **Li**, **M**., Wittek, F., Reid, N., Singh, R., Shirley, N., Ismagul, A., Eliby, S., Johnson, A., Milligan, A.S., Hrmova, M., Langridge, P. and Lopato, S. (2010). Defensin promoters as potential tools for engineering disease resistance in cereal grains. *Plant Biotechnol J*. 8 (1): 47-64.

II

Patents

1. Specific expression using transcriptional control sequences in plants (WO 2007/048207). PCT filed on October 27, 2006. Entered the National phase on April 21, 2009. One of the two inventors.

2. Transcription regulators for reproduction associated plant part tissue specific expression (WO 2007/137361 A1). PCT filed on May 19, 2007. Entered National phase in October 2008. One of the two inventors.

3. Flower active transcriptional control sequences. PCT filed on July 30, 2009. One of the three inventors.

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ABSTRACT

Cereal seeds are important source for food, feed and also provide an industrial raw material. A sound understanding of seed initiation and the control of early endosperm development will be important for increasing cereal crop yield and improving grain quality using biotechnology.

The approach taken in this project was to investigate the potential application of candidate genes and pathways for manipulating endosperm initiation and development. The candidate system chosen was the FIS class *Polycomb* group complex since there has been considerable work on the role of this complex in the transcriptional regulation of seed initiation and endosperm cellularization in *Arabidopsis*. The aims of the work described in this thesis were, first to identify and characterize novel proteins which directly interact with the known PcG members FIE and SWN. These two proteins play a key role in the regulation of endosperm proliferation in *Arabidopsis*. The second aim was to identify homologs from rice and *Arabidopsis* which potentially regulate grain size, weight and composition and to investigate the expression profiles and possible functions of these genes and isolate endosperm-specific promoters.

Several novel plant PcG complex proteins from *Arabidopsis* were identified *via* yeast two-hybrid (Y2H) screens. Several other interacting transcription factors of FIE were identified, including SWN (a.k.a. EZA1/SWINGER), AREB2 (ABA-responsive element binding factor 2), VPL (Viviparous-like) and ICE2 (Inducer of CBF Expression 2). Interacting partners of SWN were also isolated. These included AtRING-H2, an E3 type protein ubiquitin ligase, v-SNARE, and BrD (Bromodomain-containing protein), and a plant PHD finger-containing factor. The expression profile of these genes was studied using quantitative real-time PCR (qRT-PCR) in a series of *Arabidopsis* tissues prepared from wild type (WT) plants. Expression of these genes was also studied by using the predicted 5'-regulatory sequences of *SWN*, *AtRING-H2*, *AREB2*, *VPL* and *ICE2* fused to GUS reporter gene and stably transformed into *Arabidopsis*. The expression pattern of each candidate was revealed by GUS assays. Results showed that the *VPL* promoter was specific to the male gamete, while the *AtRING-H2* promoter was found to be male gametophyte- and seed-specific.

In order to elucidate the functions of the key PcG partner SWN and its possible role in the regulation of seed initiation, T-DNA insertion lines were analyzed and gene knockdown constructs were developed. Seed development was disrupted by 50% in one SWN silencing line *via* dsRNAi. A preliminary study of the *SWN* silencing plant was carried out but further characterization is needed to uncover the role of *SWN* in seed development.

Manipulation of endosperm development will be dependent on access to promoters specific to early endosperm development. *Arabidopsis END1*, a homolog of a barley endosperm marker gene was characterized in this project. *AtEND1* was identified as a member from *Arabidopsis* lipid transfer protein family. This gene was specifically expressed in gametophytes and developing seeds during free nuclear division and endosperm cellularization. A number of seed-specific *cis*-acting elements were identified in the 5'-regulatory region of *AtEND1*. The promoter-GUS construct was also transformed into canola. A less specific expression pattern was observed in transgenic canola plants. Analyses of the transgenic plants in *Arabidopsis* and canola are presented.

Three rice orthologs at *END1* (*OsPR602*, *OsPR9a* and *OsPRPI*) were identified from database searches. The expression of these genes in rice was analyzed by qRT-PCR. Transcriptional GUS fusion constructs of the predicted promoter regions were transformed into rice and barley. Analyses of transgenic barley and rice plants were performed by using PCR screening, Southern blot hybridization and histological GUS assay. These genes were predominantly expressed in the developing rice endosperm. *OsPR602* and *OsPR9a* showed similar spatial and temporal expression patterns in barley compared to their expressions in rice. Expression analysis found that *OsPRPI* directed gene expression in the vascular bundles of anthers, hulls and micropylar and chalazal poles of pistil shortly before and after anthesis and the vascular bundles of developing caryopses until 19 days after pollination in rice. There was no GUS expression in barley under the control of *OsPRPI*. The studies also revealed wounding-induced expression of this gene in the vascular traces of leaf blade, leaf sheath, stem and grains. The induction and expression level of *OsPRPI* was studied by northern blot analysis and qRT-PCR. These gene promoters will be useful tools in transgene research and in the manipulation of cereal grain yield, quality and tolerance to biotic and abiotic stresses.

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ABBREVIATIONS

ABRC	*Arabidopsis* Biological Resource Center
ACPFG	The Australian Centre for Plant Functional Genomics
AD	activation domain
AL	aleurone layer
AREB2	ABA-Responsive Element Binding factor2
ARF	auxin responsive factor
At	*Arabidopsis thaliana*
ATG	translation start site/start codon
BD	DNA binding domain
BETL	basal endosperm transfer cell layer
bHLH	basic Helix-Loop-Helix
BLAST	basic local alignment search tool
Bn	*Brassicaceae napus*
BRD	bromodomain
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CDS	coding sequence
CLF	Curly Leaf
Col-0/Col-4	Columbia-0/Columbia-4
CSE	central starchy endosperm
CTAB	Cetyl trimethylammonium bromide
CZE	chalazal endosperm
DAP	days after pollination
DDM1	Decrease in DNA Methylation1
DEK	defective kernel1
DIC	differential interference contrast
DME	DEMETER
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSC	discolored
E. coli	*Escherichia coli*
EDTA •Na_2	ethylenediaminetetraacetic acid sodium salt
EED	embryonic ectoderm development
Emb	embryo defective
EMF	Embryonic flower
EMP	empty pericarp
END1	Endosperm1
ESC	Extra Sex Combs
ESR	embryo-surrounding region
EST	expressed sequence tag
ETC	endosperm transfer cells
E(z)	Enhancer of Zeste

EZA1	Enhancer of Zeste from *Arabidopsis*1
FIE	Fertilization-Independent-Endosperm
FIS1	Fertilization-Independent Seed1
FIS2	Fertilization-Independent Seed2
FLC	FLOWERING LOCUS
FWF	Fertilization-Independent-Fruit development
GFP	green fluorescent protein
GUS	*UidA*/5-bromo-4-chloro-3-indolyl-β-D-glucuronidase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HOX	homeobox
Hv	*Hordeum vulgare*
Hyg	hygromycin B
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
LB	Lysogeny broth/Luria-Bertani broth
L*er*	Landsberg *erecta*
LMDM	laser micro-dissecting microscopy
LS	longitudinal section
LTP	lipid transfer protein
MCE	micropylar endosperm
MEA	MEDEA
MET	methyltransferase
MSI1	MULTICOPY SUPRESSOR OF IRA1
NASC	European Arabidopsis Stock Centre
NCBI	National Center for Biotechnology Information
NCD	nuclear cytoplasmic domain
NPT II	Neomycin Phosphotransferase II
Os	*Oryza sativa*
PBS	Phosphate Buffered Saline
PcG	*Polycomb* Group
PCR	polymerase chain reaction
PEN	peripheral endosperm
PHD	plant homeodomain
PHE1	PHERES1
PI	proteinase inhibitor
PRE	*Polycomb*-responsive element
qRT-PCR	real-time quantitative reverse transcriptional polymerase chain reaction
RFLP	restriction fragment length polymorphism
Rif	rifampicin
RING	Really Interesting Novel Protein/Ring-finger protein
RMS	radial microtubule systems
RNAi	ribonucleic acid interference
RO	Reverse Osmotic
RPM	rotation per minute

RT-PCR	reverse transcriptional polymerase chain reaction
PVP360	polyvinyl-pyrrolidone 360
PVPP	Polyvinylpyrrolidone
SAL	subaleurone layer
SDS	Sodium dodecyl sulfate
SIGnAL	Salk Institute Genomic Analysis Laboratory
SPEC	spectinomycin
SWN	SWINGER
Ta	*Triticum aestivum*
TAA/TAG/TGA	translation stop site/stop codon
TAIR	The *Arabidopsis* Information Resource
Td	*Triticum durum*
TF	transcription factor
TIGR	The Institute for Genomic Research
TL	transfer cell layer
TS	transverse section
TTN	TITTAN
UTR	untranslated region
VPL	Viviparous1-Like
VRN2	Vernalization2
Ws	Wassilewskija
WT	wild type
XCL1	extra cell layer1
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-GLUC	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
Y2H	yeast two-hybrid
YFP	yellow fluorescent protein
Zm	*Zea mays*

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